

**Mechanistic Studies on Human Fibrinogen
Polymorphisms that Influence Blood Clot Formation
and Lysis**

Katriona Amy Greenhalgh

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Katriona Amy Greenhalgh

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Abstract

The blood clot is composed of a mesh of fibrin fibres with cellular elements embedded in this network. The structure of the fibrin clot can determine predisposition to atherothrombotic conditions, as compact clots composed of thin fibres and small pores are associated with premature and more severe cardiovascular disease. Both genetic and environmental effects can cause qualitative and quantitative changes in procoagulant and anti-fibrinolytic proteins, altering fibrin clot formation, structure and lysis.

Three hypotheses were tested in this work: Firstly, the B β Arg448Lys fibrinogen variant affects clot structural and functional properties in T2DM patients. Secondly, α 2-AP is the protein responsible for the differences in lysis of recombinant B β Arg448Lys fibrinogen variants in the plasma environment. And lastly, I proposed the A α Trp334Cys/Asn335Tyr changes in the Birmingham II patient are responsible for her clinical presentation. Accordingly, the aims of this work were to identify any additional effect of B β Arg448Lys on fibrin network in type 2 diabetes, study the mechanisms behind the effects of the B β Arg448Lys mutation on fibrin clot structural and functional properties, and finally, investigate the effect of fibrinogen A α Trp334Cys/Asn335Tyr on clot structure and lysis and abnormal patient phenotype.

Compact clots with resistance to fibrinolysis were detected in carriers of B β 448Lys variant of fibrinogen with diabetes. A direct role of the polymorphism in the changes observed with plasma clots was identified, and this may add to an already increased level of vascular risk in women with diabetes.

In a recombinant system, differences in lysis between B β Arg448Lys variants were attributable to the interplay between porosity of the clot, the fibrin- α 2-AP interaction and plasmin generation.

Finally, we confirm the A α Trp334Cys/Asn335Tyr substitutions in the fibrinogen BII patient are responsible for a clinical presentation of bleeding and thrombosis, providing mechanistic explanations for this patient phenotype.

In summary, I extensively investigated the interplay between the fibrinogen molecule, fibrin clot structure, fibrinolysis and the environment and made a number of novel observations. Data generated has shed light on the mechanisms of increased thrombosis risk in patients with compact clots made of thin fibres, through genetic or environmental influence, which in turn will help to develop effective treatment strategies to reduce ischemic events in high risk patients.

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List of abbreviations

α_2 -AP	Alpha-2-antiplasmin
ABI	Ankle brachial index
AFM	Atomic force microscopy
AGE	Advanced glycation endproduct
AM	Activation mix
APCE	Antiplasmin precursor cleaving enzyme
BII	Birmingham II
BCS	Bovine calf serum
BSA	Bovine serum albumin
CAD	Coronary artery disease
cDNA	Complementary deoxyribonucleic acid
CFT	Clot formation time
CHO	Chinese hamster ovary
CHD	Coronary heart disease
CI	Confidence interval
COX-1	Cyclooxygenase-1
CVD	Cardiovascular disease
dBp	Diastolic blood pressure
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DVT	Deep vein thrombosis
EGF	Endothelial growth factor
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
FV	Factor 5
FVII	Factor 7
FVIII	Factor 8
FIX	Factor 9
FX	Factor 10
FXI	Factor 11
FXII	Factor 12
FXIII	Factor 13

FXIIIa	Activated factor 13
FpA	Fibrinopeptide A
FpB	Fibrinopeptide B
FITC	Flourescein isothiocyanate
G418	Geneticin 418
GM	Growth media
HDL	High density lipoprotein
IMT	Intra mural thickness
ITS	Insulin transferrin sodium selenite
IHD	Ischaemic heart disease
IVC	Inferior vena cava
Kd	Dissociation constant
LBS	Lysine binding site
LD	Linkage disequilibrium
LDL	Low density lipoprotein
LDR	Lothian diabetes register
LM	Lysis mix
LSCM	Laser scanning confocal microscopy
LSMS	Liquid sample mass spectrometry
LT	Lysis time
MA	Maximum absorbance
MES	2-morpholinoethanesulfonic acid
mg	Milligram
mg/ml	Milligram per millilitre
MI	Myocardial infarction
mmol/L	Millimole per litre
NC-AFM	Non-contact mode atomic force microscopy
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NTP	N-terminal peptide
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
PB	Permeation buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor

RCL	Reactive centre loop
RGD	Arginine-glycine-aspartate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
PTM	Post translational modification
sBP	Systolic blood pressure
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SFM	Serum free media
SNP	Single nucleotide polymorphism
SPR	Surface plasmon resonance
SPSS	Statistic package for social scientists
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TAE	Tris-acetate-EDTA
TAFI	Thrombin activatable fibrinolysis inhibitor
TBS	Tris buffered saline
TC	Total cholesterol
TF	Tissue factor
TGF β	Transforming growth factor beta
TSP-1	Thrombospondin-1
TIA	Transient ischaemic attack
TM-AFM	Tapping mode atomic force microscopy
tPA	Tissue plasminogen activator
Tris	Tris(hydroxymethyl)aminomethane
U/ml	Unit per millilitre
VSMC	Vascular smooth muscle cell
VTE	Venous thrombotic embolism
vWF	Von Willebrand factor
WT	Wildtype
γ'	Gamma prime

Publications and Presentations

Publications

Inhibition of endothelial cell Ca²⁺ entry and transient receptor potential channels by Sigma-1 receptor ligands,

MS Amer, L McKeown, S Tumova, R Liu, VAL Seymour, LA Wilson, J Naylor, **K Greenhalgh**, B Hou, Y Majeed, P Turner, A Sedo, DJ O'Regan, J Li, RS Bon, KE Porter, DJ Beech,

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K Greenhalgh,
MCRC Monthly Research Talk, University of Leeds

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in type 2 diabetes: potential implications for individualised therapy,
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K Greenhalgh, K Standeven, P Grant and RA Ajjan,
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2012, Leeds, UK

Molecular mechanisms behind differences in clot formation, morphology and lysis
secondary to the B β Arg448Lys mutation in fibrinogen,
K Greenhalgh, K Standeven, P Grant and RA Ajjan,
XXIIInd International Fibrinogen Workshop, July 2012, Brighton, UK

Fibrinogen Birmingham II - a novel variant associated with hypodysfibrinogenemia, due
to co-inheritance of Trp334Cys and Asn335Tyr in the fibrinogen A α chain,
K Greenhalgh, M Hill , N Thompson, B Gordon, RAS Ariens, M Williams, G Dolan, RA
Ajjan,
XXIV Congress of The International Society on Thrombosis and Haemostasis (ISTH),
July 2013, Amsterdam, NL

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in type 2 diabetes: potential implications for individualised therapy,
K Greenhalgh, S Alzahrani, J Price, M Strachan, P Baxter, Z Kurdee, K Standeven, PJ
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Chapter 1. Introduction

Clot formation is an essential physiological process to prevent blood loss upon vessel injury, however changes in various parameters involved in the clotting cascade can lead to pathological complications with serious clinical implications. Alterations in clot formation, morphology and lysis can increase risk of cardiovascular disease (CVD) and can result in irreversible end organ damage. Consequently it is important to understand the mechanisms behind the clotting process to elucidate the impact on atherothrombotic and CVD and to help develop preventative and therapeutic targets.

1.1 An overview of blood clot formation

Thrombus formation occurs via a series of interlinked events known as the coagulation cascade that involves interplay between cellular elements and plasma proteins. Exposure of tissue factor (TF) as a result of vessel injury leads to exposure of a prothrombotic core and activation of various clotting factors which liberate active thrombin. This thrombin activates platelets, cleaves fibrinogen to form fibrin and activates Factor 13 (FXIII). The resulting clot is made of a mesh-like network of fibrin fibres to which various clotting factors and cellular components bind and crosslink forming the mature clot which prevents continued bleeding.

1.1.1 The role of coagulation factors

Coagulation factors FXII, FXI, FIX, FVII, FVIII are essential components of the blood clotting process, being activated sequentially to activate FX and FV, resulting in the formation of thrombin, which in turn converts soluble fibrinogen into insoluble fibrin network, thereby forming the mature clot. Complex interactions and feedback mechanisms control the activity of these factors, so change or deficiency in any of these proteins has profound effects on clot formation, structure and lysis and consequently susceptibility to disease.

1.1.2 The role of platelets

Platelets, the major cellular element involved in the blood clotting process, become activated at sites of vessel injury where collagen is exposed and there are increased levels of clotting factors such as thrombin. Once activated, platelets can bind many coagulation factors (Von Willebrand factor (vWF), thrombin, FXI, fibrinogen and prothrombin) via a number of exposed receptors, leading to their adherence to the site of injury and aggregation¹. Platelet activation, adherence and aggregation act to form a platelet plug using vWF and fibrinogen as a connecting agent. This platelet plug not only helps to stop the initial bleeding but helps wound repair by the secretion of growth factors by the activated cell².

It is the interplay between all of these factors that determine how the clot behaves and thus risk of pathogenic consequences. Both quantitative and qualitative changes in the cellular and acellular components can be caused by genetic and environmental factors, which increase susceptibility to vascular disease.

1.2 The coagulation cascade

The fibrin network represents the backbone of the blood clot with cellular elements, including platelets and red and white blood cells, embedded in this network. Thrombus formation represents the last step in the atherothrombotic process and occurs via a series of interlinked events that progress through steps known as the coagulation cascade. Traditionally, this has been divided into two independent cascades that converge to a common pathway; the intrinsic, composed of FXII, XI, IX and VIII and the extrinsic composed of TF which activates FVII converging to activate FX, FV, thrombin and fibrinogen. However, this is more of an *in-vitro* model and does not necessarily represent *in-vivo* coagulation, particularly as thrombus formation also relies on the interplay between these factors and platelets¹. Therefore, *in vivo* clot formation is a more complex process that involves cellular elements and plasma proteins. Exposure of TF as a result of vessel injury (secondary to atherosclerotic plaque rupture) leads to exposure of a prothrombotic core and activation of FVII/TF complexes and consequent FIX, FX and FV activation. The latter two factors liberate active thrombin from its precursor, prothrombin. This activates platelets adhered to the site of injury via interaction with vWF and collagen³. This interaction works to fully activate the platelets leading to their degranulation and consequent FV release which is later activated by FXa and thrombin. FVIII also plays a role in thrombus formation by interacting with FIXa as a result of thrombin activation, increasing coagulation. This complex feedback mechanism generates enough thrombin to cleave fibrinogen to form fibrin and to activate FXIII, enhancing fibrin crosslinking and thus clot stability. The coagulation cascade is summarised in Figure 1-1.

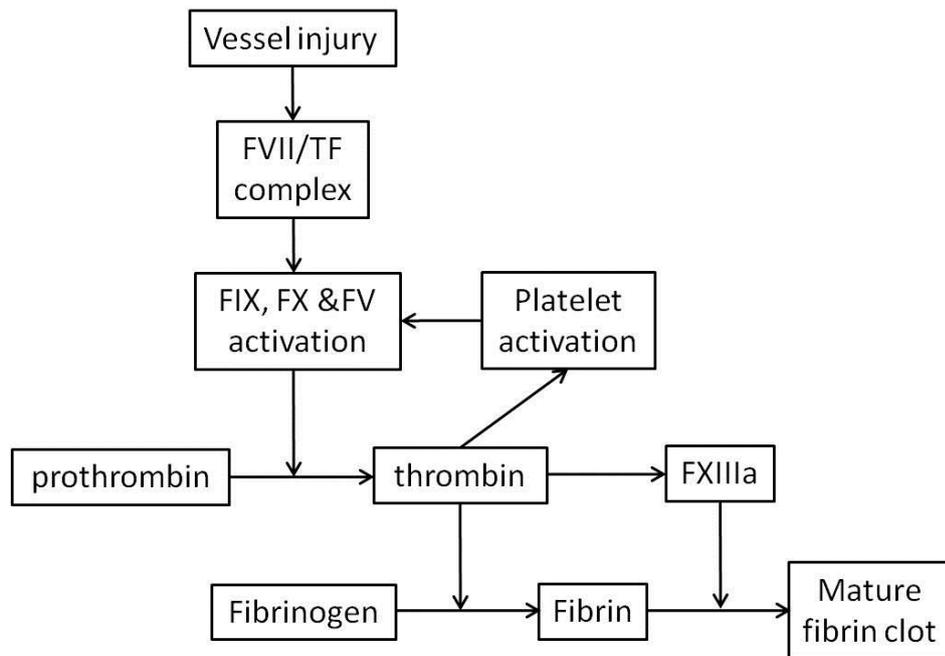


Figure 1-1. Schematic representation of the coagulation cascade resulting in fibrin clot formation

Vessel injury results in activation of coagulation factors, cleavage of prothrombin to thrombin, platelet activation and resultant conversion of fibrinogen to fibrin, FXIIIa crosslinking and clot stabilisation.

Most of the proteins involved in the clotting process belong to the serine endopeptidase family which are characterised by their ability to cleave a specific peptide bond in their substrate and contain a serine residue in their active site. The substrates of these enzymes are usually endopeptidase precursors, resulting in amplification of the pathway via consecutive enzyme activation⁴. The resulting clot is made of a mesh-like network of fibrin fibres to which various clotting factors bind and crosslink forming the mature clot which prevents continued bleeding. Whilst platelets have a crucial role in clot formation, my thesis concentrates on the acellular arm of coagulation, in particular the fibrinogen molecule, which will be the focus of my work.

1.3 Serine proteases

Serine proteases are a family of enzymes characterised by their ability to cut certain peptide bonds in proteins due to their unique active site featuring a preserved serine residue. They are abundant in the digestive system and complement cascade, as well as being involved heavily in blood clotting. Digestive enzymes such as trypsin, chymotrypsin and elastase, secreted by the pancreas, are all serine proteases, sharing the same position of serine in their active site. Despite this similarity they have specificities for varying substrates and different peptide bonds. In plasma, several of

the clotting factors are serine proteases, including FX, FXI and FXII as well as thrombin and plasmin.

These proteins circulate in the plasma in an inactive proenzyme state from which they must be activated by specific proteolysis to their active catalytic state. They are multidomain proteins, consisting of a serine protease domain and a series of other domains such as kringles, fibronectin- and epidermal growth factor-like domains. Two anti-parallel β barrels are surrounded by four helical segments to make up the enzymatically active serine protease domain, the active cleft of which is formed by a 'catalytic triad' of histidine, asparagine and serine residues. The other domains facilitate the efficient activity of the enzyme by aiding the binding of the enzyme and target⁵. The structure of these proteins is illustrated in Figure 1-2.

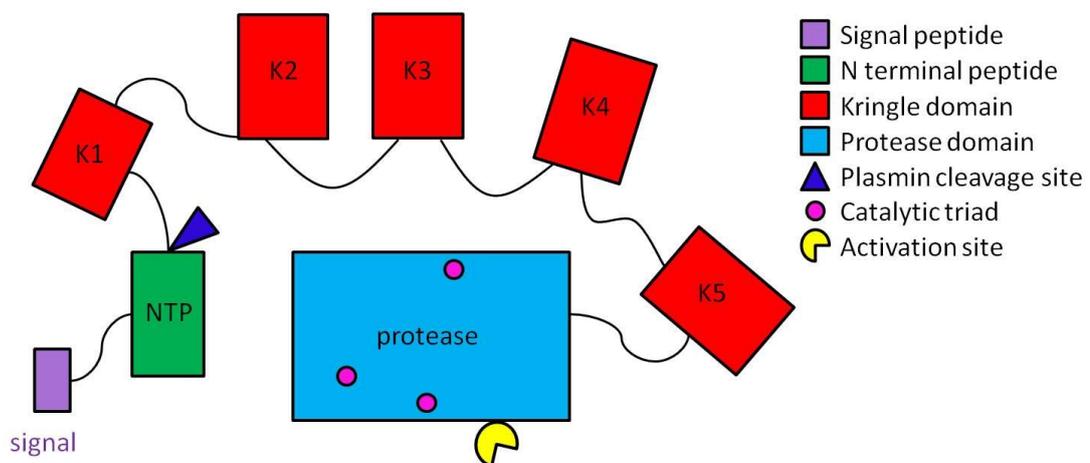


Figure 1-2. Schematic representation of the primary structure of a human serine protease (plasminogen)

The signal peptide, the N terminal peptide, the plasmin cleavage site (Lys77-Lys78), kringles 1-5, the protease domain, the catalytic triad (His603, Asp 646 and Ser741) and the activation site (Arg561-Val562) are shown.

1.4 Serine protease inhibitors

Serine protease inhibitors (serpins) regulate many physiological processes such as coagulation, fibrinolysis, inflammation, angiogenesis and apoptosis by irreversibly inhibiting serine proteases in these systems⁶. Three β -sheets (A-C), nine α -helices (hA-hI) and a relatively mobile reactive centre loop (RCL) make up the standard serpin structure, with the scissile bond of the serine protease recognition site lying in the RCL at the surface of the molecule. Serpins are capable of adopting several different conformations as a result of their mobile RCL; the native form, the latent reactivatable form (with the RCL inserted into the A β sheet, with the scissile bond shielded), the

substrate form (with cleaved RCL), the protease-complex form and an inactive polymerised form (RCL inserted into the A β -sheet of another serpin molecule)⁷.

The inhibition by serpins is said to be 'suicide inhibition' due to the fact they are consumed and inactivated during the inhibition reaction. This is a multi-step process, the first of which is fast and reversible, while the remaining steps proceed slowly and are irreversible. First a non-covalent 1:1 Michaelis-like complex is formed with the serine protease, followed by the cleavage of the reactive peptide scissile bond in the RCL due to the insertion of the RCL into the β -sheet A. This results in the movement of the RCL 71 Å to the opposite side of the serpin, thus transferring the bound protease with it. Consequent attachment of the serpin to the catalytic triad serine residue of the serine protease via a covalent bond then completely inactivates the protease^{5, 6}. The protease can now no longer be released from the serpin, where it is proteolytically destroyed⁶.

In plasma, the action of these serpins, which make up nearly 20% of the proteins in blood, is hugely important. The large scale rapid amplification of a relatively small initiation reaction in the clotting cascade is essential to the blood clotting process. However it is equally important for there to be a mechanism by which this amplification can be controlled and stopped when the need for it is over.

1.5 Fibrinogen

1.5.1 Structure of fibrinogen

The primary structure of fibrinogen, a 340,000 Dalton multi-domain protein, has been fully sequenced. It is formed by three pairs of polypeptide chains, A α , B β and γ , made of 610, 461 and 411 amino acid residues respectively, noted as (A α , B β , γ)₂. The protein is said to consist of 3 nodular regions; two lateral domains (D domains) which are 67200 Da each, composed of globular carboxyl termini of B β and γ chains, and one dimeric central domain (E domain) to which the carboxyl termini of the A α chains are folded. The domains are linked by arms or 'coiled coils' formed from one each of the 3 A α , B β and γ chains supercoiled as α helices. Cyanogen bromide cleavage has revealed 5 'disulfide knots' (or fibrinogen-cyanogen-bromide fragments) containing all 29 disulfide bridges present in the protein. In this way, it has been shown disulfide bonds hold the coiled coils' supercoiled α helix structure in place as well as forming disulfide rings as the attachment to the central and lateral domains⁸. At the amino terminal, 11 disulfide bridges directly link the pair of γ chains that make up the so called 'backbone' of the protein, to which the A α and B β chains are covalently linked to form the central domain⁹.

Of the 29 disulfide bonds, 17 are interchain while the remaining 12 are intrachain connections, 6 in each half-molecule. These form loops in the carboxyl terminal of each of the chains ($A\alpha$ Cys442-Cys472; $B\beta$ Cys201-286, 211-440 and 394-407 and γ Cys153-182 and 326-339)¹⁰. These structures are depicted in Figure 1-3. The amino-terminals of the $A\alpha$ and $B\beta$ chains are thickened due to the presence of fibrinopeptides A and B respectively which are cleaved by plasmin to form fibrin, noted $(\alpha\beta\gamma)_2$.

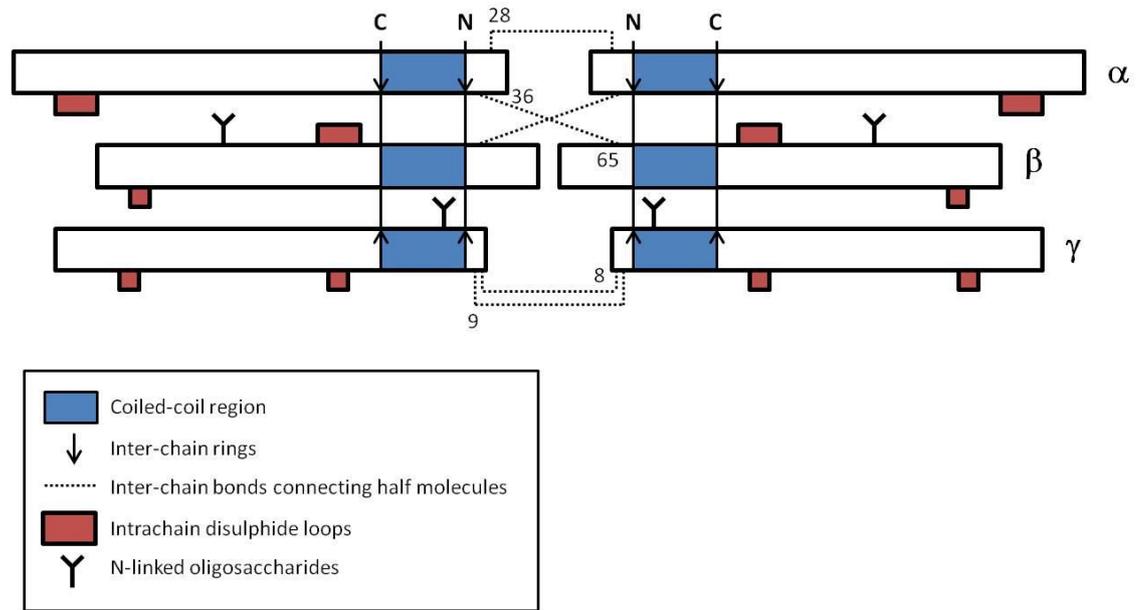


Figure 1-3. Schematic representation of the structure of fibrinogen

The locations of the interchain rings ($A\alpha$ 28, α 36- β 65 and γ 8- γ 9) and bonds and intrachain disulfide loops ($A\alpha$ Cys442-Cys472; $B\beta$ Cys201-286, 211-440 and 394-407 and γ Cys153-182 and 326-339) in relation to the coiled-coil region and the N-linked oligosaccharides are shown. Adapted from Zhang & Redman (1996)¹⁰.

The amino acid residue sequence of the coiled coils is such that the inside is hydrophobic and the outside polar, along which there are 3 clusters of half cysteine residues⁸. Four carbohydrate side chains (2500 Da each), made up of sialic acid, galactose, mannose and N-acetylglucosamine, exist which are linked at the N-terminal to asparagine residues on the lateral domains of the β chains and near the central domain area of the γ chains¹¹.

Acuna et al. (1987)¹² showed the protein to be essentially rigid in the 10-1000 ns time range, they estimated it to be a 47 x 10.5 nm elongated cylinder, carrying 4 g of water per 1 g protein. This confirms the intrinsic rigidity and stable conformation of the molecule in solution, with no free movement of the bulky segments. This fits in nicely with the requirement of rigidity to facilitate the non-covalent interactions formed in the

half-staggered structure of protofibrils and to form a bridge in platelet aggregation to crosslink individual activated platelets.

The γ chain is subject to alternative splicing at its C-terminus, producing either γA or γ' (gamma prime). The majority of γ' circulates in a heterodimer with the more common γA as $\gamma A/\gamma'$. The difference occurs due to polyadenylation at various sites on the messenger RNA transcript. The 411 amino acid γA chain is composed of 10 exons and 9 introns due to polyadenylation occurring downstream of exon 10. However when polyadenylation occurs at an alternative site, in intron 9, intron 9 is not spliced out and γ' is secreted with an extra 20 amino acid extension coded by intron 9 instead of amino acids $\gamma A_{408-411}$ from exon 10¹³.

1.5.2 Assembly and secretion of fibrinogen

Fibrinogen is assembled in a stepwise process from the formation of $A\alpha$ - γ and $B\beta$ - γ complexes from single chains, to the addition of a third chain to form half-molecules ($A\alpha.B\beta.\gamma$) which then dimerise at the amino-terminal (Figure 1-4). These connections rely entirely on disulfide bonds: Symmetrical interchain disulfide bonds form at the amino terminal between the two half-molecules at adjacent Cys $A\alpha_{28}$, Cys γ_8 and Cys γ_9 residues as well as Cys $B\beta_{65}$ and Cys $A\alpha_{36}$ residues becoming disulfide linked. Interchain disulfide rings on the carboxyl terminal of the coiled coil region, though not playing a crucial part in chain assembly, are required for proper protein folding. In addition, $B\beta$ and γ intrachain loops affect the addition of the third chain to $A\alpha$ - γ and $B\beta$ - γ complexes in cooperation with the amino acid sequence of the α helix and the flanking interchain disulfide rings, by allowing proper alignment of the additional chain¹⁰. This process takes place independently of hepatocyte specific machinery via information inherent in the protein chains themselves. The protein is secreted by a general secretory mechanism from hepatic parenchymal cells¹⁴ after which it has a half life of approximately 4 days in vivo, circulating at 2-4 mg/ml (6-12 μM) in plasma³.

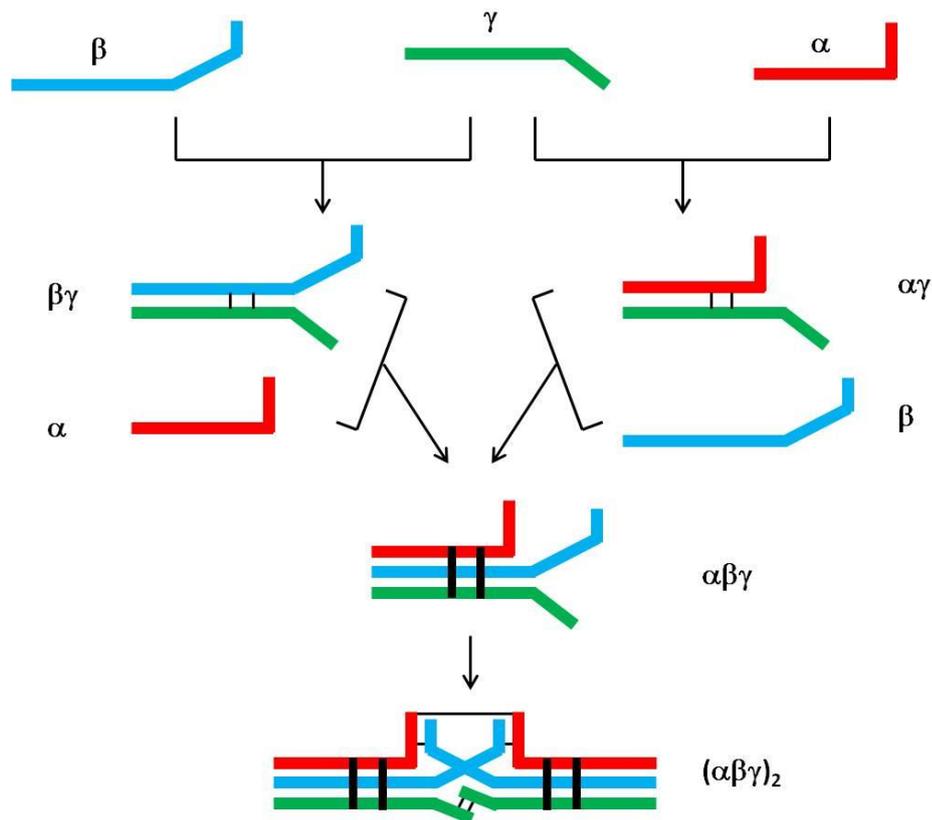


Figure 1-4. Schematic representation of the assembly of the 3 fibrinogen chains into a single fibrinogen molecule ($A\alpha.B\beta.\gamma$)₂

Either $\beta\gamma$ or $\alpha\gamma$ polymers are formed, then the α or the β chain is attached to form the half molecule. These are then joined to form the fibrinogen molecule.

1.5.3 Conversion of fibrinogen to fibrin

The conversion of fibrinogen to fibrin is catalysed by thrombin, a two chain disulfide linked 39k DA enzyme homologous with trypsin and chymotrypsin. Thrombin is a very specific enzyme, hydrolysing only Arg-Glu bonds at the N-termini of the $A\alpha$ and $B\beta$ chains of fibrinogen, leaving the rest of the molecule intact¹⁵.

1.5.3.1 Fibrinopeptide A release

Thrombin binds to the E domain of fibrinogen, hydrolysing bonds to first release the 16 residue fibrinopeptide A (FpA) from the $A\alpha$ chain, exposing the so called 'a-site' of the N terminus. This newly exposed site is able to interact with an 'A-pocket' already exposed in the D domain of the γ chain, allowing the E domain a-site of one molecule to covalently interact with the D domain A-pocket of another, resulting in elongation of the fibrin chain, leading to the formation of half staggered, double stranded protofibrils.

1.5.3.2 Fibrinopeptide B release

Secondary hydrolysis of Arg-Glu bonds in the B β chain N-terminal results in the release of the 14 residue fibrinopeptide B (FpB) and exposure of an E domain 'b-site'. In this case, the b-site interacts with a site in the D domain of another fibrinogen molecule's B β chain known as the 'B-pocket'. This in turn allows lateral growth of the polymer, thickening the fibrin fibre¹⁶. The initial linear growth of the polymer, before FpB release is represented as a 'lag' phase during clot formation in which there is no turbidity increase. The role of Fibrinopeptide A and B release in protofibril formation and fibrin polymerisation is depicted in Figure 1-5. Lateral association and interaction of two protofibrils to form a fibrin fibre is depicted in Figure 1-6.

Using a fibrinogen variant which causes significantly reduced and delayed FpA release, the role of FpB and the impact of delayed FpA release in clot formation and structure has been further investigated. Clots made from this variant fibrinogen have similar clottability and fibre thickness/branch points compared with those of wildtype (WT) fibrinogen, but form more slowly. This implies the ionic environment, rather than the order of peptide release, determines fibre size and that although FpA release supports polymerisation of fibrin more strongly, FpB release is still sufficient to support normal polymerisation and clot formation, but at a slower rate¹⁷.

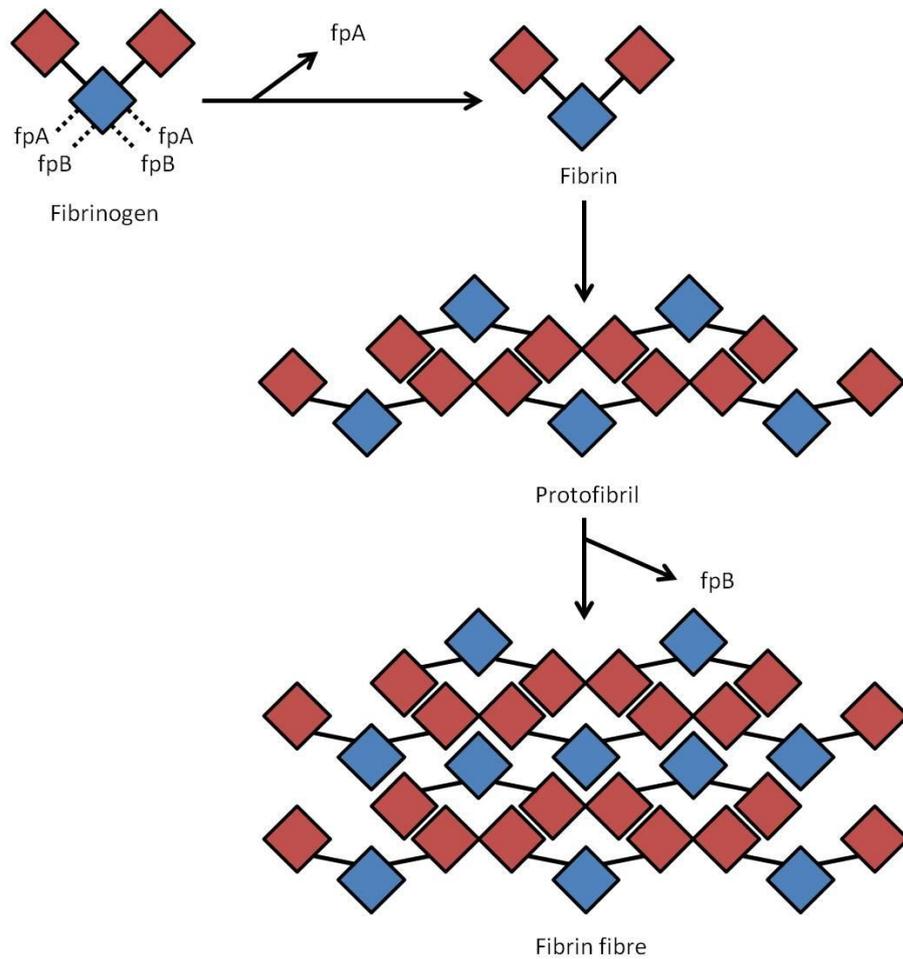


Figure 1-5. The role of fibrinopeptide A and B release on formation of the intermediary protofibril and fibrin fibre

FpA release allows the formation of the early protofibril. Following FpB release allows lateral growth of these fibrils into fibrin fibres.

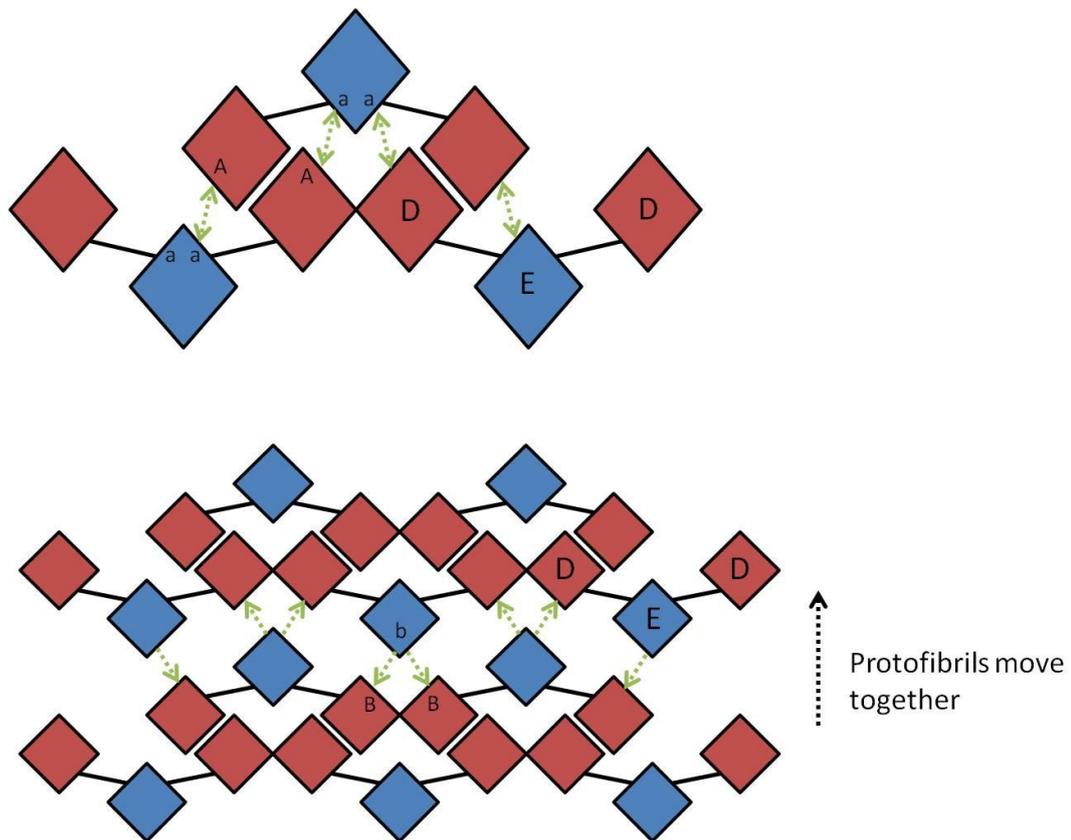


Figure 1-6. Lateral association of protofibrils to form fibrin fibres

The a-sites of the E domain of one molecule interact with the A-pockets of the D domain of another while the E domain b-sites and the D domain B- pockets of opposite molecules interact.

1.5.3.3 Lateral aggregation and the α C domains

The C terminal parts of the A α chains form two compact structures (α C domains) which strongly interact intramolecularly with each other and the central E domain. They are relatively mobile structures compared to the bulk of the molecule due to their connection to the molecule terminus through extended flexible polypeptide junctions. A conformational change occurs when fibrinogen is converted to fibrin; the α C domains dissociate from each other and the central E domain and are thus free to interact intermolecularly with other fibrin molecules. Their role in fibrin polymerisation is promotion of the ordered assembly of the complex fibrin, helping orientate the fibres with their binding partners. It also appears the interactions α C domains form are critical in lateral aggregation; clots formed from α C domain truncated fibrinogen (A α 251) are made up of densely packed thinner fibres, with more branch points and reduced permeability than WT fibrinogen. Truncation of the α C domains also leads to clots that are much less stiff, showing α C domains to be important in determining the viscoelastic and mechanical properties of fibrin and promoting clot stability. They also show less

resistance to lysis than clots made from WT fibrinogen implicating the α C domains in fibrinolysis susceptibility^{18, 19}.

1.5.4 Fibrinogen α chain

The mature 66 kDA fibrinogen α -chain is made up of 610 amino acids with a 16 residue signal peptide at the 5' end, so called fibrinopeptide A. An additional 15 amino acids at the carboxyl terminal of the polypeptide chain is predicted by the complementary deoxyribonucleic acid (cDNA) sequence which is not observed by protein sequencing of plasma fibrinogen, presumed to be due to removal during assembly, secretion or circulation by limited proteolysis²⁰.

1.5.5 Fibrinogen β chain

The entire 461 amino acid sequence of the human β chain of fibrinogen has been described. It is 52 kDA and has one carbohydrate chain. The percentage of cDNA in the liver represented by the β fibrinogen chain (2.6%) is significantly greater than found for the α or γ chains (1.3 and 1.7% respectively). This may be due to increased levels of β chain messenger RNA (mRNA) in liver or higher cloning efficiency of the β chain from existing mRNA. Of the 10 kilobases of DNA coding the β chain, only 20% of the DNA constitutes coding sequence, with eight exons and 7 introns, thought to separate the gene into regions in relation to the proteins structural domains. Exon 1 (aa 1-9) contains the signal peptide and part of FpB, the second (9-72) contains the thrombin cleavage and fibrin polymerisation sites while the third and fourth (73-133 and 134-204) contain the interdomainal connecting superhelical structures²¹.

1.5.6 Fibrinogen γ chain

The 46.5 kDA γ chain of fibrinogen consists of 411 amino acids with one carbohydrate side chain linked at Asn52. The carboxyl-terminal plays important roles in fibrin polymerisation and FXIII catalysed covalent cross-linking of various molecules²².

The main functional activities of each of the fibrinogen chains are summarised in Table 1-1.

Table 1-1. The main functional activities of the three fibrinogen chains

Chain	Main functional activities
Alpha	<ul style="list-style-type: none"> • Thrombin binding and fpA release; initiation of fibrin assembly^{15, 17} • Alignment for end-to-middle staggered fibril formation¹⁰ • Promotion of lateral fibril associations (αC domain)^{18, 19} • tPA and plasminogen binding (αC-domain - Aα148-160); enhanced plasminogen activation^{5, 8, 23, 24} • Platelet interactions (Aα95-98 & 572-575)^{8, 25} • FXIII crosslinking^{3, 26, 27} <ul style="list-style-type: none"> ○ α-α crosslinking ○ γ-α crosslinking ○ α2-AP crosslinking (αC-connector - Lys303) ○ PAI-2 crosslinking ○ Fibronectin crosslinking • α2-AP (αC-domain) and fibronectin binding²⁸
Beta	<ul style="list-style-type: none"> • Thrombin binding and FpB release; lateral growth of the fibre¹⁵⁻¹⁷ • Alignment for end-to-middle staggered fibril formation¹⁰ • Alignment for FXIII crosslinking – increasing clot strength^{3, 27} • Platelet interactions (fibrin Bβ15-42)^{8, 25}
Gamma	<ul style="list-style-type: none"> • Fibrin polymerisation and crosslinking¹⁰ • Alignment of crosslinking regions in fibrin¹⁰ • Binding and regulation of FXIII (Lys406)^{3, 8, 26, 29} <ul style="list-style-type: none"> ○ FXIIIB binding ○ γ-γ crosslinking ○ γ-α crosslinking • tPA binding and enhanced plasminogen activation (γ312-324)²⁴ • Platelet interactions (γ400-411)⁸

1.5.7 Mechanical properties of fibrin

The mechanical properties of a clot are very important in determining its ability to stop bleeding and to withstand the pressure of blood flow. Clots are capable of being deformed to a great extent and still show complete recovery, clearly demonstrating that fibrin is an elastic protein³⁰. Viscoelastic properties can determine the fate of the clot; to what extent blood flow will cause it to deform, reversibly or irreversibly, and whether it will rupture or embolise. This implicates these properties as important in clinical

outcome as tight, rigid clots are likely to be more friable and have a greater tendency to embolise³¹. It is accepted that it is the properties of the individual fibres making up the clot that dictate the mechanical properties of the structure. Three domains of the fibrin monomer have been proposed as areas of interest: the coiled coil connectors, the folded globular nodules and the α C regions.

Weisel and colleagues³² showed an initial linear increase in the force required to stretch a clot accompanied by fibre alignment, bundling and a decrease in clot volume. They proposed the expulsion of water from the clot following exposure of hydrophobic residues as a result of unfolding of the coiled-coil connectors. Work by others has demonstrated four abrupt changes in the fibrin molecule when external force is applied to a clot. They propose the first change to be the breakage of an unknown interaction close to the A-a bond, the next two events comprising unfolding of the D region. They suggest rupture of the A-a bond secondary to weakening by prior unfolding of the molecule accounts for the 4th event³³. Further studies have shown individual fibre extensibility to vary with the length of the α C region, showing a concomitant rise in extensibility with α C connector length implicating this region in playing a key role in fibre extensibility³⁴. It is likely that a mixture of all of these areas contribute to the mechanical and viscoelastic properties of the clot. Clots with a looser architecture and fewer branch points will have more room for deformation or compaction as their fibres will be more extensible thanks to fewer 'junctions' along their length. Changes in any of the contributing areas, especially the flexible α C region of fibrin could greatly affect the clot's mechanical strength.

Many variables can alter the mechanical properties of a forming clot. Clot stiffness has been shown to be greater in the presence of transglutaminase FXIIIa, which can also crosslink proteins into the clot affecting its mechanical structure and resistance to lysis. Fibrinogen gene mutations such as Fibrinogen Dusart (A α 554) can affect viscoelastic properties by interfering with lateral aggregation of fibrin monomers, leading to stiff clots made of thin fibres and many branch points³⁵.

1.6 FXIII

FXIII is able to form covalent γ - γ and γ - α crosslinks which reinforce inter-fibre contacts, increasing fibre elasticity and resistance of the clot to lysis through various mechanisms discussed below³.

1.6.1 Structure

Plasma FXIII is a tetrameric protein of 320 kDa made up of 2 A- and 2 B-subunits with each monomeric chain of 75 kDa and 88 kDa respectively. The globular A-subunits contain the active site of the protransglutimase while the inert B subunits

function as carriers in plasma. These subunits are bound by noncovalent bonds, though unbound B subunits are also found in plasma, and play a key role in preventing spontaneous activation of A-subunits and thus preserving molecule half life. In contrast, cellular FXIII is present only as A-subunit dimers³⁶.

1.6.2 Synthesis and secretion

FXIII B-subunits are synthesised in hepatocytes and secreted through the classical secretory pathway. The major source of FXIII A-subunits is cells from the bone marrow, with relatively insignificant contributions made by hepatocytes. Unlike the B-subunit, A-subunits are released as a result of cell destruction. The two subunits then combine upon contact in the plasma circulation²⁷ and circulate at 21.6 µg/ml (67.5 nM). Practically all FXIII in circulation is bound to fibrinogen (Kd $\sim 10^{-8}$ M) as fibrinogen is at a 20-100 fold excess, displaying an even higher affinity for fibrin. Platelets are also carriers of FXIII A-subunits in plasma; it is only upon activation and degranulation of these platelets that these A-subunits become free in the plasma, where they are able to associate with B-subunits already in circulation. This degranulation of platelets thus results in a FXIII 'blast'²⁷.

1.6.3 Activation

Plasma FXIII is activated by thrombin via cleavage of the Arg37-Gly38 peptide bond of the FXIII A-subunit. This separates a 37 residue activation peptide (4 kDa) from the FXIII A N-terminal³⁷ which remains in place occluding the FXIII active site, weakening the bond between the A- and B-subunits. Binding of 2 Ca²⁺ ions to the FXIII A-subunits leads to dissociation of the B-subunits from the partially activated A-subunits and exposure of the active site cysteine of the catalytic core of FXIII, releasing fully active FXIII (Figure 1-7). This activation is accelerated in the presence of increased Ca²⁺ and fibrin(ogen), an effect requiring the FXIII B-subunits for orientation of FXIII in the fibrin-FXIII matrix, this acceleration can be as much as 100-fold in the presence of polymerised fibrin. This is because thrombin cleaved FXIII becomes attached to fibrin polymers, on the surface of which the dissociation of the subunits takes place, after which the B-subunits detach from the fibrin surface. The cleavage and release of only one of the FXIII activation peptides is sufficient to expose the active thiol group of both cleaved and uncleaved A-subunits. In contrast cellular FXIII is activated via a slow non-proteolytic mechanism initiated by influx of Ca²⁺ into the cell²⁷.

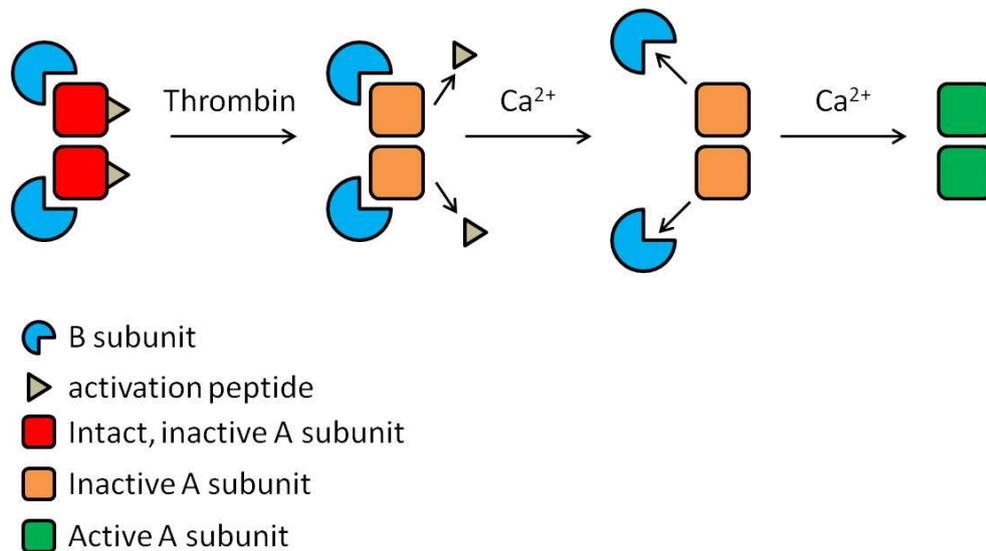


Figure 1-7. Activation of plasma FXIII by thrombin

Thrombin cleaves the Arg37-Gly38 peptide bond of the FXIII A-subunit separating an activation peptide. Binding of 2 Ca^{2+} ions to the FXIII A-subunits leads to dissociation of the B-subunits from the partially activated A-subunits and exposure of the active site cysteine of the catalytic core of FXIII, releasing fully active FXIII.

1.6.4 Crosslinking action

Once converted to an active transglutaminase, FXIIIa is able to stabilise fibrin clots by catalysing formation of E-(γ -glutamyl)lysine isopeptide bonds. Intermolecular γ - γ , γ - α and α - α bonds are formed between fibrin chains. Carboxyl terminal D domains of γ chains are cross-linked rapidly, forming γ - γ dimers resulting from bridges between Lys406 of one chain and Glu398/399 of another γ chain aligned anti-parallel⁸. The relatively slower process of α - α crosslinking results in oligomers and large α -polymers, forming bridges between glutamine residues at A α 328 and 366 and lysine residues at A α 508, 556 and 562²⁶. FXIIIa's enzyme activity relies on the transfer of an acyl group from the transglutaminase to an acyl-acceptor ϵ -amino group from a lysine residue on a molecule of fibrinogen, which then becomes linked to a γ -glutamyl residue on another fibrinogen molecule, releasing ammonia²⁷. This crosslinking leads to the formation of thick fibres with increased elasticity and rigidity that are more resistant to lysis in the plasma environment. It is thought to be the interchain crosslinks themselves conferring resistance to lysis, as well as inhibiting the binding of plasminogen and increasing inhibitor crosslinking.

1.6.5 Control of crosslinking

Since FXIII crosslinking confers resistance to fibrinolysis, it follows that uncontrolled crosslinking of fibrin fibres and of plasma proteins into a clot may result in

a fibrinolysis-resistant thrombus, which may have adverse effects in individuals with vascular disease. Inhibition of FXIII crosslinking activity within a thrombus has been demonstrated, though the mechanisms of inactivation are unclear. As no protein inhibitor has been identified for FXIIIa, it is likely inactivation is by way of proteolytic cleavage³⁸.

1.6.6 Other substrates of FXIII

FXIII is also capable of crosslinking other factors into the clot, such as α 2-AP, vWF, thrombospondin and fibronectin (Table 1-2) which can further protect the clot from proteolysis while enhancing mechanical strength, elasticity and viscosity^{8, 36}.

Table 1-2. Substrates of FXIII in the circulation

Protein	Substrate
Coagulation factors	Fibrin(ogen) α chain ⁸ Fibrin(ogen) γ chain ²⁶
Fibrinolytic proteins	α 2-AP ²⁸ Plasminogen ⁵ TAFI ³⁹ PAI-2 ⁴⁰
Adhesive/ matrix proteins	Thrombospondin ⁴¹ Vitronectin ⁴² Fibronectin ⁴³⁻⁴⁶ Osteopontin ⁴⁷ vWF ^{48, 49}
Cytoskeletal proteins	Actin ⁵⁰ Myosin ⁵⁰
Others	α 2-Macroglobin ⁴⁴

1.6.6.1 Anti-fibrinolytic proteins

α 2AP, the primary substrate for FXIII besides fibrin, is crosslinked to fibrin α -monomers and polymers (discussed in more detail in Section 1.9.4) and protects the fibrin network from lysis by inhibiting activity of plasmin. PAI-2, an inhibitor of uPA, can be crosslinked to fibrin(ogen) by FXIIIa where activity is retained and inhibition of fibrinolysis by uPA is achieved. This crosslinking may also serve to compartmentalise the PAI-2 in the clot⁴⁰. TAFI can be both polymerised and crosslinked to fibrin by FXIIIa, it is thought that crosslinking of TAFI may help activation of the enzyme,

stabilise its activity and prevent proteolytic degradation that would otherwise happen in the milieu³⁹. When activated to TAFIa, by thrombin and thrombomodulin, it works to remove C-terminal lysine residues that become exposed on fibrin upon plasmin degradation. This reduces the number of sites for tPA and plasminogen binding thus reducing plasminogen activation and suppressing fibrinolysis. High molecular weight multimers of plasminogen and fibronectin-plasminogen heteropolymers can also be formed by the crosslinking action of FXIIIa⁴³.

1.6.6.2 Adhesive proteins

Fibronectin, a glycoprotein present in connective tissue and basement membrane, is a substrate of FXIIIa, which is crosslinked to fibrin and collagen^{44, 45}. Fibronectin on the surface of, and within, fibroblasts can also be crosslinked into assembling extracellular matrices by FXIIIa⁴⁶. vWF in its monomeric form only can be crosslinked by FXIIIa to form polymers, whilst both monomeric and polymeric vWF can be crosslinked to the fibrin α -chain and collagen. Incorporation of amines into vWF is also catalysed by FXIIIa^{48, 49}. Vitronectin and osteopontin are both substrates for FXIIIa, promoting cell attachment and spreading, and mediating osteoclast attachment to bone respectively^{42, 47}. Thrombospondin, secreted during platelet activation, can act as a FXIIIa substrate wherein FXIII polymerises thrombospondin to homopolymers and heteropolymers with fibrin⁴¹.

1.6.6.3 Cytoskeletal proteins

Both platelet and skeletal muscle actin and myosin are substrates for FXIIIa, which catalyses the incorporation of amines into myosin to form myosin polymers⁵⁰.

1.6.7 Clinical relevance

1.6.7.1 Wound Healing

FXIII plays a major role in wound healing, since deficiency of the clotting factor leads to delayed wound healing, incomplete re-epithelisation, persisting necrotised fissure and abnormal scarring⁵¹, with substitution of the FXIII restoring wound healing to control. FXIII's effect on promotion of wound healing is multifactorial, affecting fibroblasts, macrophages, the extracellular matrix and angiogenesis. Migration and adherence of fibroblasts to the site of injury is enhanced by FXIIIa crosslinking of fibrin α chains⁵² and the incorporation of extracellular matrix protein (e.g. fibronectin) into the clot by FXIIIa⁵³. Similarly, FXIIIa enhances macrophage and monocyte migration to the site of injury and inhibits their apoptosis, providing an anchoring mechanism for the cells at the injury site⁵⁴. Cellular FXIII also contributes to tissue repair by enhancing

receptor mediated phagocytosis of monocytes and removal of cell debris and apoptotic cells⁵⁵.

1.6.7.2 Angiogenesis

FXIIIa has a pro-angiogenic effect, a product of the enhanced endothelial cell migration and proliferation, as well as decreased apoptosis in its presence, allowing new capillaries to form at the site of injury to reperfuse the tissue. This effect seems to work through down regulation of TSP-1 (Thrombospondin-1), a glycoprotein involved in the inhibition of angiogenesis by inhibiting endothelial cell migration and proliferation and promoting apoptosis⁵⁶.

1.6.7.3 Pregnancy

FXIII is essential for maintaining pregnancy, with studies showing FXIIIa deficient patients to suffer pregnancy loss in the first trimester⁵⁷ and replacement therapy of FXIIIa allowing these patients to come to full term⁵⁸. Fibrin(ogen) and fibronectin have been identified as important substrates of FXIII in this situation as they are prevalent in the Nitabuch's fibrinoid membrane, the layer of tissue the placenta detaches itself from at birth⁵⁹. It is thought FXIIIa crosslinks fibrin(ogen) and fibronectin, thus stabilising the membrane and conferring resistance to lysis. The pro-angiogenic effect of FXIII also plays a crucial role in normal placenta development.

1.6.7.4 Cardiac complications

Studies on FXIIIa deficient mice have shown FXIIIa to have a protective effect against cardiac rupture⁶⁰. Deficient mice die within 5 days of left coronary artery ligation induced myocardial infarction (MI) due to left ventricle wall rupture compared to WT mice or FXIIIa deficient mice supplemented with FXIIIa, in which there are no deaths. The inflammatory response at the site of injury is reduced in FXIIIa deficient mice and this is due to impaired migration of monocytes to the lesion, a process requiring active FXIII⁶¹. The actions of FXIIIa are summarised in Figure 1-8.

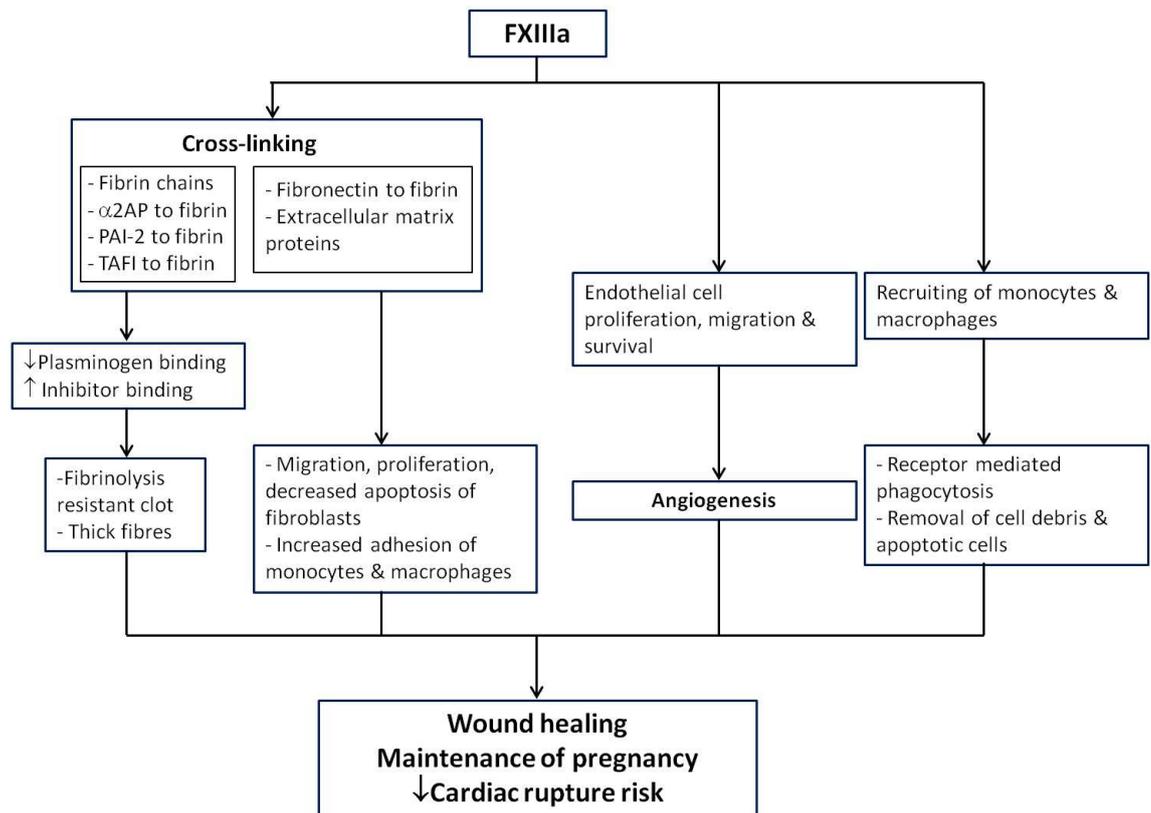


Figure 1-8. Mechanisms of action of activated FXIII

FXIIIa crosslinks various proteins to fibrin and forms interchain links between fibrin fibres thus producing clots resistant to lysis and causing changes in adhesion, migration, proliferation and apoptosis of fibroblasts, monocytes and macrophages. It also plays a role in angiogenesis and wound healing.

1.7 Platelets

In a healthy individual, platelets circulate in their inactive form at 150,000-450,000 per μL of blood, showing no binding to clotting proteins in the blood and a lifespan of 5-9 days. However at sites of vessel injury where collagen, vWF and TF are exposed and where there are increased levels of clotting factors, such as thrombin, platelets become activated. They can also become activated by high shear stress in conditions such as arterial stenosis²⁵. Once activated, platelets can bind many coagulation factors via a number of exposed receptors; GpIb-IX-V is able to bind vWF/VII complexes, thrombin and FXI while GpIIb-IIIa binds fibrinogen and prothrombin, in this way coagulation factors promote platelet stimulation and aggregation¹. Platelet activation, adherence and aggregation act to form a platelet plug using vWF and fibrinogen as a connecting agent. This platelet plug not only helps to stop the initial bleeding but helps wound repair by the secretion of growth factors (platelet derived growth factors (PDGF) and transforming growth factor beta (TGF β)) by the activated cells. These stimulate invasion of fibroblasts to the site of injury and

deposition of extracellular matrix. Upon clot lysis, platelets are cleared by phagocytosis².

GpIIb-IIIa is a calcium-dependent heterodimer of the integrin family of adhesion receptors which can bind fibronectin, vWF, vitronectin as well as fibrinogen when exposed on the surface of activated platelets. The receptor undergoes a conformational change following platelet activation involving receptor binding by thrombin or ADP triggering initiation of the phosphoinositide signal transduction pathway leading to exposure of multiple binding sites on the extracellular domains of the receptor⁸. The dissociation constant of the binding of fibrinogen to GpIIb-IIIa on stimulated platelets is around 100 nM, approximately 100-fold lower than the circulating fibrinogen concentration suggesting immediate fibrinogen binding upon platelet activation in plasma. It is thus evident that tight regulation of GpIIb-IIIa-fibrinogen binding is essential to prevent spontaneous and deleterious platelet aggregation. Three areas of the fibrinogen molecule have been suggested to bind to the GpIIb-IIIa receptor; the carboxyl-termini of the γ -chains, two regions of the α chain that contain an RGD motif (Arg-Gly-Asp-X) and sequences located between residue 15-42 of the β -chain. It is thus clear that platelets play a major role in blood clot formation, cessation of bleeding and wound repair, with low levels of platelets or their activation causing excessive bleeding, while over-activation and excessive platelet count leading to a thrombotic environment.

1.8 Fibrinolysis

It is essential that once the blood clot is no longer needed, it is removed safely from the circulation as clots that remain can embolise, causing stroke and other organ ischaemia. This is achieved by fibrinolysis, progressive cleavage of fibrin polymers that can be cleared safely. Fibrinolysis is initiated by the release of tissue plasminogen activator (tPA) from endothelial cells upon the conversion of fibrinogen to fibrin and the consequent generation of plasmin from plasminogen. Plasmin causes fibrin degradation via cleavage at multiple sites in the fibrin molecule resulting in the formation of fibrin degradation products. This process is mainly inhibited by α 2-AP and PAI-1, which bind free plasmin and inhibit plasminogen activation respectively²³. The mechanisms of fibrinolysis are summarised in Figure 1-9.

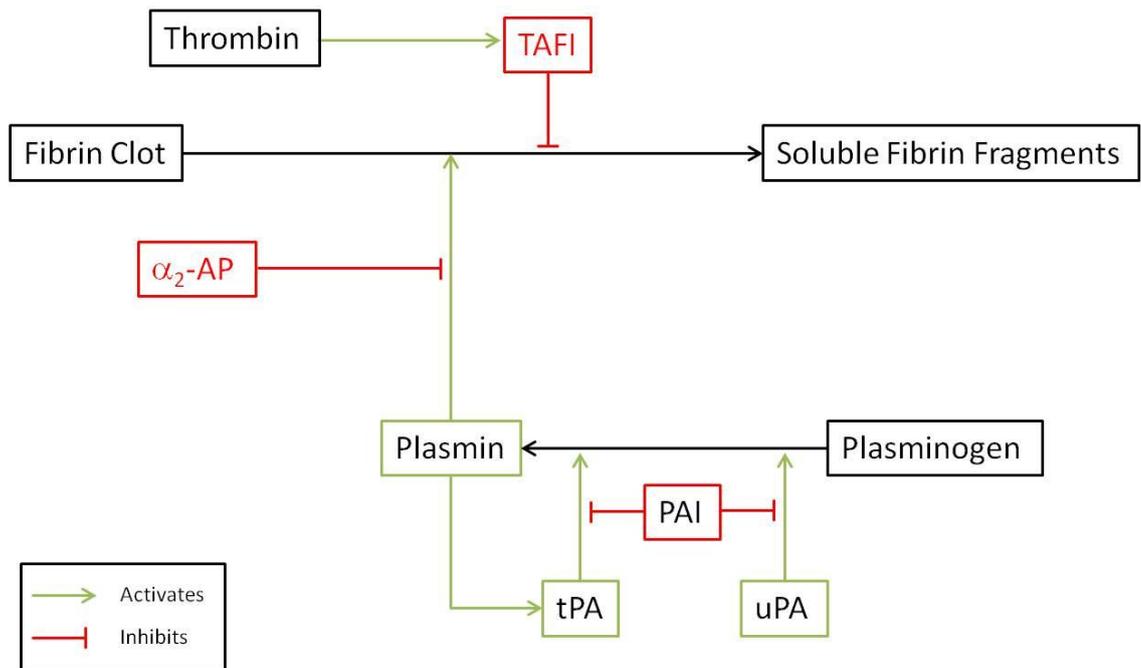


Figure 1-9. The fibrinolytic system

The fibrin clot is dissolved by plasmin which is converted from plasminogen by tPA and uPA. Various feedback mechanisms exist to control this process, including inhibitory proteins such as α_2 -AP which directly inhibits plasmin action on fibrin.

1.8.1 Plasminogen

1.8.1.1 Structure

Plasminogen is a 90 kDA single chain glycoprotein made up of 791 amino acid residues and belongs to the serine peptidase family of proteins. During secretion a 19-residue signal peptide is cleaved from a pre-cursive 810 amino acid protein to release mature Glu-plasminogen, the pro-enzyme of plasmin. Plasminogen has a multidomain structure composed of an N-terminal peptide (NTP), 5 kringle (triple loop structures stabilised by disulfide bridges) and 3 serine protease domains composing the catalytic triad His603, Asp646, Ser741. In circulation, plasminogen binds histidine-rich glycoprotein which serves as a high-affinity receptor to immobilise plasminogen to cell surfaces at sites of tissue injury⁵. Plasminogen circulates in two N-terminal variant forms, Glu- and Lys-plasminogen. Lys-plasminogen is formed by the release of a 76 residue preactivation peptide from Glu-plasminogen by plasmin cleavage of the Lys76-Lys77 bond of Glu-plasminogen. Lys-plasminogen is more readily activated by plasminogen activators and has a higher binding affinity to fibrin⁶². The increased fibrin binding affinity of Lys-plasminogen can be explained by the conformational change that Glu-plasminogen undergoes when it is proteolytically modified to Lys-plasminogen. While Glu-plasminogen has a compact spiral conformation, Lys-plasminogen and

plasmin exhibit an open, elongated structure⁵. This suggests the cleavage of the NH-terminal peptide of Glu-plasminogen elicits a conformational change that exposes the high affinity fibrin binding site present in Lys-plasminogen⁶².

1.8.1.2 Synthesis and Secretion

Human plasminogen is synthesised in the liver and circulates in plasma at a relatively high concentration of around 2 μ M.

1.8.1.3 Activation

Cleavage of the Arg561-Val562 peptide bond of plasminogen by tPA (and uPA), and the release of a 77 residue activation-NTP lead to plasminogen conversion to active plasmin. Consequent autocatalytic cleavage within the plasmin molecule of the 78-79 residue peptide bond results in a two-chain disulfide linked molecule. A heavy chain (from residue 79-561, 483aa) contains all kringles (K1-5), while a light chain (from residue 562-791) contains the catalytic triad. Each kringle except K3 contain lysine binding sites (LBS), which mediate binding to fibrin(ogen) and α 2-AP^{5, 63}.

1.8.1.4 Action

Plasminogen binds intact fibrin throughout the clot upon clot formation and it is this plasminogen that is responsible for initiation of lysis. Plasmin cleaves polypeptide chains at lysine residues so new carboxyterminal lysine residues are revealed in the clot, producing further binding sites for plasminogen on fibrin. Fibrinolysis is greatly accelerated by both the consequent increase in local plasminogen concentration and increased plasminogen reactivity due to plasminogen binding to these sites. This increased reactivity is due to altered conformation leading to increased sensitivity to activation⁶⁴. Two binding sites with different affinities for fibrin exist in plasminogen, both located on fibrin fragments D and E. The low affinity site is capable of activating 5 times more plasminogen upon fibrin binding than the high affinity⁸.

Plasmin specifically hydrolyses arginyl and lysyl bonds in the coiled-coils of fibrin. Initial cleavage breaks the Lys583-Met584 then the Lys206-Met207 and Lys230-Ala231 bonds of the A α chain, releasing a 4 kDA C terminal fragment and fragment X (260 kDA). Cleavage of fragment X across all three chains releases singular fragments Y (160 kDA) and D (100 kDa) and the consequent cleavage of this fragment Y releases a second D fragment and one fragment E (60 kDA). This allows the transformation of insoluble fibrin polymers into soluble fragments and ultimate dissolution of the clot⁵, summarised in Figure 1-10.

This progressive fragmentation leads to increased fibre diameter, bending of the fibres and splaying of the ends of the fibre before release of each fragment. It is now known this occurs by transverse cutting across the fibre rather than the previous theory of progressive cleavage uniformly around the fibre²³.

Plasmin has a broad specificity *in-vivo*, acting as a proteolytic factor not only in fibrinolysis but also extracellular matrix degradation, angiogenesis, wound healing and pathogen cell invasion. It can also inactivate and degrade other coagulation factors such as FVa, vWF and thrombospondin⁵.

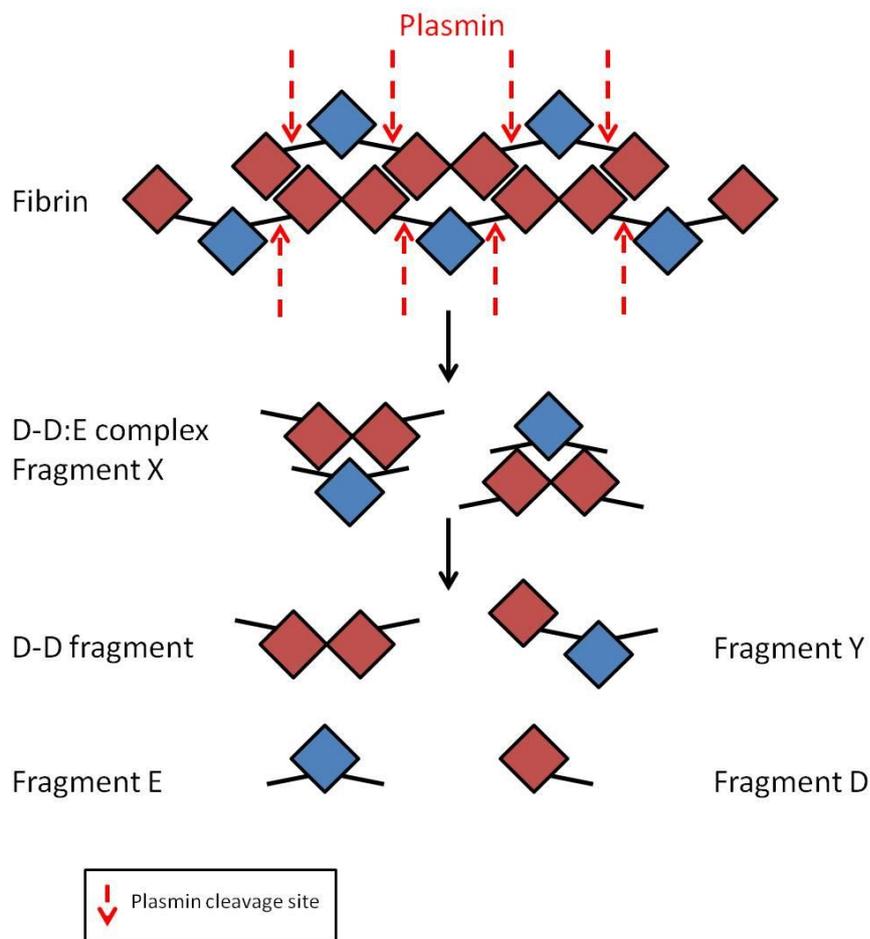


Figure 1-10. Dissolution of the fibrin clot by plasmin

Plasmin cleaves the fibrin fibre to produce soluble fibrin fragments; firstly D-D:E complexes, then smaller D-D, E, Y and D fragments.

1.8.1.5 Plasminogen activators

Plasminogen is converted into its active form, plasmin, by tPA in plasma, while uPA works pericellularly to produce plasmin for intercellular processes. As mentioned above, they release active plasmin by cleaving at Arg561-Val562 of plasminogen to remove a 77 residue activation-NTP.

1.8.1.5.1 tPA

tPA is a serine protease synthesised mainly in endothelial cells which circulates in plasma at 5-10 µg/ml (71-142 nM) as a single chain glycoprotein of 527 amino acid residues (70 kDa). This proenzyme form is converted to a two-chain completely active form by plasmin itself as well as FXa by cleavage of the Arg275-Ile276 bond and formation of an interchain disulfide bridge at Cys264-Cys395. The multidomain structure comprises one fibronectin type, one endothelial growth factor (EGF)-like and two kringle domains in one chain and the serine protease domain in the other.

Binding of tPA to fibrin enhances the activation of plasminogen by tPA and consequently exposes C-terminal lysine and arginine residues by proteolysis by plasmin, these are capable of binding the LBS in K2 of tPA, increasing its local concentration and accelerating lysis⁵. Fibrin thus works to co-localise tPA and plasminogen on the its surface, working as both a template and a substrate for the two²⁴.

1.8.1.5.2 uPA

uPA is a serine protease synthesised in the lung, kidney and endothelial cells which circulates in plasma at 5-10 µg/ml (91-182 nM) as a single chain glycoprotein of 411 amino acids (55 kDa). Similarly to tPA, it is converted to its active two-chain form after cleavage of the Lys158-Ile159 bond by plasmin and FXIIa and formation of a interchain disulfide bridge. Its multidomain structure consists of one EGF-like and one kringle domain in one chain and a serine protease domain in the other chain⁵.

1.9 Inhibition of fibrinolysis

Though the lysis of clots is essential to prevent embolism and organ ischaemia and to facilitate wound healing, it is important that the clot remains at the site of injury long enough to prevent continued bleeding. There is thus tight control over promotion and inhibition of lysis in the circulation. A number of different proteins (e.g. α2-AP, (PAI)-1 and 2 and TAFI) inhibit lysis of the fibrin clot, contributing to the intricate balance of the coagulation system⁵.

1.9.1 PAI-1

Human PAI-1 is a serpin secreted from endothelial, smooth muscle, liver and fat cells which circulates at ~2-20 µg/L (40-400 nM) in plasma. It is a 50 kDa, 379 amino acid, single chain glycoprotein. Both uPA and tPA are inhibited by PAI-1, thereby reducing plasmin generation from plasminogen and modifying fibrinolysis rates. PAI-1

also inhibits thrombin, plasmin, trypsin and active protein C but at a much slower rate than it does tPA and uPA⁵.

1.9.2 PAI-2

PAI-2, also a serpin, is only detectable in plasma during early pregnancy or in pathological conditions when it circulates at around 5 µg/ml (83 nM). It is secreted from epithelial cells, monocytes, macrophages and keratinocytes as a mature single chain 60 kDA, 415 amino acid glycoprotein. FXIIIa can catalyse the formation of isopeptide bonds between Gln83, 84 and 86 of PAI-2 and lysine residues in the fibrinogen A α -chain where it is an inhibitor of tPA and uPA, playing a role in maintenance of the placenta and embryonic development⁵.

1.9.3 TAFI

TAFI is a 401 amino acid, 60 kDA glycoprotein which circulates in plasma at ~75 nM. Once activated by thrombin, TAFI inhibits fibrinolysis by its carboxypeptidase B activity; removing C-terminal lysine residues from degrading fibrin thus reducing the available tPA and plasminogen binding sites. FXIIIa is able to crosslink TAFI to fibrin which facilitates its activation, stabilises its enzymatic activity and stops degradation of the active enzyme as the carboxypeptidase activity is unstable when in serum ($t_{1/2}$ ~ 10 min)^{64, 65}.

1.9.4 Antiplasmin

1.9.4.1 Structure

α 2-AP, a 67 kDA single chain glycoprotein of the serpin family, is the main inhibitor of plasmin in human blood. In plasma, Met- α 2-AP, a 464 residue protein with a methionine residue at the N-terminus is cleaved to produce a 452 residue protein with an asparagine N-terminus, named Asn- α 2-AP. The 97 kDA proteinase responsible for this cleavage is antiplasmin-cleaving enzyme (APCE) which cleaves the Pro12-Asn13 bond of Met- α 2-AP⁶⁶. Met- and Asn- α 2-AP circulate at a ratio of 30:70 respectively in the plasma⁶⁷.

1.9.4.2 Synthesis and secretion

α 2-AP is synthesised in the liver and circulates in plasma at concentrations around 1 µM.

1.9.4.3 Action

α 2AP is able to directly inhibit free plasmin in the circulation as well as fibrin bound plasmin, regulating fibrinolysis, however α 2AP incorporation into the clot by first noncovalent binding and then covalent FXIII-mediated crosslinking results in increased clot resistance to lysis.

1.9.4.4 Binding

Tsurupa et al. (2010)²⁸ showed α 2-AP to bind non-covalently only to polymeric fibrin D regions and α C domains, or fibrin adsorbed to a membrane, displaying no binding to fibrinogen in solution or monomeric fibrin. This suggests that α 2-AP binding sites that are cryptic in fibrinogen become exposed upon fibrin polymerisation (or surface adsorption). The dissociation constant (Kd) of the noncovalent α 2-AP-polymeric fibrin interaction is 45-68 nM, far below the physiological α 2-AP concentration, demonstrating the high affinity of the two molecules *in vivo*, suggesting binding of α 2-AP can regulate fibrinolysis. Using surface plasmon resonance (SPR), this binding occurs Lys-independently to the D region (D1 and D-D fragments) and the α C domain (A α 221-610 fragment), which are cryptic in fibrinogen and become exposed in fibrin. This noncovalent binding may serve to orientate the α 2-AP molecule on fibrinogen, enhance crosslinking and inhibit plasmin in the early stages of fibrinolysis, before covalent crosslinking by FXIII takes place. The affinity of α 2-AP binding to D-D fragments is greatly increased in the presence of plasminogen, while the affinity to other binding fragments is unaffected by its presence. This may be due to plasminogen-fibrin binding induced conformational changes in the fibrin D-region exposing α 2-AP binding sites or the ability of α 2-AP to bind to plasminogen, thus facilitating simultaneous binding of α 2-AP to fibrin and fibrin-bound plasminogen²⁸.

1.9.4.5 Crosslinking

The binding sites of the serpin to fibrin (in the α C domain and D regions) are separate to the FXIIIa crosslinking site which lies in the α C connector. FXIIIa crosslinks the 42 amino acid N-terminal of α 2-AP to fibrin forming covalent bonds between Gln2 (of Asn- α 2-AP) or Gln14 (of Met- α 2-AP) and Lys303 in the α C connector of the A α -chain of fibrinogen²⁸. Crosslinking of α 2-AP into the fibrin clot causes inactivation of plasmin and thus inhibition of fibrinolysis due to formation of an inactive 1:1 stoichiometric complex with plasmin⁶⁷. Asn- α 2-AP is crosslinked to fibrin ~13 times faster than Met- α 2-AP and consequently Asn- α 2-AP exhibits greater inhibition of plasmin induced fibrinolysis⁶⁶. This is thought to be due to the extra N-terminal 12 residue peptide of Met- α 2-AP limiting accessibility of FXIIIa to the Gln14 crosslinking

site⁶⁸. Though $\alpha 2$ AP does not show significant binding to fibrinogen in solution, it has been shown that the two can be crosslinked by FXIII, but due to the lack of preceding binding, this process is much less efficient than the crosslinking to fibrin²⁸. The binding and crosslinking sites of $\alpha 2$ -AP to fibrin are depicted in Figure 1-11.

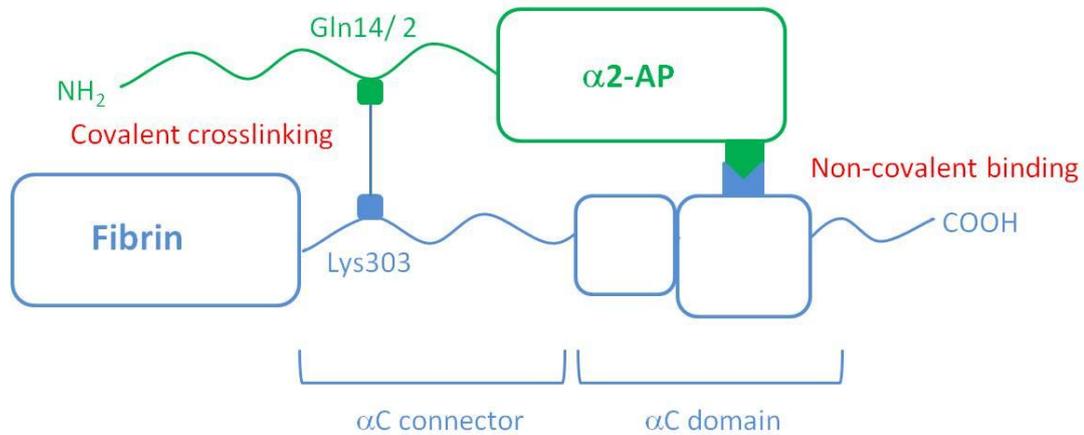


Figure 1-11. Sites of non-covalent binding and covalent crosslinking by FXIIIa of $\alpha 2$ -AP to fibrin

$\alpha 2$ -AP binds non-covalently and Lys-independently to fibrin D regions (D1 and D-D fragments) and αC domains (A α 221-610 fragment). FXIIIa crosslinks the N-terminal of $\alpha 2$ -AP to fibrin between Gln2 (of Asn- $\alpha 2$ -AP) or Gln14 (of Met- $\alpha 2$ -AP) and Lys303 in the αC connector of the A α -chain of fibrinogen.

1.9.4.6 Inhibition of lysis

Clot lysis is slowed in direct proportion to the circulating level of $\alpha 2$ -AP and homozygous deficiency of the $\alpha 2$ -AP gene is associated with a bleeding tendency, emphasising its physiological relevance in haemostasis⁶⁶. The inhibition of plasmin by $\alpha 2$ -AP mimics that of all serpins, with the C-terminal domain of $\alpha 2$ -AP and kringle structures of plasmin interacting so that the Arg364 of $\alpha 2$ -AP and the active serine in plasmin covalently bond resulting in the cleavage of the reactive site peptide bond in $\alpha 2$ -AP (Arg376-Met377) and formation of an inactive $\alpha 2$ -AP-plasmin complex^{67, 69}. The C-terminal extension of $\alpha 2$ -AP from Asn410 to Lys464 consists of 55 amino acids, 5 of which are conserved lysine residues (418, 427, 434, 441, 448 and 464). These are instrumental in the interaction of $\alpha 2$ -AP and plasmin kringle domains (K1 and K4) enhancing the $\alpha 2$ -AP-plasmin interaction 30-60 fold⁷⁰. Lys464 and Lys448 of $\alpha 2$ -AP have been identified as the most important in initiating binding and increasing the rate of interaction with plasmin kringle domains, although all lysine residues contribute to this process. This has been shown by the progressive loss of plasmin association, binding and inhibition by $\alpha 2$ -AP with consecutive mutation of the conserved lysine residues, and the same level of binding and inhibition as C-terminally truncated $\alpha 2$ -AP

when all lysine residues are mutated. Though these lysine residues are clearly of great importance, even C-terminally truncated α 2-AP can associate with and bind to plasmin. Exosites in the α 2-AP core, away from the reactive scissile bond, and the active site of plasmin interact, contributing to target protein recognition and specificity⁷¹.

Alongside the fibrin-binding and the active site of α 2-AP, it also has a site capable of binding plasminogen, thereby preventing its binding to fibrin, providing another mechanism by which α 2-AP affects clot lysis⁶⁷. It is interesting to note that tPA and plasminogen bind to the fibrin D and α C regions without disturbing α 2-AP binding, possibly due to their different nature of Lys-dependent binding. The ability of fibrin to simultaneously accommodate all 3 proteins in the same vicinity allows the fine control of fibrinolysis through intricate and closely controlled crosstalk between the proteases and serpin²⁸.

1.10 Factors influencing fibrin structure

Fibrin clot structure can be influenced by both quantitative and qualitative changes in fibrinogen and these can be due to both genetic and environmental influences. These changes have marked effects on clot permeability and elasticity, lysis rates and platelet binding and thus risk of clotting complications, it is therefore important to understand factors that affect clot structure.

1.10.1 Quantitative changes in clotting factors

Circulating levels of plasma fibrinogen are influenced by genetic and environmental factors with evidence suggesting up to 50% of variability is determined by genes. High concentrations of fibrinogen are associated with dense and tight clots³; they can also affect blood viscosity and platelet aggregability.

1.10.1.1 Genetic Interactions

Twin studies have revealed that levels of coagulation factors are influenced by an individual's genetic background; one study showed that genes account for 51% of the variance in plasma levels of fibrinogen⁷². Several single nucleotide polymorphisms (SNPs) in the genes encoding all 3 fibrinogen chains are associated with variation in plasma fibrinogen levels. One example of which is the Gly445Ala SNP in the promoter region of the fibrinogen β gene which causes increased fibrinogen levels; this genetic variant has distinct nuclear protein binding properties affecting the expression of the β chain and therefore synthesis of intact fibrinogen. This may be due to the presence of (IL)-6 responsive sequences in the promotor regions of this gene resulting in genetically determined sensitivity of fibrinogen expression as (IL)-6 is the main

mediator of acute phase fibrinogen synthesis⁷³. These changes in fibrinogen concentration affect clot structure as discussed above.

1.10.1.2 Environmental interactions

Hypertension, diabetes, hyperlipidemia, obesity, sedentary life style, alcohol consumption and smoking all lead to increased levels of many clotting factors such as TF, fibrinogen, tPA, PAI-1 and FXIII leading to a more thrombotic environment¹. This can be due to endothelial dysfunction, increased inflammatory markers and/or hyperglycaemia itself.

1.10.2 Qualitative changes in clotting factors

Not only can levels of proteins in the circulation change, but the form in which they circulate can be modified by various mechanisms, discussed below.

1.10.2.1 Genetic interactions

Genetic variations in clotting factors can induce qualitative changes in the protein, without affecting plasma levels, consequently altering clot structure. For example, the substitution of a threonine for an alanine at position 312 in the coding region of the fibrinogen α -chain, results in increased stiffness of clots and decreased fibrin density⁷⁴. FXIII crosslinking and lateral aggregation are affected due to the polymorphism's position in the α C domain resulting in the formation of thick fibrin fibres while levels of plasma fibrinogen are not affected⁷⁵. A substitution of valine with leucine at position 34 in FXIII can also affect clot structure. Possession of the FXIIIVal34Leu mutation has been shown to increase FXIII activation by thrombin, causing earlier cross-linking, and reduction in lateral aggregation and thus clots with thin fibres³⁶.

1.10.2.2 Environmental interactions

Environmental changes affecting the blood such as increased glucose or oxidative stress can cause changes in fibrinogen after it is secreted.

1.10.2.2.1 Posttranslational modifications of fibrinogen

After translation of each fibrinogen chain in the hepatocytes, the molecule can be modified by various post-translational modifications (PTMs). These can change the functional properties of the protein by covalent addition of functional groups to amino acid side chains.

1.10.2.2.1.1 Glycation

One of the most common PTMs of fibrinogen is the non-enzymatic glycation by reducing sugars such as glucose, forming fructosamines and advanced glycation endproducts (AGEs). The initial reaction is between an amino group in the protein and the aldehyde group of glucose forming amadori products of the N-substituted (1-deoxyketos-2-yl)amine arrangement, AGEs are only formed after consequent oxidation and dehydration⁷⁶. Glycation adducts can also be formed by the proteins reaction with α -oxoaldehydes such as glyoxal and methylglyoxal. Glycation by α -oxoaldehydes takes place on arginine residues whilst that by glucose is directed on lysine residues^{77, 78}.

Clots made from glycated fibrinogen result in a tight and rigid network, resistant to lysis, this may be because certain areas of the fibrinogen molecule corresponding to plasminic cleavage sites and crosslinking sites are preferentially glycated, thus impairing crosslinking and plasminic digestion. It has also been shown that glycoaldehyde, generated during inflammation and hyperglycaemia can react with arginine and lysine residues to impair protein function delaying plasma coagulation and fibrin polymerisation, with clots made of thinner fibres⁷⁹.

Other circulating factors are also susceptible to glycation; recently, plasminogen has been shown to be more extensively glycated in diabetes. This causes impaired fibrinolysis through decreased plasmin generation and impaired functional protein activity as a result of decreased plasmin generation and reduced enzyme catalytic activity upon conversion from plasminogen in diabetes. These changes are reversible with moderate improvement of glycemic control, directly implicating circulating glucose levels in this PTM in diabetes⁸⁰.

1.10.2.2.1.2 Oxidation

Reactive oxygen species (ROS) and AGEs, levels of which are increased in oxidative stress, inflammation and diabetes can lead to oxidation of circulating proteins⁸¹. Oxidation of fibrinogen, usually on lysine residues, results in the protein being more readily transformed to fibrin, enhanced platelet aggregation and decreased plasminogen activation by tPA thus creating a prothrombotic environment. The oxidised form of fibrinogen also modifies blood viscosity and reduces erythrocyte deformability. Acetylation of fibrinogen can inhibit this oxidation and thus prevent the reduction in catalytic efficiency induced^{82, 83}.

1.10.2.2.1.3 Acetylation

It has been shown that aspirin is capable of acetylating lysine residues on the α -chain of fibrinogen. Work in our laboratory has shown that fibrinogen can be acetylated by aspirin, resulting in clots composed of thick fibres and large pores with reduced

rigidity which are less resistant to lysis. This may be because the acetylation leads to changes in charge distribution and fibrin conformation⁸⁴. A recent study has shown that Lys539, 429, 224 and 208 are some of the acetylation sites on fibrinogen, interestingly these are all residues involved in FXIIIa crosslinking thus implicating acetylation of these residues in FXIII crosslinking and final fibrin structure⁸⁵.

1.10.2.2.1.4 Phosphorylation

Fibrinogen circulates in its phosphorylated form in plasma, modified in the A α -chain at Ser3 and Ser345. It is of particular interest that the phosphorylated Ser3 lies within fpA and it has been shown the degree of modification of this residue, increased in acute phase reaction conditions, modulates fpA release by enhancing thrombin binding. A concomitant increase in resistance to plasmin-induced fibrinolysis is also evident with the degree of protein phosphorylation⁸⁶.

1.10.2.2.1.5 Homocysteinylation

Sulfhydryl groups can be introduced into fibrinogen on lysine residues by homocysteine thiolactone, a metabolite of homocysteine, this leads to fibrin that is not able to support plasminogen activation by tPA and thus clots that are resistant to lysis. Since fibrinolytic proteins bind at lysine residues as well as them being the sites of plasmin cleavage it is not surprising modification of these residues would have this effect⁸⁷.

1.10.2.2.1.6 Nitration

Fibrinogen can be nitrated in vivo on tyrosine residues 292 and 422 of the β chain leading to accelerated clot formation, altered clot morphology, increased FXIII crosslinking, fibrin stiffness, increased permeability and resistance to lysis⁸⁸.

1.10.3 Other factors affecting clot structure

Several other factors influence the formation, structure and lysis of a fibrin clot, including pH, ionic strength, and concentrations of calcium, dextran or anticoagulants. Clots made of thicker fibrin fibres are seen in the presence of high plasma calcium levels when compared with those formed in the absence of calcium due to its requirement for the assembly of procoagulant complexes and thrombin generation. Antithrombin prolongs time to clot formation by effectively lowering plasma thrombin concentration, resulting in thicker fibred clots. In contrast, plasma proteins such as albumin and haemoglobin shorten the onset of fibrin clot formation³. Dietary factors such as increased fibre levels result in clots which are more permeable and less rigid due to decreased cholesterol and lipoprotein levels and decreased rate of fibrinogen

conversion⁸⁹, while increased incorporation of inflammatory molecules, due to high plasma protein levels, such as C3 results in clots with thin fibres and impaired lysis⁹⁰.

1.10.4 Effect of fibrin structure on fibrinolysis potential

Dense clots made of thin fibres are more resistant to lysis in the plasma environment than those made of loosely woven thick fibrin fibres²³. However at the level of individual fibrin fibres, thick fibres are more resistant to lysis. This phenomenon is explained by the difference in distribution and movement of fibrinolytic components through the fibrin network showing fibrin network architecture to govern clot lysis rather than fibre diameter. Loosely woven clots made of thick fibres have a decreased fibrin density and number of fibres for the same amount of total protein. This allows fibrinolysis components such as tPA to bind more broadly across the clot and move quicker through the fibrin network. It has also been suggested plasminogen binding and activation may be inhibited in clots made of fine fibres^{23, 84}. This indicates not only rate of plasmin generation but its movement through the clot is rate limiting for fibrinolytic activity and that it is not fibrin fibre diameter, but fibrin network structure, that governs the distribution of fibrinolytic proteins²³. Both quantitative and qualitative changes in clotting factors can determine fibrin clot structure and susceptibility to fibrinolysis as discussed above.

1.11 Cardiovascular disease

Cardiovascular disease is an important cause of morbidity worldwide and results in approximately one third of global mortality, with around 200,000 deaths per year in the UK alone (www.heartstats.org). The formation of an obstructive thrombus in a blood vessel is the last step in the atherothrombotic process, leading to organ ischaemia, which if prolonged results in irreversible tissue damage manifesting clinically as MI or stroke. Atherosclerosis often progresses from endothelial dysfunction leading to permeation of the vascular wall with lipid particles and inflammatory molecules allowing the formation of foam cells, which are cholesterol laden macrophages that form fatty streaks, a pathology that can be found at a very early age. The inflammation caused by this reaction leads to increased levels of cytokines and chemo-attractant proteins, exacerbating the situation. Mature atherosclerotic plaques are formed from chronic inflammation products and fibrous tissue deposition into the fatty streaks. When mature and unstable plaques rupture, exposing a prothrombotic core, the lumen of the vessel can become occluded due to thrombus formation secondary to platelet and coagulation factor activation⁸⁴.

1.11.1 Clot structure and susceptibility to cardiovascular disease

The structure of the clot governs susceptibility to atherothrombotic complications. Ex vivo fibrin networks with thin fibres, small pores and dense structure are associated with premature and more severe atherothrombotic disease^{91, 92}.

1.11.1.1 Effect of quantitative changes in clotting factors on CVD risk

Increases in many clotting factors have been associated with various CVD; plasma concentrations of fibrinogen, vWF, PAI-1 and lipoprotein were found in coronary artery disease (CAD) patients, with fibrin stiffness and fibrin architecture being predictive of CAD⁹². As discussed above, high concentrations of fibrinogen are associated with dense, tight clots made of thin fibrin fibres; this confers increased risk of cardiac disease. The Northwick Park Heart study showed an 84% increase in MI development with an increase of one SD in fibrinogen levels (equivalent to 0.6 g/L). On top of the risk imparted by the development of clots with a tight network structure, the accumulation of fibrinogen directly integrated into atherosclerotic lesions is proportional to plasma fibrinogen concentration. The problem is exacerbated by the fact atherosclerosis can provoke acute phase fibrinogen synthesis before clinical incidents are detected through activation of the coagulation cascade and resultant increased local production of fibrinogen degradation products such as fragment D. These degradation products can facilitate the release of IL-6 from macrophages, increasing not only the hepatic synthesis of fibrinogen, but other acute phase proteins⁹³. The presence of fragment D in arterial lesions further exacerbates CVD risk through stimulation of smooth muscle cell proliferation and its chemotactic influences on monocytes⁹⁴.

1.11.1.1.1 Genetic interactions

The role of genetic variation in the fibrinogen gene in predisposition to CVD has not been elucidated despite some of the polymorphisms effect on protein levels appearing to predispose to cardiovascular events⁹².

1.11.1.1.2 Environmental interactions

Regular exercise and a diet high in vegetables, fish and dairy and low in red meats correlate with low plasma fibrinogen levels and reduction in CAD risk. Conversely smoking leads to increased fibrinogen levels and concomitant risk in CAD risk secondary to the inflammatory reaction in the lungs in response to smoke inhalation.

1.11.1.2 Effect of qualitative changes in clotting factors on CVD risk

1.11.1.2.1 Genetic interactions

Vascular risk is influenced by genetic factors, with twin studies revealing increased concordance of death from coronary heart disease (CHD) in monozygotic twins. Other studies show altered fibrin clot structure in relatives of patients with CAD, implicating genetic factors affecting fibrin in determining predisposition to CVD. For example, the fibrinogen A α Thr312Ala polymorphism discussed above is associated with increased post-stroke mortality⁹⁵, venous thrombosis, pulmonary embolism and chronic thromboembolic pulmonary hypertension. This may be due to the increased stiffness of the clots formed from this variant and consequent risk of fragmentation and embolisation⁹⁶. Though *in vitro* work has shown the FXIIIVal34Leu polymorphism causes clots to form with thin fibres, a typically prothrombotic phenotype, possession of the FXIII34Leu allele is associated with decreased risk of atherothrombotic disease. A clear gene-environment interaction can be seen in that with increased insulin resistance, the protective effect of the polymorphism is lost.

1.11.1.2.2 Environmental interactions

Environmental risk factors such as diabetes, hyperlipidaemia and life style factors such as smoking also influence atherothrombotic risk. PTMs change the functional properties of the protein as discussed above, altering clot structure, in this way they too can modify CVD risk. Hyperglycemia as a result of diabetes causes increased glycation of fibrinogen, consequently resulting in the formation of a compact clot with reduced susceptibility to lysis⁹⁷, potentiating the development of atherosclerosis and related complications. AGE residues can be used as risk markers and factors of disease development, with increased plasma concentrations found at sites of vascular complications *in vitro*, and increased levels of the residues implicated in vascular complication in diabetes, uraemia and aging *in vivo*. Levels of the adducts of fibrinogen homocysteinylation are associated with risk of stroke and coronary heart disease⁸⁷ and increased levels of nitrated fibrinogen are associated with CAD and atherosclerotic lesions^{88, 98}.

1.11.2 Effect of fibrinolysis potential on CVD risk

The increased risk of disease associated with dense clots made of thin fibres is partially due to more difficult lysis of clots composed of such structure²³, secondary to slower rate of tPA induced plasmin generation in the presence of thin compared with thick fibres and increased risk of embolisation. Fibrin stiffness and fibrin architecture

are also predictive of CAD, partly due to their effect on fibrinolysis⁹². A significant predictive value of inactive tPA/PAI complexes, indicating elevated PAI activity and impaired fibrinolysis, has been shown for MI, ischemic heart disease (IHD) and stroke. tPA levels have also been identified as a risk factor due to the protein's mitogenic effect on smooth muscle cells and its ability to liberate active plasmin from plasminogen which is able to activate proteases which can weaken atherosclerotic plaques and cause them to rupture⁹⁴.

1.12 Fibrinogen variants

Fibrinogen variants provide a useful tool to investigate the effect of small changes in the fibrinogen gene on clot structure and susceptibility to disease. They allow us to see how a change in a specific part of the protein can affect its function, shedding light on the native function of that area. The more common changes are termed 'polymorphisms' while those that are extremely rare are called 'dysfibrinogenemias'. Some of these variants are discussed below.

1.12.1 Fibrinogen polymorphisms

Several SNPs (single nucleotide polymorphisms) in the genes encoding all 3 fibrinogen chains are associated with variation in plasma fibrinogen levels. The Gly445Ala SNP in the promoter region of the fibrinogen β gene causes increased fibrinogen levels and distinct nuclear protein binding properties affecting the expression of the β chain and therefore synthesis of intact fibrinogen⁷³. These changes in fibrinogen concentration affect clot structure as discussed above.

The AcThr312Ala mutation in the coding region of the fibrinogen α -chain, results in increased stiffness of clots and decreased fibrin density⁷⁴. FXIII crosslinking and lateral aggregation are affected due to the polymorphism's position in the α C domain resulting in the formation of thick fibrin fibres while levels of plasma fibrinogen are not affected⁷⁵. This polymorphism is associated with increased post-stroke mortality⁹⁵, venous thrombosis, pulmonary embolism and chronic thromboembolic pulmonary hypertension⁹⁶. The linkage disequilibrium of this polymorphism with the fibrinogen B β Gly455Ala SNP, itself a predictor of venous thrombotic embolism (VTE), confounds the deleterious properties of this mutation. A common mutation in the B β chain of fibrinogen (B β Arg448Lys) results in compact clots, composed of thin fibres, small pores, increased stiffness and resistance to lysis, a pathology leading to predisposition to CAD, triple vessel pathology, venous thromboembolic disease, hypofibrinolysis and embolisation^{99, 100}.

1.12.1.1 Fibrinogen B β Arg448Lys

The main focus of this study, a mutation lying in the coding region of the carboxyl terminal of the fibrinogen β -chain, is associated with increased risk of CAD, triple vessel pathology, venous thromboembolic disease, hypofibrinolysis and embolisation^{99, 100}. The mutation is in strong linkage disequilibrium (LD) with two other mutations, the Gly445Ala and the Cys148Thr in the 5' flanking region and the promoter region of the B β -chain fibrinogen gene. Several mutations in the fibrinogen β -chain (e.g. Gly445Ala) are linked to plasma fibrinogen levels and this has been attributed to both sensitivity of this mutation to IL-6, the main acute phase protein that induces fibrinogen production, and the fact that the β -chain is rate limiting for the synthesis of the fibrinogen molecule⁷³. This LD may affect transcription rate of the β -chain polypeptide by interaction with an IL-6 responsive element close to the mutation site which controls binding of nuclear proteins, consequently contributing to higher plasma fibrinogen levels in the B β 448Lys variant¹⁰¹. Added to this, a functional role of the mutation has been suggested by its link with increased risk of stroke in females independent of plasma fibrinogen levels¹⁰¹.

The B β Arg448Lys mutation results from a mutation at position 1236 of the gene causing a guanine base to be replaced with an adenine, and consequently an arginine residue to be replaced with a lysine at position 448. It occurs at a frequency of ~25% in the white population, causing significant changes in clot formation, structure and lysis when studied in the plasma environment¹⁰². When compared with B β 448Arg, clots formed from B β 448Lys variant have a more compact structure composed of thin fibres and small pores with increased stiffness; a pathology leading to predisposition to cardiovascular and atherothrombotic disease. This altered clot structure also predisposes to disease via interactions with endothelial cells and fibroblasts, impairing their reorganisation into microtubules, affecting angiogenesis and promoting atherosclerosis¹⁰².

Fibrinolysis rates are significantly slower in the B β 448Lys variant when compared to B β 448Arg in the plasma environment. However, in a purified system, no difference between lysis rates of clots of the two variants is seen, explained by the increased number of fibres in B β 448Lys variant being compensated for by quicker lysis of thin fibres. In the presence of plasma proteins, the incorporation and crosslinking of different proteins into the clot may vary between the variants, hence affecting fibrinolysis rates¹⁰².

Clot stiffness in the B β 448Lys variant is greater than that of the B β 448Arg variant, an effect that is even more pronounced in the presence of FXIII, with B β 448Lys final stiffness reaching more than 3-fold that of B β 448Lys and continuing to increase

after 3 hr. Continual structural rearrangement of the fibrin fibres with B β 448Lys has been proposed as a mechanism for this phenomenon.

The clots formed from B β 448Lys variant being composed of thinner fibres suggests the mutation has an effect on lateral aggregation. Molecular modelling suggests loss of 3 hydrogen bonds in an area of the β -chain thought to be involved in lateral aggregation and stabilisation of the coiled-coil caused by the mutation could be responsible by affecting an area of the α C domain and causing conformational changes in the B β chain. It seems the B448ArgNH1-B315GluOE2 hydrogen bond cannot form between B448LysNZ-B315GluOE2 which could destabilise the B β 398-A α 157 interaction in the coiled coil. Residues B β 397Glu and A α 398Asp appear more mobile in B β 448Lys, these residues are involved in B-B interactions during fibrin polymerisation and may thus affect the fibrin network structure¹⁰². While both arginine and lysine residues are polar and as such sit on the outside of proteins, lysine residues are only capable of forming one hydrogen bond due to their single amino group, whereas arginine residues are capable of making multiple hydrogen bonds.

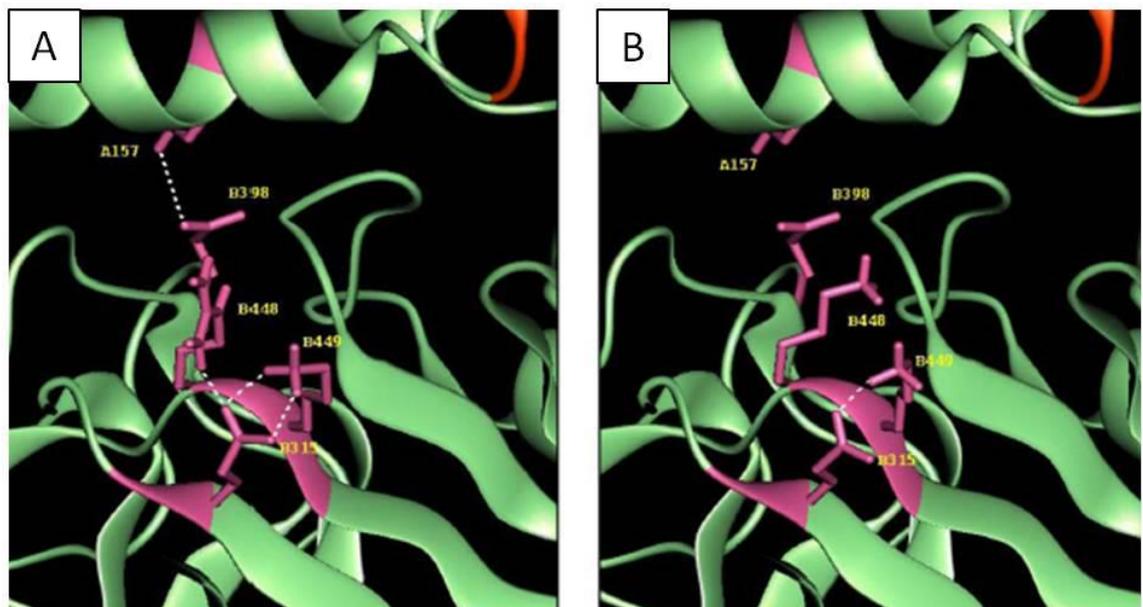


Figure 1-12. Molecular modelling showing the effect of BBArg448Lys variant on position of the fibrinogen BB448 residue

(A) BB448Arg and (B) BB448Lys are shown. Potential loss of a hydrogen bond between BB448 and BB315 could destabilise the B β 398-A α 157 interaction in the coiled coil. Figure adapted from Ajjan et al. (2008)¹⁰².

The mechanisms behind the observed changes in fibrin structure, clot rigidity and fibrinolysis rate of B β 448Arg and B β 448Lys variants need further exploration, though many hypotheses for the influence of the mutation have been suggested. The proximity

of the B β Arg448Lys to 3 important sites in the β -chain that influence clot structure, formation and lysis (a β -chain polymerisation site, an interaction site for the C-terminal of the α -chain and a calcium binding site on the β -chain) may implicate these areas in the observed changes. The effects of the B β 448 arginine to lysine substitution are summarised in Figure 1-13.

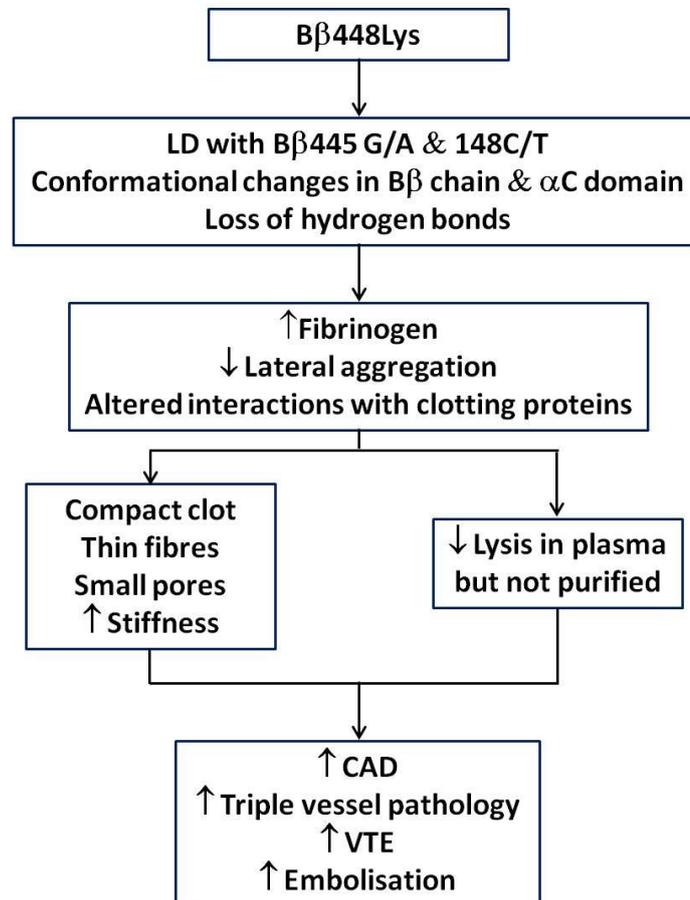


Figure 1-13. Changes seen due to the B β Arg448Lys polymorphism

B β 448Lys causes hyperfibrinogenaemia and formation of compact clots with thin fibres and small pores that are more resistant to lysis in the plasma environment. It predisposes to increased risk of CAD, VTE and embolisation.

1.12.2 Dysfibrinogenemias

Dysfibrinogenemia, a disease in which an abnormality in the fibrinogen molecule is inherited, results in defective fibrin clot formation, manifesting in potentially life-threatening symptoms¹⁰³. These conditions provide a useful tool to investigate the effect of small changes in the fibrinogen gene on clot structure and susceptibility to disease. Nonsense/missense mutations, insertions/deletions and chain truncations in the fibrinogen genes have all been identified as responsible for various fibrinogenemias resulting in modifications of fibrinopeptide release, fibrin polymerisation, fibrin

crosslinking and/or fibrinolysis. The position in which the mutation lies governs whether the dysfibrinogenemia will present as a bleeding or prothrombotic disease. Missense mutations often lead to the development of hypofibrinogenemia (low plasma fibrinogen concentrations) due to their effect on intracellular transport mechanisms and consequent accumulation in the endoplasmic reticulum (as in the γ Gly284Arg polymorphism) where the variant molecules are not expressed in plasma. Truncations can result in hypofibrinogenemia due to a complete lack of expression of the mutant molecules in plasma as the truncated polypeptide cannot be assembled with the other fibrinogen chains (as in the β -chain Gly-40 truncation)¹⁰⁴.

The severity of the condition can depend on whether the variant is homo- or heterozygous. Very few homozygote dysfibrinogenemias have been identified, but these result in a homodimeric fibrinogen molecule with two mutant chains. The by far more common heterozygous forms can result in one of two homodimers (either two normal or two abnormal chains) or a heterodimer with one normal chain and one abnormal chain. Heterozygotes that have 50% normal fibrinogen molecules are usually asymptomatic as this amount of normal fibrinogen molecules is able to maintain normal blood coagulation. Symptoms appear when the dysfibrinogen interferes with functional components of the fibrinogen molecule³⁵.

One of the most prevalent dysfibrinogenemias results from a single amino acid substitution in the fibrinogen A α chain, causing an arginine at position 16 to be replaced by a cysteine or histidine. Prolonged clotting time, impaired polymerisation and hemorrhagic complications result from decreased fibrinopeptide A release by thrombin as in Ledyard¹⁰⁵, Milano IV¹⁰⁶ and Bern III¹⁰⁷ dysfibrinogenemia.

Another phenotypically important mutation is fibrinogen Dusart, which is associated with thrombophilia and results from heterozygosity for a single base change (cytosine to guanine) in the A α -chain gene, causing amino acid 554 of the chain to be changed from arginine to cysteine. The functional defect of recurrent thrombosis is associated with reduced plasminogen binding, impaired plasminogen activation by tPA and abnormal fibrin polymerisation, causing thinner fibres due to defective lateral association of fibres and disulfide-linked fibrinogen-albumin complexes³⁵. In this study we look at a newly identified dysfibrinogenemia, the Fibrinogen A α Birmingham II mutant, discussed further in section 1.12.2.1.

1.12.2.1 Fibrinogen A α Birmingham II mutant

While B β Arg448Lys mutation is relatively common, a rare novel fibrinogen variant has been detected in a 34 year old woman, which is associated with hypodysfibrinogenemia¹⁰⁸. Clinically, the individual presented with a bleeding tendency associated with venous thrombotic events. A familial history of hypofibrinogenemia with

a bleeding tendency was recorded but there were no cases of thrombosis. This is the only case that has been identified, showing compound heterozygosity for Trp334Cys and Asn335Tyr mutations in the α -chain of fibrinogen. Two mutations in the polypeptide have been identified at positions 1001 and 1002 causing a guanine and an adenine respectively to be both replaced by thymine. SIFT analysis suggests these changes could affect protein function and polyphen analysis predicts them as damaging. However, no studies have been conducted to analyse whether these amino-acid changes cause alterations in clot structure/lysis that explain the clinical phenotype. The asparagine to tyrosine substitution results in the replacement of a polar residue that is comfortable at the surface of proteins and is often involved in active and binding sites with an easily phosphorylated, hydrophobic residue that likes to be hidden in the centre of the protein structure. Tryptophan, a hydrophobic residue is also replaced with a cysteine; a residue that is often found in enzyme active sites and capable of forming disulfide and covalent bonds. The amino acid residues affected by the candidate mutations are both highly conserved residues in the α C domain of fibrinogen, which have a role in crosslinking during fibrin polymerisation. Changes in binding of fibrinolytic modulators, altered clot structure and increased resistance to lysis have been linked to other mutations in this region.

1.13 Diabetes

Diabetes is a complex multifactorial condition in which both genetic and environmental factors play a role. It is associated with qualitative and quantitative changes in procoagulant and anti-fibrinolytic proteins leading to fibrin clots with tight network structure and resistance to lysis, consequently predisposing to a thrombotic environment^{102, 109, 110} (Figure 1-14).

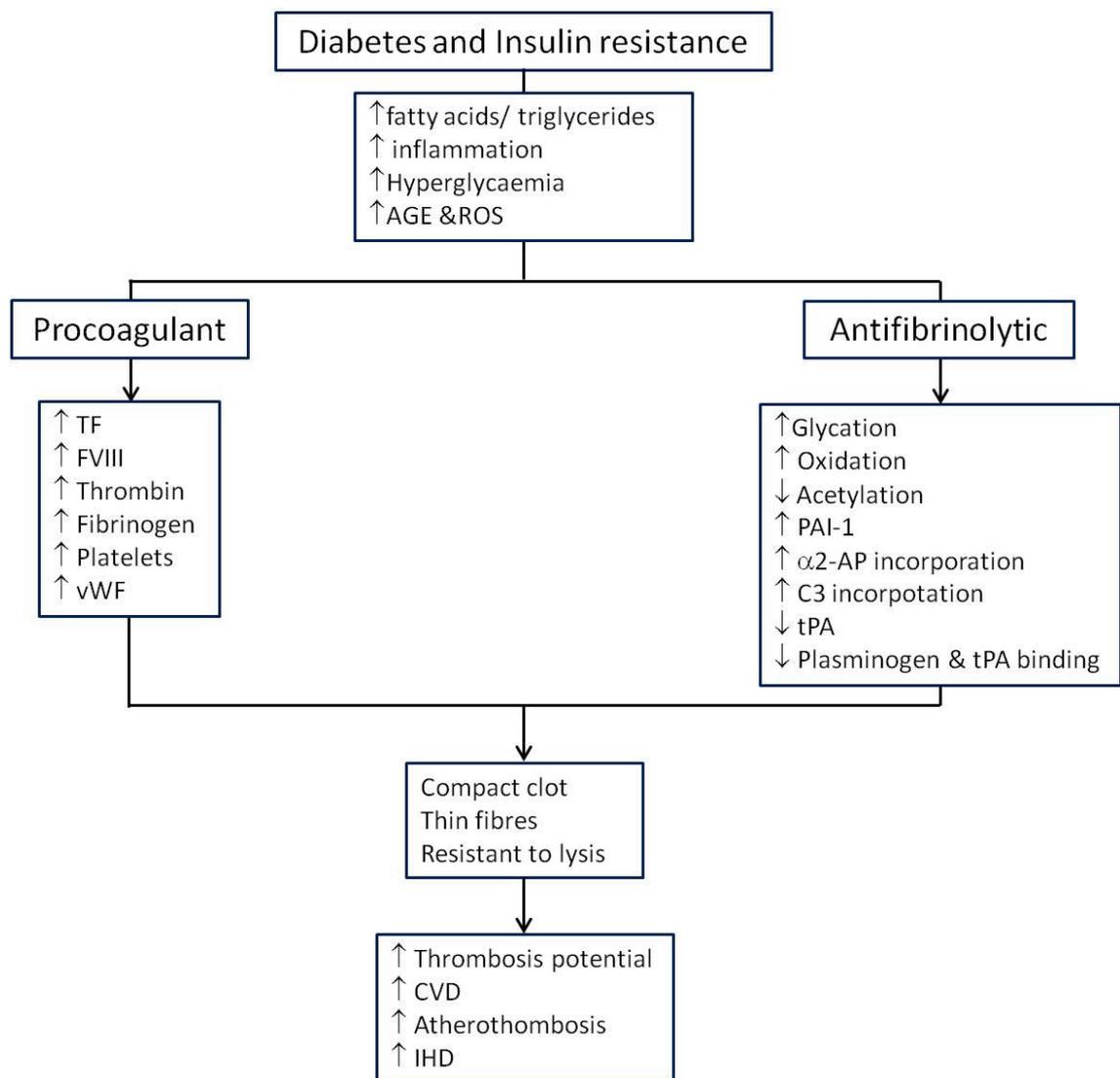


Figure 1-14. Mechanisms by which diabetes and insulin resistance effect the coagulation system and cardiovascular disease

Increased levels of procoagulant and antifibrinolytic proteins in diabetes lead to compact clots made of thin fibres that are more resistant to lysis. This results in an increased thrombosis potential and increased risk of CVD, atherothrombosis and IHD.

1.13.1 Diabetes and the prothrombotic environment

Diabetes causes increased levels and activity of procoagulant and antifibrinolytic proteins (e.g. platelets, tissue factor, thrombin, PAI-1 and fibrinogen), qualitative changes in clotting factors and increased levels of inflammatory proteins. This predisposes to a thrombotic environment and premature atherosclerosis^{109, 110}.

1.13.2 Mechanisms of compact clots and hypofibrinolysis in diabetes

Clots made from plasma purified fibrinogen of type two diabetes mellitus (T2DM) patients are characterised by thinner fibres, increased branching, tight network

structure and resistance to lysis¹¹¹. Plasma clots also show reduced permeability compared to controls, indicating a more compact structure⁹¹. This is due to qualitative and quantitative changes in clotting factors in the blood in this condition.

1.13.2.1 Quantitative changes in coagulation factors

1.13.2.1.1 Fibrinogen

Hyperfibrinogenaemia is evident in both T1DM and T2DM with Framingham data¹⁰⁹ showing a concomitant rise in fibrinogen concentration throughout a range of blood sugar levels. However others have shown insulin sensitivity to be the only variable able to predict fibrinogen values, confirming a negative correlation between the two. Other mechanisms explaining hyperfibrinogenaemia in DM have been proposed to be due to increased circulating free fatty acids and enhanced inflammation. Free fatty acid release is increased in both T1DM and T2DM which can lead to stimulation of hepatic fibrinogen synthesis whilst cytokines such as TNF- α and IL-6, released during increased inflammatory events in these conditions, can also cause hepatic fibrinogen synthesis promotion¹¹². It is most likely interplay of all of these conditions occurring in diabetes that lead to the increase in circulating fibrinogen concentration rather than one alone.

1.13.2.1.2 Tissue Factor

Tissue factor expression is up-regulated in T2DM due to increased exposure of VSMC (vascular smooth muscle cells), which constitutively express TF, secondary to plaque rupture and enhanced stimulation by the inflammatory response. It appears baseline circulating levels of TF are also increased by the high levels of both glucose and insulin that appear in T2DM and the increased levels of AGEs of ROSs activating nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), further enhancing TF production. Studies have shown that inducing hyperglycaemia in healthy subjects leads to increased TF levels, while improving glycaemic control in T2DM reduces levels, implicating TF as a factor in the thrombotic condition in T2DM^{113, 114}.

1.13.2.1.3 FVII

FVII levels are increased in T2DM which is associated with the increased circulating triglycerides due to poor glycaemic control in the condition, with reduction in triglyceride levels causing a concomitant reduction in FVII levels, suggesting better glycaemic control may lead to reduced FVII levels. It has also been shown that hyperglycaemia can increase FVII levels independent of triglycerides¹¹⁵.

1.13.2.1.4 Thrombin

Hyperglycaemia causes increased thrombin production causing thrombin levels to be increased in T1 and T2DM through low grade activation of the coagulation cascade, enhancement of thrombin generation persists even when glycaemia is normalised¹¹⁶.

1.13.2.1.5 Fibrinolytic proteins

Diabetes and specifically hyperglycaemia also affects fibrinolysis potential by influencing PAI-1 and tPA levels. PAI-1 levels are increased while tPA levels decrease in hyperglycaemia, leading to a prothrombotic and anti-fibrinolytic environment.

FXIII crosslinking of α 2-AP to fibrin is enhanced in T2DM¹¹⁷ which also works to increase the resistance of the clot to lysis, the mechanism by which this occurs is not clear but structural alteration of the fibrin molecule in diabetes may lead directly to enhanced crosslinking, or indirectly promote activation of FXIII by thrombin. The increased resistance to lysis of diabetic clots is also related to reduced plasminogen and tPA binding and consequent impaired plasmin generation¹¹⁷.

1.13.2.1.6 Inflammatory proteins

C3 levels are increased in the associated low grade inflammation present in diabetes and are predictive of thrombotic events. Though complement proteins are traditionally regarded as inflammatory proteins, it appears they also interact with the coagulation system with work showing clot lysis time to concomitantly rise with plasma C3 levels, independent of fibrinogen and PAI-1 levels. Recent work has shown C3 to prolong lysis of clots made from diabetic fibrinogen compared with control and that this may be due to enhanced C3 incorporation into the clots of T1DM patients. They suggest C3 may interfere with tPA and plasminogen binding, increase mechanical resistance of the clot, or, as a competitive substrate of plasmin, affect plasmin binding to fibrin and thus compromise fibrinolysis. They showed clots formed of thinner fibres with C3 incorporation, suggesting it may affect lateral aggregation⁹⁰.

1.13.2.2 Qualitative changes in clotting factors

Post-translational modifications in fibrinogen caused by changes in the blood environment in diabetes can also affect clot structure and fibrinolysis.

1.13.2.2.1 Glycation

Glycation of fibrinogen secondary to hyperglycaemia has been demonstrated and fibrinogen glycation correlates well with capillary glucose in T2DM^{78, 110}. Clots made from glycated fibrinogen result in a tight and rigid network, resistant to lysis, as discussed in section 1.10.2.2.1.1. It has also been shown that glycoaldehyde, generated during inflammation and hyperglycaemia can react with arginine and lysine residues to impair protein function delaying plasma coagulation and fibrin polymerisation, with clots made of thinner fibres⁷⁹.

1.13.2.2.2 Oxidation

ROS and AGEs, levels of which are increased in oxidative stress, inflammation and diabetes can lead to oxidation of circulating proteins. Impaired fibrin polymerisation and thrombin induced clot formation to complete loss of clottability can result from oxidation of fibrinogen⁸¹.

1.13.2.2.3 Acetylation

Aspirin is regularly prescribed in diabetes due to its cardioprotective role through its action on platelet cyclo-oxygenase-1 (COX-1). Here it inhibits COX-1s activity, so decreasing levels of thromboxane-A2 and reducing platelet aggregation potential. Acetylation of lysine residues on the α -chain of fibrinogen can occur in the presence of aspirin resulting in clots composed of thick fibres and large pores with reduced rigidity which are less resistant to lysis. In patients with diabetes, the clinical efficacy of aspirin appears to be reduced, which is often termed clinical aspirin resistance. Compromised platelet inhibition by aspirin, secondary to reduced platelet protein acetylation, has been suggested as one mechanism, although this remains controversial¹¹⁸. A competition between acetylation and glycation of fibrinogen has also been suggested as another mechanism, but studies have mainly been conducted in vitro and definitive conclusions are still awaited⁸⁵.

1.13.3 Diabetes as an independent risk factor for disease

Diabetes is a major health problem and cardiovascular complications remain the main cause of mortality in this population. The condition currently affects 347 million people worldwide and is projected to be the 7th leading cause of death by 2030 (www.who.int). The rapidly increasing global prevalence of diabetes is a significant cause for concern as the risk of mortality in diabetes subjects due to atherothrombotic complications is equal to that of nondiabetic individuals with a history of ischemic heart

disease^{109, 110, 119}. Not only are individuals with diabetes at higher risk of cardiovascular disease, their prognosis following vascular ischaemia is significantly worse than individuals with normal glucose metabolism. The reasons for this are probably multifactorial, including more extensive vascular pathology, increased thrombosis potential and high prevalence of heart failure, which is partly related to atherothrombotic complications.

Diabetes predisposes to development of atherogenic risk factors, such as obesity, increased fibrinogen concentration, hypertriglyceridemia and raised blood pressure. However these factors have been shown to be of no greater significance in predicting atherogenic risk in diabetics than nondiabetics¹⁰⁹. It follows that diabetes adds extra, independent, risk factors for cardiovascular risk above those 'traditional' risk factors such as obesity and fibrinogen concentration¹¹⁰. An independent effect of glucose intolerance on cardiovascular risk, after all standard risk factors, and fibrinogen, have been adjusted for has been identified¹⁰⁹. And even in the pre-diabetes stage, when normoglycaemia remains but a state of insulin resistance is present, an increased risk of atherothrombotic events is evident¹²⁰.

Hyperglycaemia itself can also cause atherosclerosis by enhancing the non-reactive enzymatic glycation of other plasma proteins and lipids as a result of oxidative stress leading to increased levels of circulating AGEs. These AGEs can trigger inflammatory processes, blood-vessel wall perturbation and oxidised low density lipoprotein (LDL) formation, all of which can initiate atherosclerotic processes¹¹⁰.

1.13.4 Glycaemic control

Improving glycaemic control and insulin sensitivity go some way to reduce the hypercoagulable and atherothrombotic environment in diabetes and the use of drugs such as metformin and statins also show improvement in risk of cardiovascular events in these patients.

1.13.5 Diabetes and B β Arg448Lys

Taken together, both diabetes and B β Arg448Lys variants of fibrinogen modulate cardiovascular risk and induce thrombotic changes in fibrin networks. Despite the relatively high prevalence of both conditions and their role in atherothrombotic conditions, whether B β Arg448Lys directly affects clot structure and lysis in a complex condition such as T2DM is unclear.

1.14 Aims of the work

From the evidence presented above, it is clear that fibrinogen is essential in the blood clotting process, which in itself is crucial for preventing excessive bleeding upon injury. Disturbances at any point in the clotting system can lead to chronic symptoms and death. While a number of studies have investigated various fibrinogen mutations, the common B β Arg448Lys mutation remains only partially characterised and the role of this polymorphism in high risk vascular conditions, such as diabetes, remains unknown. Equally, the novel A α Trp334Cys/Asn335Tyr mutation, which is associated with a peculiar clinical presentation, is yet to be fully investigated. Understanding the functional role of common and rare mutations in fibrinogen will unravel new mechanisms that will enhance our knowledge and will help in the long-term to identify novel therapeutic targets. Firstly, I hypothesise that the B β Arg448Lys fibrinogen variant will affect clot structural and functional properties in T2DM patients. Secondly, I suggest α 2-AP is the protein responsible for the differences in lysis of recombinant B β Arg448Lys fibrinogen variants in the plasma environment. Lastly, I propose the A α Trp334Cys/Asn335Tyr changes in the Birmingham II patient are responsible for her clinical presentation. Therefore my work is aiming to:

- 1) Identify any additional effect of B β Arg448Lys on fibrin network in a high risk vascular condition with complex pathogenesis, such as T2DM.
 - a. Analyse the effect of B β Arg448Lys on ex vivo clot structure/fibrinolysis in subjects with T2DM.
 - b. Analyse of the role of the polymorphism on previous and existing vascular pathology in this population.
- 2) Study the effects of the common B β Arg448Lys mutation on fibrin clot structural and functional properties.
 - a. Analyse the effects of B β Arg448Lys mutation on early protofibril formation, FXIII activation and cross-linking.
 - b. Elucidate potential differences in binding and crosslinking of fibrinolytic proteins to B β 448Arg and B β 448Lys variants of fibrinogen.
- 3) Investigate the role of the rare variant of fibrinogen A α Trp334Cys/Asn335Tyr on clot structure and lysis.
 - a. Analyse the effects of this variant of fibrinogen on clot structure and lysis in plasma, purified and recombinant systems.
 - b. Establish whether clinical presentation of the patient is due to abnormality in the fibrinogen molecule resulting in altered clot structure and/or lysis.

Chapter 2. Materials & Methods

Materials

General Materials

Material	Source
Chinese Hamster Ovary cells (CHO cells)	Susan Lord
DMEM/ F12 1:1 liquid	Invitrogen
BD NuSerum supplement	VWR
Bovine calf serum	ThermoScientific
Penicillin-Streptomycin	Sigma
Trypsin/ EDTA	Sigma
DMSO	ThermoScientific
Cytodex microcarrier beads	Sigma
NaCl	Sigma
KCl	Sigma
Na ₂ HPO ₄ ·2H ₂ O	Sigma
KH ₂ PO ₄	Sigma
Boehringer Mannheim's ITS	Roche
Aprotinin solution	Sigma
PMSF	Sigma
Ammonium sulphate	ThermoScientific
ε-amine n-caproic acid	Sigma
Benzamidine	Sigma
Pepstatin	Sigma
Leupeptin	Sigma
MES-buffer	Sigma
Tris	Sigma
EDTA	Sigma
Soybean trypsin inhibitor	Sigma
CaCl ₂	Sigma
IF-1 monoclonal antibody to fibrinogen	Kamiya Biomedical
Sodium acetate	Sigma
SDS-Page sample buffer/ reducing agent	Invitrogen
MES SDS running buffer	Invitrogen
Perfect protein marker	Novagen
GelCode blue stain reagent	ThermoScientific
Thrombin	Millipore

Sodium acetate trihydrate	Sigma
Sodium 5,5 diethylbarbituate	Sigma
HCl	Sigma
NaH ₂ PO ₄ ·2H ₂ O	Sigma
Calbiochem fibrinogen	Millipore
Tween20	Sigma
OPD tablets	DAKO
H ₂ O ₂	Sigma
H ₂ SO ₄	Sigma
Tissue plasminogen activator	Technoclone
Factor 13	ZLB Behring
Plasminogen	ERL
Antiplasmin	Millipore
Glycine	Sigma
Methanol	Sigma
Skimmed milk	Marvel Foods
Supersignal west pico luminal	ThermoScientific
Supersignal west pico stable	ThermoScientific
488 Alexa labelled fibrinogen	Invitrogen
Cadolydic acid	Sigma
Gluteraldehyde	Sigma
Acetone	Sigma
NiCl ₂	Sigma
Acetate sample buffer	Expedeon Ltd
DTT	Sigma
HEPES running buffer	Expedeon Ltd
Alexa fluor 594 fibrinogen	Invitrogen
BSA	Sigma
Biotin	ThermoScientific
Streptavidin alkaline phosphatase	Sigma
P-nitrophenol phosphate	Sigma
Diethonamine	Sigma
MgCl ₂	Sigma
NaOH	Sigma
S2251 substrate	Sigma
Dpn I restriction enzyme	Agilent

XL-1-Blue supercompetent cells	Agilent
SOC broth	Sigma
Metaphor agarose powder	Lonza
Acetic acid	Sigma
Ethidium bromide	Sigma
DNA loading buffer	Bioline
High molecular weight bioladder	Bioline
DH5 α cells	Invitrogen
Geneticin 418	Invitrogen
L-histidinol	Sigma

Antibodies

Antibody	Source
Rabbit anti-fibrinogen	Agilent Technologies
Goat polyclonal anti fibrinogen HRP	Abcam
Goat anti-human α 2-AP HRP	ERL
Rabbit anti-albumin HRP	Abcam
Streptactin HRP	Bio-Rad

Equipment

Equipment	Source
5702R centrifuge	Eppendorf
Nunclon petri dish	ThermoScientific
Falcon tube	VWR
Cryovial	VWR
850 cm ³ polystyrene roller bottles	Corning
Roller bottle incubator	VWR
Filter paper	GE Healthcare
0.2 μ m bottle top membrane filter	ThermoScientific
Stratus thermostatic water bath	Northern Media
Sorvall RC-5B refrigerated super speed centrifuge	ThermoScientific
Automated chromatography system	Biocad Sprint
100,000 MWCO vivapsin concentrator tubes	Generon
Dialysis tubing	Sigma
ND-1000 spectrophotometer	Nanodrop
NuPAGE Novex Bis-Tris gel	Invitrogen

Novex mini-cell tank	Invitrogen
KC10 Amelung coagulometer	Amelung
Clauss reference plasma	Biomereux
Nunc-immuno microwell maxisorp plate	Sigma
MRX microplate reader	Dynex Technologies
Clear polystyrene flat bottom plate	Grenier Bio-One
ELx-808 IU ultramicroplate reader	Labtech International
Immobilon-P transfer polyvinylidene difluoride membrane	NEN™ Life Sciences
Western blot mesh	Bio-Rad
Whatman filter paper	SLS
Mini-PROTEAN 3 cell	Bio-Rad
Kodak image station 2000R	Eastman Kodak
Ibidi confocal slide	Applied Biophysics
LSM 510META microscope	Zeiss
E300 critical point dryer	Quorum Technologies
208HR high resolution sputter coater	Cressington
Quanta 200F FEGESEM electron microscope	FEI
Ultimate 3000 nano LC system	Dionex
PepMap C18 analytical column	Dionex
Time of flight mass spectrometer	Synapt HDMS
Alexa Fluor 488 labelling kit	Invitrogen
DB-5A dri-block heating block	Techne
QuickChange site directed mutagenesis kit	Statagene
PTC-200 Peltier Thermal Cycler	MJ Research
QIAprep spin miniprep kit	Qiagen
MIKRO 2000 centrifuge	Hettich
Multimage™ light cabinet	Alpha Innotech
Plasmid MaxiPrep kit	Qiagen

Methods

2.1 Fibrinogen production and purification

2.1.1 CHO cell culture

2.1.1.1 Thawing of cells

A 1 ml vial of the desired Chinese Hamster Ovary (CHO) cells were removed from liquid nitrogen storage and rapidly thawed at 37 °C. When thawed, these cells were re-suspended in 9 ml growth media (GM – DMEM/ F12 1:1 liquid (Invitrogen, Carlshead, CA, USA), 5% BD NuSerum supplement (VWR, Arlington Heights, IL, USA), 5% bovine calf serum (ThermoScientific Waltham, MA, USA), 0.01% Penicillin-Streptomycin (Sigma, St Louis, MO, USA) and centrifuged at 1000 rpm for 10 min in a 5702R centrifuge (Eppendorf, Hamburg, Germany) to wash the cells of DMSO and produce a cell pellet. The media was removed from the pellet by aspiration and the pellet re-suspended in 20 ml GM. The cells were then transferred in 2 ml aliquots to 100 mm Nunclon petri dishes (ThermoScientific) containing 8 ml GM.

2.1.1.2 Growth to confluence

Cell media was changed every 2-3 days and cells split when they reached confluence. To do this, all media was removed from the petri dish and 2 ml trypsin/EDTA (Sigma) added. The dish was incubated at 37 °C for 2 min and a microscope used to check if the cells had become loose from the dish. When cells were no longer adhered to the dish, 8 ml GM was added and the cells split between 5 fresh petri dishes containing 8 ml GM each.

2.1.1.3 Freezing down cells

The media was removed from the cells by aspiration, 2 ml trypsin/EDTA added and incubated until the cells became loose of the plate. 8 ml GM was added to the plate and the cells suspended by pipetting up and down before transferring to a 15 ml falcon (VWR). The cells were then spun down into a pellet by centrifugation in a 5702R centrifuge at 1000 rpm for 10 min. The supernatant was discarded and the cells re-suspended in 2 ml of a 90%GM, 10%DMSO (ThermoScientific) solution. 1 ml of cells was transferred to each of two cryovials (VWR), which were labelled with the cell line and date of freezing. The cyrovials were then transferred to a pot containing isopropanol which was stored at -80°C for 10-24 hr before being moved to liquid nitrogen storage.

2.1.1.4 Transfer to Roller Bottles

After reaching confluence, cells were split with trypsin/EDTA and transferred to 850 cm³ polystyrene roller bottles (Corning, Tewksbury, MA, USA) containing 200 ml GM. One confluent 100 mm plate of cells was used per roller bottle; the media were removed from the plates by aspiration and 2 ml trypsin/EDTA added to each plate. For 10 roller bottles, the cells were loosened and pooled in 50 ml GM. 5 ml of these pooled cells were added to each roller bottle containing 200 ml GM. The bottles were rotorted horizontally several times to distribute the cells evenly on the surface then placed in the roller bottle incubator (VWR; Figure 2-1) and grown until the cells were confluent (5-7 days).



Figure 2-1. Roller bottles in roller bottle incubator

Cells were grown in roller bottles in a 37 °C roller bottle incubator. Cytodex microcarrier beads were added to increase cell surface area.

2.1.1.4.1 Addition of cytodex microcarrier beads

Once the cells were confluent, to increase the surface for adherent cell growth, cytodex microcarrier beads (Sigma) were added to each confluent roller bottle. For each bottle, 1 g of beads was needed. For 10 roller bottles; 10 g beads was suspended in 350 ml Phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄·2H₂O, 2 mM KH₂PO₄, pH 7.4) in a 500 ml bottle and autoclaved on a fixed media cycle. After cooling, the PBS was removed by aspiration with care taken not to disturb the beads. The PBS was replaced with GM and the bottle shaken and left till the beads settled to the bottom of the bottle. This GM was removed by aspiration in the

same way and the washing with GM repeated to remove all traces of PBS. 500 ml GM was then added to the beads and 100 ml of GM from each roller bottle removed and replaced with 100 ml of fresh GM. The beads were swilled to distribute them within the 500 ml of GM and 50 ml of the bead mix added to each roller bottle. Each roller bottle was then rotorted horizontally to coat the side with beads before being placed back in the incubator without media change until cells covered the whole surface of the beads (5-6 days).

2.1.1.4.2 Change to serum free media

At this point the GM in each bottle was exchanged for serum free media (SFM; DMEM/ F12 1:1 liquid, 0.01% Penicillin-Streptomycin). All the media in the roller bottles were discarded and 50 ml SFM added to each roller bottle and the bottles rotorted several times to rinse. The rinse media were replaced with 100 ml SFM and cells washed overnight in the roller bottle incubator. Next day all SFM were removed and replaced with 200 ml fresh SFM.

2.1.1.5 Harvesting media

5 days after transfer to SFM, 100 ml media were harvested. These 100 ml were replaced with 100 ml fresh ITS media (DMEM/F12 1:1 liquid, 0.01 mg/ml Boehringer Mannheim's ITS (Roche, Basel, Switzerland), 4 µg/mL aprotinin solution (Sigma), 0.01% Penicillin-Streptomycin)). ITS added essential nutrients to the DMEM/F12 solution, facilitating the growth of the CHO cells in a serum free environment whilst the aprotinin in the media protected the secreted fibrinogen from proteolysis.

The roller bottles were removed from the incubator and placed in the sterile hood; 100 ml media from each bottle was removed and poured into a plastic funnel lined with filter paper (GE Healthcare, Little Chalfont, United Kingdom) into a 1 L bottle to remove all detached beads and cells. 100 ml fresh ITS media was added to each roller bottle which were then put back into the roller bottle incubator. The collected filtered media were transferred into bottles labelled with the date and number of harvest. From each batch of harvest a 1 ml aliquot was removed and stored at -20 °C to be used to determine fibrinogen content of the harvested media by enzyme-linked immunosorbent assay (ELISA) as in section 2.3. Periodically the fibrinogen content was determined by ELISA. When fibrinogen concentration began to decrease significantly, harvesting was stopped and the bottles thrown away. Media were generally harvested over 6-8 weeks, every 2-3 days. 150 µl of 100 mM PMSF (Sigma) was added per 100 ml of harvested media to prevent proteolytic degradation during storage. The harvested media were then stored at -40 °C until protein purification.

2.1.2 Ammonium sulphate precipitation of fibrinogen

2.1.2.1 Preparation of ammonium sulphate

A saturated solution of ammonium sulphate was prepared by addition of 1520 g ammonium sulphate (ThermoScientific) to 2 litres of dH₂O in a 5 litre beaker. The mix was placed on a hot plate and a stirrer added. The mix was heated while stirring until all ammonium sulphate dissolved. The hot solution was then filtered through a 0.2 µm bottle-top membrane filter (ThermoScientific) into 2 litre bottles. The bottles were stored at 4 °C overnight or until crystals formed.

2.1.2.2 Fibrinogen precipitation

Media were thawed in a 37 °C Stratus thermostatic water bath (Northern Media, Hessle, United Kingdom); fibrinogen from 4 litres of medium was precipitated per day. In the cold room, thawed media were pooled and 2 litres poured into each of two 5 litre beakers that already contained 5 mM ε-amine n-caproic acid (Sigma), 5 mM benzamidine (Sigma), 1 µM pepstatin (Sigma), 1 µM leupeptin (Sigma), 100 µM PMSF and 0.7 M MES-buffer (Sigma).

Leupeptin, a synthetic inhibitor of serine and cysteine proteases, inhibits plasmin, trypsin, papain, and cathepsin B. Pepstatin A is a potent inhibitor of acid proteases including pepsin, rennin and cathepsin D, and PMSF protects the fibrinogen in solution against a broad range of proteases. The pH of the solution was brought to 5.6 by the addition of 0.7 M MES buffer to increase the precipitation yields as this is the isoelectric point of fibrinogen. This is the pH at which a protein has no net electrical charge meaning it will precipitate out of solution.

The fibrinogen in media was then precipitated with cold saturated ammonium sulphate solution. The amount of ammonium sulphate solution required was given by the volume of media multiplied by 0.67 (e.g. 1340 ml for 2 L media). A stirrer was added to the beakers and the media gently stirred (50 rpm). A funnel lined with filter paper was held above each beaker and the ammonium sulphate solution allowed to drop slowly through the filter into the media. When all ammonium sulphate had filtered through, the beakers were wrapped with saran wrap and left overnight at 4 °C without stirring.

2.1.2.3 Centrifugation

The next day the Sorvall GS3 rotor was cooled to 4 °C in a Sorvall RC-5B refrigerated super speed centrifuge (ThermoScientific) during which time the precipitated media/ammonium sulphate mixture was distributed between in 6 of 400 ml

Sorvall centrifuge cups which were balanced equally in pairs. These were placed in the cool GS3 rotor, the exactly weighted tubes opposite each other in the rotor to ensure perfect balance, and the lid of the rotor secured. Media was then centrifuged for 30 min at 9000 rpm, 4 °C with the brakes off.

During this time, a pellet buffer was prepared containing; 200 mM Tris, 0.3 M NaCl, 1 mM EDTA, 5 µM leupeptin, 5 µM pepstatin, 100 µM PMSF, 10 U/ml soybean trypsin inhibitor (Sigma), 5 mM ε-amine n-caproic acid and 5 mM benzamidine which was kept on ice until use.

When the centrifugation was complete and a weakly attached yellow pellet had formed on the inner wall of the centrifuge cups, they were removed and taken back to the cold room with care so as not to disturb the pellet. The media were discarded and any excess removed from the tube with a pastette. The precipitated fibrinogen in each bottle was dissolved with 1.2 ml pellet buffer. The solution was pipetted up and down and along the walls of the bottle to give the fibrinogen time to dissolve. The dissolved pellet was pooled into a SS34 rotor tube and kept on ice until all precipitate had been collected, after which it was kept on ice for 30 min. During this incubation, the SS34 rotor was cooled in the Sorvall centrifuge. The tube was balanced against a balance tube and centrifuged at 19,000 rpm for 30 min at 4 °C with the brakes on. The supernatant was transferred to a clean 15 ml falcon tube and put at 4 °C if loaded onto the column within the next 2 days or frozen at -80 °C.

2.1.3 Protein purification

2.1.3.1 Preparation of samples for fibrinogen purification

2.1.3.1.1 Plasma

To prepare the citrated plasma pools for purification on the IF-1 column, 1.2 ml plasma was added to a final volume of 5 ml of 100 U/ml heparin and 0.02 M CaCl₂ in equilibration buffer (0.02 M Tris, 0.3 M NaCl, 1 mM CaCl₂, pH 7.4).

2.1.3.1.2 Recombinant

If frozen, the supernatant from the Sorvall steps was thawed in a 37 °C water bath and kept on ice until use. 1 M CaCl₂ was added to the sample to a final concentration of 10 mM, which was then mixed and left on ice for at least 30 min.

2.1.3.2 IF-1 monoclonal antibody and BioCad

Purification of fibrinogen was performed by affinity chromatography using calcium dependent IF-1 monoclonal antibody (Kamiya Biomedical, Seattle, WA, USA) and an

automated chromatography system (Biocad sprint, Applied Biosystems, Warrington, UK). After loading the sample, the column underwent washing using 6 column volumes 0.02 M Tris, 1 M NaCl, 1 mM CaCl₂, pH 7.4 and 0.05 M sodium acetate, 0.3 M NaCl, 1 mM CaCl₂, pH 6. Elution of fibrinogen was achieved by adding 7 column volumes 0.02 M Tris, 0.3 M NaCl, 5 mM EDTA, pH 7.4. The chromatograph produced was used to estimate a peak of the fibrinogen eluted. Those tubes of elutant within this peak were tested by spectrophotometry as in section 2.1.3.5. All those fragments with a protein concentration >0.05 mg/ml, assessed by a ND-1000 spectrophotomer (Nanodrop, Wilmington, DE, USA), extinction coefficient 15.1, were pooled and stored at -80 °C.

2.1.3.3 Concentration

To concentrate the pooled fibrinogen eluted from the BioCad, 100,000 MWCO Vivaspin20 concentrator tubes (Generon, Maidenhead, Berkshire, UK) were spun at 2000 g (3000 rpm) until the volume in the filter was 1 ml. The fibrinogen was transferred to a microcentrifuge tube in 200 µl steps making sure to wash the filter surface thoroughly with the solution to dislodge all fibrinogen caught in the membrane.

2.1.3.4 Dialysis

Dialysis tubing (Sigma) was prepared by boiling the tubing in 1 M EDTA. The tubing was rinsed with ddH₂O and each of the fibrinogen samples dialysed against 50 mM Tris, 100 mM NaCl pH 7.4 at 4 °C for 2 consecutive hr and overnight with stirring (50 rpm).

2.1.3.5 Concentration of fibrinogen determination

Protein concentration was determined using a ND-1000 spectrophotomer (Nanodrop, Wilmington, DE, USA), extinction coefficient, 15.1.

2.1.3.6 SDS Page

A sodium dodecyl sulphate (SDS) polyacrylamide gel (Invitrogen) was run to determine the quality and purity of the dialysed fibrinogen. 15 µg samples of fibrinogen were prepared in 10 µl sample buffer and 4 µl reducing agent (Invitrogen) and made up to 26 µl with ddH₂O and reduced at 70 °C for 10 min. The samples were run on a 4-12% NuPAGE Novex Bis-Tris gel (Invitrogen) at 200 V for 35 min using MES SDS running buffer in a Novex mini-cell tank (Invitrogen). 5 µl of Perfect Protein marker (Novagen, Madison, WI, USA) was added to one well as a molecular weight marker.

2.1.3.7 GelCode staining

After electrophoresis, the gel was rinsed with dH₂O for 15 min, replacing with fresh water every 5 min. The gel was then stained with GelCode Blue Stain Reagent (ThermoScientific) solution for 1 hr with shaking. The gel was subsequently de-stained 4 times for 15 min on each occasion with dH₂O. The gel was visualised using Chemi-imager software (Alpha Innotech, San Leandro, CA, USA) using a trans-white light. Three bands were expected (for the α , β and γ chains of fibrinogen), and correct sizes verified using an appropriate ladder (66, 56 and 48 kDa respectively) as shown in Figure 2-2.

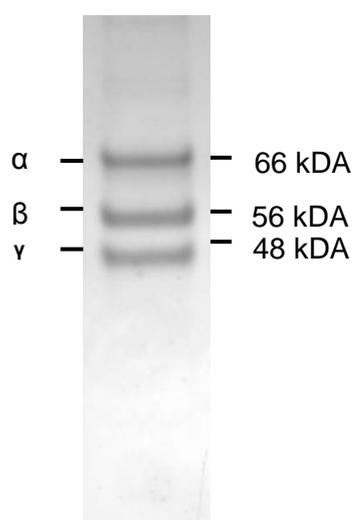


Figure 2-2. SDS-PAGE gel showing purified fibrinogen

Each band is well defined and separated at the expected molecular weights. There is no smearing or any unexpected bands.

Once the integrity and purity of the protein had been confirmed, the samples were pooled together, swirled to mix and concentration determined by spectrophotometry. The stock was then diluted to the desired concentration by addition of permeation buffer (PB; 0.1 M NaCl, 0.05 M Tris, pH 7.4) and aliquoted into eppendorf tubes. The purified fibrinogen was stored at -80 °C.

2.1.3.8 Clottability assay

The protein concentration of sample of fibrinogen was assessed by spectrophotometry as in section 2.1.3.5. 10 μ l of an activation mix (AM) was added to 90 μ l of each sample to final concentrations of 5 U/ml thrombin (Millipore, Billerica, Massachusetts, USA), 5 mM CaCl₂. The samples were allowed to clot for 1 hr at 37 °C after which they were centrifuged at 13,000 rpm for 10 min. The protein concentration

in the supernatant was then measured by spectrophotometry and used to make an estimate of the percentage of protein that did not clot.

2.2 Clauss Method for plasma fibrinogen level determination

Plasma was diluted 1:10 in veronal buffer pH 7.35 (0.026 M sodium acetate trihydrate, 0.026 M sodium 5,5 diethylbarbiturate, 0.11 M NaCl, 0.016 M HCl in H₂O) and incubated at 37 °C with 5 U/ml thrombin in a KC10 Amelung Coagulometer (Amelung, UK) with stirring. The time taken for the clot to form was compared to a calibration curve of reference plasma (Biomereux, Hampshire, UK) with a known level of fibrinogen and the fibrinogen concentration deduced.

2.3 Fibrinogen ELISA

A Nunc-Immuno MicroWell 96 well MaxiSorp flat bottom plate (Sigma) was coated with DAKO anti-fibrinogen antibody A0080 (Agilent Technologies, Santa Clara, CA, USA). 100 µl of 1.15 µg/ml of antibody in buffer A (0.0025 M NaH₂PO₄·2H₂O, 0.0075 M Na₂HPO₄·2H₂O, 0.145 M NaCl) was added to each well, the plate covered with film and left at 4 °C overnight. A standard curve of known concentrations (0.5 µg/ml, 0.25, 0.125, 0.0625, 0.03125, 0.015625) of calbiochem fibrinogen (Millipore) was made in buffer B (0.0025 M NaH₂PO₄·2H₂O, 0.0075 M Na₂HPO₄·2H₂O, 0.5 M NaCl, 2% Tween20). The collected samples of recombinant protein from the roller bottles were thawed and prepared neat, 1:10 and 1:20 in buffer B. The plate was washed by emptying the wells into the sink, reverse pipetting 200 µl buffer B into each well then pouring off into sink again, 3 times. The fibrinogen samples were loaded onto the plate; 100 µl of each sample per well, each in duplicate. Two 'blank' buffer B wells were also added to the standard curve. The plate was incubated for 2 hr with shaking at 300 rpm at room temperature, after which it was washed 3 times with buffer B. The second antibody was then loaded; 100 µl of 0.625 µg/ml goat polyclonal antibody anti fibrinogen HRP (Abcam, Cambridge, England) in buffer B to each well. The plate was incubated for 1 hr at room temperature with shaking at 300 rpm before washing 3 times with buffer B. 4 DAKO OPD Tablets (2 mg) were dissolved in 12 ml water to a concentration of 0.66 mg/ml with 5 µl H₂O₂. 100µl of this solution was loaded into each well. 100 µl 2 M H₂SO₄ was added to each well to stop the reaction when most wells had reached ~0.5 OD at 490 nm. The plate was read at 490 nm on a MRX Microplate Reader (Dynex Technologies, Ashford, Middlesex, UK).

2.4 Analysis of clot formation and lysis

2.4.1 Turbidity

The turbidity method provides a dynamic measurement of clot formation. As the clot starts to form, optical density increases; this is measured with a plate reader which estimates the amount of light absorbed by the clot when light of a set wavelength is directed through it (Figure 2-3A).

2.4.2 Turbidity and lysis

The lysis method provides a dynamic measurement of clot formation and lysis. As the clot lyses, optical density, and absorbance, decrease until they reach a baseline when the clot is fully dissolved (Figure 2-3B).

2.4.3 Turbidimetric parameters

Several clot formation and lysis parameters can then be calculated from this trace (Figure 2-3):

Lag phase represents the initial stage of clot formation, when fibrinogen is converted to fibrin monomers which start to form protofibrils. During this phase, optical density remains at baseline. The end of lag phase is taken at the time point when there is an exponential increase in optical density above baseline. Short lag times have been associated with thrombogenic clots *ex-vivo*, although not all studies agree^{92, 119, 121}.

Maximum absorbance represents the stage at which the clot is fully formed; optical density increases from baseline as lateral aggregation of the protofibrils formed in lag phase occurs. MA was recorded as the highest reading of optical density from the turbidity trace. This measurement reflects both the thickness of the fibre fibres (purified system and in plasma) and can also measure compactness of plasma clots²³. Clinically, a higher MA of clots prepared from plasma samples indicates a more thrombogenic clot^{23, 119, 121}.

Clot formation time represents the time between the end of the lag phase and the point at which MA is reached. This indicates the rate at which the fibrin fibres form, with long CFT indicating the formation of clots with higher final turbidity or ineffective fibrinolysis. A recent study has shown that longer CFT is associated with worse clinical outcome¹²².

Lysis time represents the time from MA to the time at which 50% of the clot has lysed (i.e. 50% MA on the descending limb of the turbidity curve). This is an indicator of how susceptible the clot is to fibrinolysis, with longer LT associated with increased risk of CVD^{122, 123}.

Lysis area is a complex measure of clot formation, clot maximum absorbance and lysis times. Hence, larger lysis area is associated with more thrombotic clots.

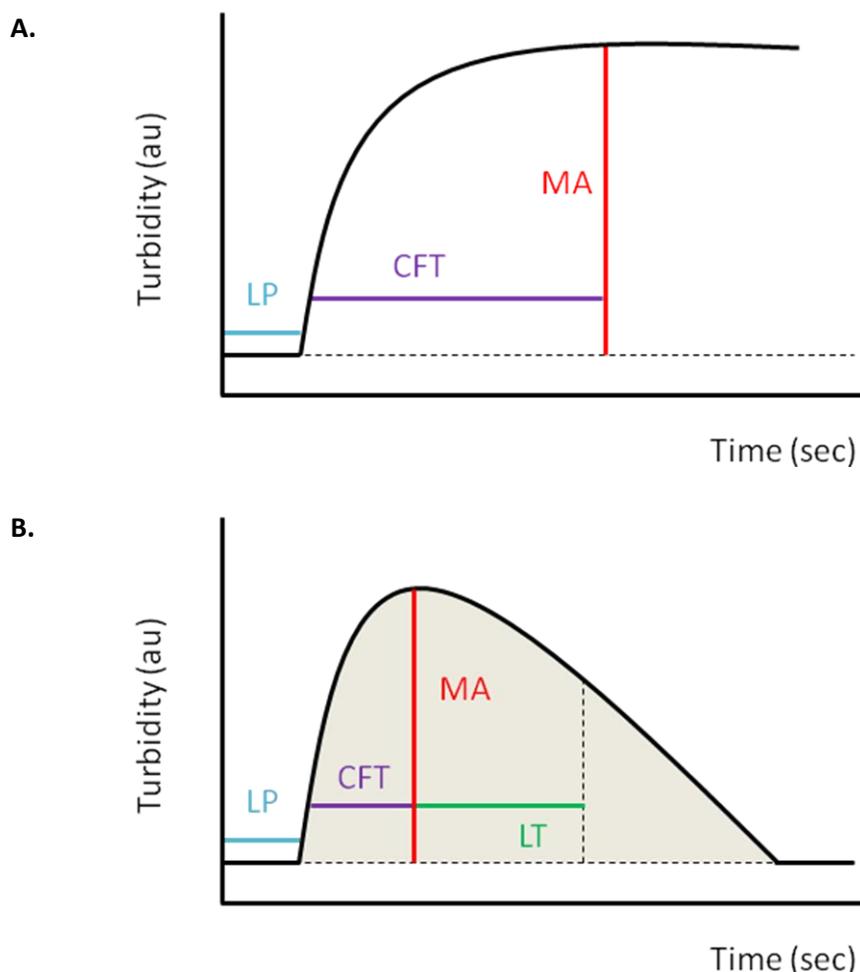


Figure 2-3. Illustration of the turbidimetric and lysis parameters generated
(A) Turbidity only (B) Turbidity and lysis. Lag phase (LP), clot formation time (CFT), maximum absorbance (MA), lysis time (LT) and lysis area (shaded area) are shown.

2.4.4 Clots made from plasma samples

Clot turbidity and lysis measurements were conducted as previously described¹²². A total of 25 μ l of each plasma sample was mixed with 75 μ l lysis mix [LM; 100 mM NaCl, 50 mM Tris, 83 ng/ml tissue plasminogen activator (tPA) (Tecnoclone, Vienna, Austria), pH 7.4], at ambient temperature in a 96 well clear polystyrene flat bottom microplate (Grenier Bio-one, Gloucester, UK). Polymerisation was initiated by addition of 50 μ l of AM [50 mM Tris, 100 mM NaCl, 0.09 U/ml thrombin, 22.5 mM CaCl₂, pH 7.4] and increase in turbidity at 340 nm continuously monitored every 12 sec on a ELx-808 IU ultramicroplate reader (BIO-TEK Instruments INC, Winooski, VT, USA) over a period of 60 min then every 2 min for 9 hr. Four replicates were performed for each variant.

Turbidimetric assays of individual plasma clots were conducted by another member of the team.

2.4.5 Clots made from plasma purified or recombinant protein

Clot turbidity and lysis measurements were conducted as described before¹⁰². A total of 50 µl of purified/recombinant fibrinogen, at 0.5 mg/ml, was mixed with 50 µl LM [1.1 µg/ml FXIII, 18.75 µg/ml plasminogen (ERL, South Bend, IN, USA), in the presence and absence of 10 µg/ml calbiochem antiplasmin (Millipore) in 100 mM NaCl, 50 mM Tris, pH 7.4] in a Greiner 96 well clear polystyrene flat bottom microplate in duplicate. Polymerisation and lysis were initiated by the addition of 50 µl AM [1.5 U/ml thrombin, 0.23 µg/ml tPA and 7.5 mM CaCl₂ in 100 mM NaCl, 50 mM Tris, pH 7.4], at ambient temperature. Four replicates were performed for each variant. Increase in turbidity at 340 nm was continuously monitored every 12 sec on ELx-808 IU ultramicroplate reader over a period of 120 min.

2.5 Western Blotting

A NuPAGE Novex Bis-Tris gel (Invitrogen) was run as described in section 2.1.3.6 and the gel incubated in transfer buffer (0.025 M Tris, 0.2 M glycine, 20% methanol, pH 8.4) with shaking for 10 min. Immobilon-P transfer Polyvinylidene Difluoride membrane (NENTM Life Science Products, Boston, MA, USA), pore size 0.45 µm was cut in the top left-hand corner to orientate on the gel and soaked in methanol (15 sec), ddH₂O water (2 min) and transfer buffer (2 min). A blot mesh (Bio-Rad, Hercules, CA, USA) was opened and onto the black mesh was placed a sponge, an 8x8 cm piece of whatman filter paper (SLS, Nottingham, UK), the gel, the membrane, another filter paper, and then another sponge (all pre-soaked in transfer buffer). A syringe was used to wet each surface with transfer buffer and bubbles rolled out between each surface with a roller. A 'blank' mesh was made if only running one blot by inserting 2 filter papers instead of the gel and membrane. The meshes were then closed and secured before loading onto a rig in a Mini-PROTEAN 3 Cell (Bio-Rad) contained in a plastic tub. An ice brick was added to the tank which was then topped up to the rim with transfer buffer. The transfer was run for 1 hr at 100 V while stirring. Once run the rig was dismantled and the membrane washed in Tris-buffered saline (TBS; 40 mM Tris, 140 mM NaCl, pH 7.4)/ 0.05% Tween20 for 10 min at room temperature with shaking. The membrane was then incubated in blocking solution (5% skimmed milk in TBS/ 0.05% Tween20) overnight at 4 °C and at room temperature for 1 hr. It was washed 4 x 10 min with TBS/ 0.05% Tween20 at room temperature then incubated in blocking buffer with the antibodies in Table 2-1 for 1 hr at room temperature depending on which protein was to be visualised:

Table 2-1. Combinations of antibodies used for western blot analysis

Protein	1°	2°
α2-AP	Goat anti-Human α2-AP HRP (ERL) (1:1000)	N/A
Fibrinogen	Rabbit polyclonal anti-Fibrinogen (DAKO) (1:1000)	Goat anti-rabbit HRP (DAKO) (1:1000)
Albumin	Rabbit anti-Albumin HRP (Abcam) (1:1000)	N/A
Molecular Weight Marker	Streptactin HRP (BioRad) (1:5000)	N/A

If two antibodies were used, the blot was washed 4 times with TBS/ 0.05% Tween20 between the 1° and 2° antibody. The blot was washed in the same way after antibody application and before development. The blot was developed with a 1:1 mix of Supersignal West Pico Luminol (Thermo scientific) and Supersignal West Pico Stable (Thermo scientific) for 5 min after which it was transferred to acetate and chemiluminescence captured using a Kodak Image Station 2000R (Eastman Kodak Company, New Haven, CT, USA).

2.6 Laser scanning confocal microscopy

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

2.6.1 Preparing a clot for LSCM

For plasma clots, 7.5 µl plasma and 1.5 µl 488 Alexa labelled fibrinogen (0.5 mg/ml) (Invitrogen) were mixed with 21 µl PB. 28.5 µl purified/recombinant fibrinogen (0.5 mg/ml) and 1.5 µl 488 Alexa labelled fibrinogen (0.5 mg/ml) were mixed together. An AM of 0.035 M CaCl₂ and 0.35 U/ml thrombin was prepared in PB. 5 µl AM was then added to 30 µl of plasma/fibrinogen mix, mixed thoroughly and 30 µl added to a well of an Ibidi confocal slide (Applied Biophysics, New York, USA). A cover was placed on to the slide which was stored in a humid dark box until imaging.

2.6.2 Imaging fluorescent samples

The slide was visualised with LSM510 software on the LSM 510META microscope from Zeiss (Oberkochen, Germany), using the 488 Argon laser, green reflector and 40x 1.3 oil ph3 objective lens. The slide was loaded onto the specimen stage. Scan mode was then selected (LSM) and single track 'CY2 alexa 488' chosen. Detector gain was set to 999, offset to -0.217 and pinhole to 94. To view the same section of each clot the focus wheel was used to find the top of the clot using the 'fast xy' scanning mode and then the view moved 1.5 fine focus turns into the clot. For static images a 1024² image size was used and the resolution set to 8. For static Z stacks the resolution was reduced to 4. 3 Z-stacks of each clot were taken, with range 10, 20 and 50 μM and intervals 1, 2 and 5 μM respectively.

2.6.3 LSCM clot lysis

Time series function was used with 400 scans with 1 sec between scans. The gain, offset and pinhole were set as above, with resolution at 8 pixels and picture size at 512². 20 μl LM (100 $\mu\text{g}/\text{ml}$ plasminogen, 10 $\mu\text{g}/\text{ml}$ tPA in PB) was added to the Ibidi well and the slide tapped gently for 30 sec to encourage the LM into the clot channel. The slide was then left for 30 sec in a vertical position before loading onto the stage and clot visualisation. The lysis front was identified using fast xy scanning mode and a time series taken at 4 min after addition of LM so that the lysis front just approached the edge of the first frame of the time series. A 'Lysis 2' time series was also taken 30 sec after the lysis front passed the frame of the first lysis. Images were viewed, edited and analysed using LSM image browser (Zeiss).

2.6.4 Clot density by LSCM

A macro was developed to assess clot density in collaboration with Gareth Howell of the Bioimaging Flow Facility at the University of Leeds. This macro works by processing the confocal clot image in Image J (National Institute of Mental Health, Bethesda, Maryland, U.S) in such a way that a single fibre becomes a single black line against a white background, as in Figure 2-4A-B. The macro then places a 20x20 grid (Figure 2-4C) on top of this image from which it can calculate how many times the fibres cross the gridlines. This gives an accurate estimate of how many fibres bundles are present in the image, and thus clot density. Development of a similar macro to measure density of fibres from EM images was attempted. However Image J could not process the image in the same way to produce the clot 'skeleton' as there is not a strong enough contrast between fibrin and background in these images, rather a transition through shades of grey.

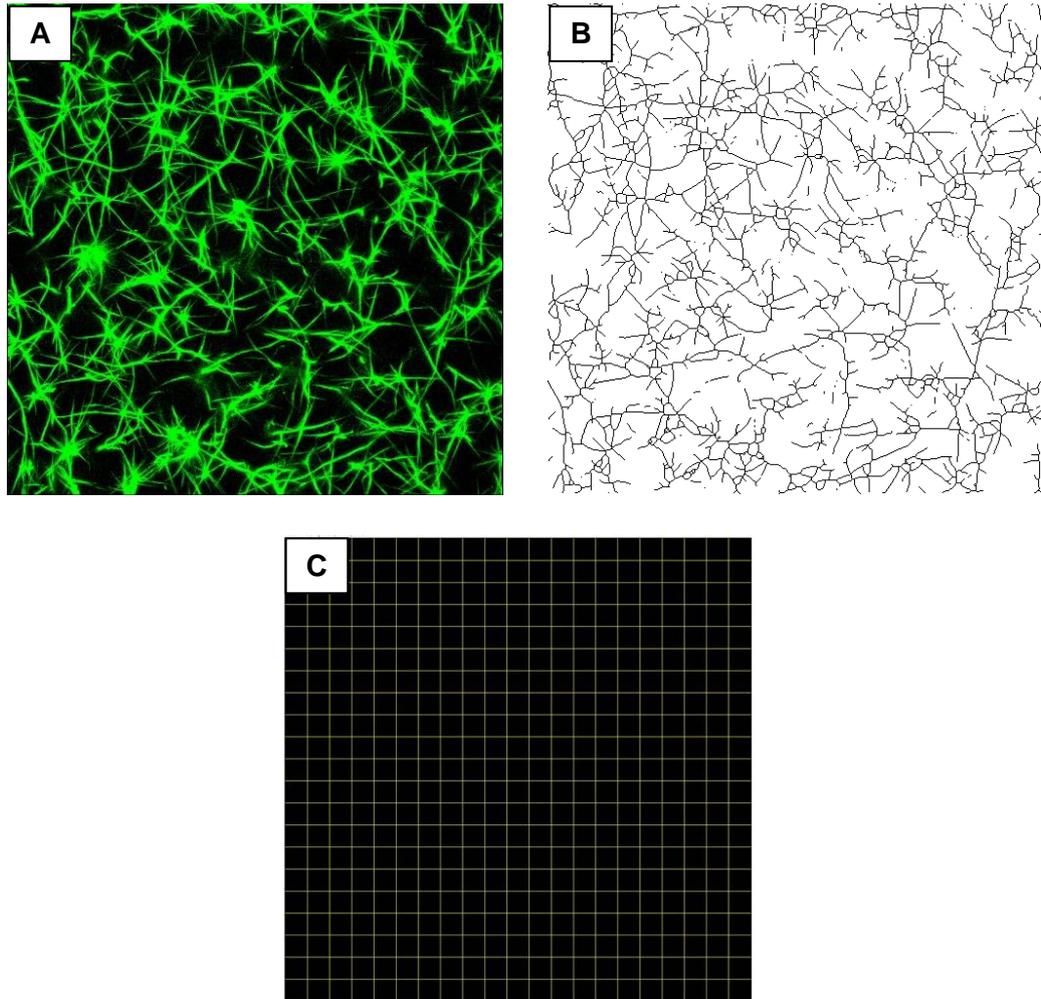


Figure 2-4. Macro processing of LSCM clot images

(A) LSCM image of confocal clot (B) Macro processed image (C) 20x20 image grid. Each time a line on B crosses a line on C a fibre is counted.

2.7 Scanning Electron Microscopy

2.7.1 Preparing a clot for electron microscopy

To prepare a clot for electron microscopy (EM), the lid of a 0.5 ml eppendorf tube was cut off and holes pierced in the lid with a needle and labelled with pencil. The bottom of the lid was wrapped with parafilm to cover the holes.

2.7.1.1 Clot preparation

For purified fibrinogen 45 μ l of fibrinogen (0.5 mg/ml) was mixed with 5 μ l of AM (0.025 M CaCl_2 , 5 U/ml thrombin in PB). For a plasma clot, plasma was diluted 1:2 with PB and 5 μ l AM (0.05 M CaCl_2 , 11 U/ml thrombin in PB) added. The mixture was pipetted up and down with a 200 μ l tip with the end chopped off to evenly distribute

AM. The clots were then left to form in the lids for 2 hr in a humidity chamber before washing.

2.7.1.2 Fixing and dehydration

The parafilm was removed from the bottom of the lid and the clots placed in a beaker of sodium cacodylate buffer (0.078 M Cacodylic acid (Sigma), pH 7.4) for 10 min, the buffer was replaced with fresh buffer and left for another 10 min. The clot was fixed by transferring the lid to 2% Glutaraldehyde (Sigma) in sodium cacodylate buffer for 30 min then washed 2 x 10 min in sodium cacodylate buffer. The buffers were stirred gently with a flea while washing and fixing. The clots were then dehydrated by sequentially transferring them from 30% to 50% to 70% to 80% to 90% to 95% acetone for 10 min each. They were then transferred to fresh 100% acetone 3 times for 10 min before being transferred to fresh 100% acetone ready for critical point drying.

2.7.1.3 Critical point drying

Critical point drying was carried out by Martin Fuller at the University of Leeds using a E3000 critical point dryer (Quorum Technologies Ltd, UK). This technique dehydrates the fibrin structure so that it can be coated with platinum in order to be detected under the EM. Since EM analyses the surface morphology of biological specimens, it is imperative that the surface of the fibrin is not disturbed in the drying process. This means air drying is unsuitable for this application as it can cause deformation and collapse of protein structures. This is because water has a high surface tension to air and thus the specimen is subject to considerable forces at the boundary between the water in the sample and the surrounding air as the liquid in the sample evaporates, at the so called 'phase boundary'. In CPD, this surface tension is reduced by replacing the water in the sample with liquid CO₂, using acetone as an intermediate, progressively lowering the surface tension between the sample liquid and surrounding air. It is then possible for the liquid CO₂ to pass from liquid to gas without an abrupt change in state, thus avoiding damage to the surface of the sample.

2.7.1.4 Sputter coating

The clots were then mounted onto aluminium specimen mounts covered in a carbon film using tweezers. The clots were then coated in with a 7 nm thick conducting layer of platinum palladium applied in a 208HR high resolution sputter coater (Cressington, UK). Covering the sample in a thin layer of conducting metal prevents the sample from charging under the electron beam.

2.7.2 Imaging sample

Samples were viewed and photographed using a Quanta 200F FEGSEM field-emission scanning electron microscope (FEI, Oregon, USA) in 3 different areas of each clot. Images were captured using FEI's own software, designed to run with the EM. Fibre diameters of all clots were measured with image analysis software package ImageJ 1,23y.

2.8 FXIII purification

It was necessary to purify FXIII as the group stock ran out midway through my research. The commercial FXIII that is available has a large amount of albumin and other impurities in that can affect the clotting process. To eliminate the effect of any of these contaminants, pure FXIII was isolated from this product by affinity chromatography for use in my studies. A sepharose 6B 200 ml size exclusion column was attached to the BioCad ensuring that there was no air in any part of the tubing. The column was equilibrated for 2 hr with PB at a flow rate of 0.5 ml/min. Two vials of Fibrogammin P (ZLB Behring, King of Prussia, PA 19406, United States) (250 U) were reconstituted in 5 ml of pure water by gentle mixing. The sample was injected onto the BioCad and buffer flow resumed at 0.5 ml/min collecting fractions every 3 ml over 1.2 CV (240 ml). The purified FXIII_{A₂B₂} was eluted in the second of four peaks on the chromatograph. The fractions within this peak were immediately kept on ice to avoid protein activation or precipitation. An aliquot of each fraction was analysed on a non-reducing SDS-PAGE gel to determine sample purity. Those fractions of sufficient purity were pooled and concentrated by centrifugation using vivaspin MXCO100 spin columns, 4,500 rpm for 5 min and final concentration determined using a Nanodrop ND-1000 spectrophotometer, extinction coefficient 1.31. Pure FXIII_{A₂B₂} was aliquoted into appropriate volumes to avoid freeze-thawing and stored at -80 °C. Activity of the purified protein was tested using the biotin labelled pentylamine incorporation FXIII activity assay as in section 4.1.9.1.

2.9 Atomic Force Microscopy

2.9.1.1 Sample preparation

To visualise individual fibrinogen molecules before polymerisation a 15 µl volume of 0.17 mg/ml fibrinogen was diluted in 1530 µl MilliQ water. 50 µl of this mixture was then pipetted on to a mica and allowed to incubate for 2 min. Mica is graphite like material which is attached to the stage on which the sample is mounted by superglue. This mica material is used for its layered texture, these extremely thin layers can be

split and separated to reveal a new clean layer beneath, which is chemically inert and very smooth. Before the sample is loaded onto the mica, the top layer is cleaved by attaching and then removing sellotape to the surface, taking the top layer with it. Since the mica and fibrinogen molecule are both negatively charged, a new layer of positively charged material is required to help attract fibrinogen to mica. This was done by treating the freshly cleaved mica with 2 mM NiCl_2 for 10 min. The surface was then washed by flooding with water and dried with pressurised nitrogen after which it was ready for sample loading. Once the sample had incubated for 2 min, the surface was rinsed with MilliQ water 10 times and dried under nitrogen. To visualise the early stages of fibrin polymerisation, when the protofibrils are beginning to form it was necessary to look at the turbidity curves produced. As the stage we are interested in visualising is the lag phase, an appropriate concentration of thrombin to be used had to be determined. This concentration would allow normal clot formation, but a long enough lag phase to stop the clotting reaction before gel point, when fibrin fibres begin to assemble (when OD begins to rise).

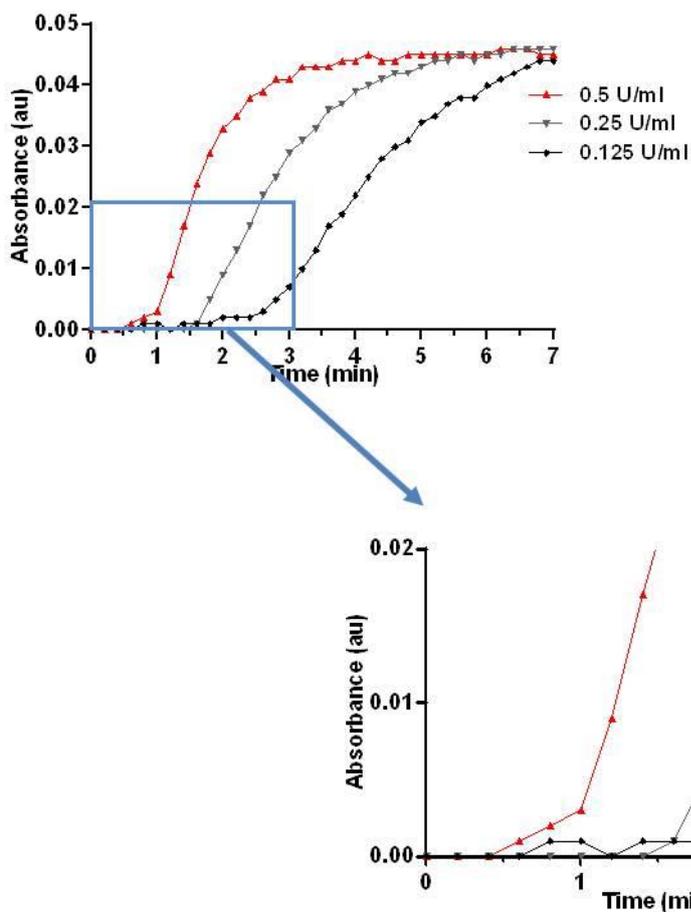


Figure 2-5. Effect of thrombin concentration on lag time of fibrin polymerisation

A lag time of 90 sec before OD increased was obtained with a final thrombin concentration of 0.25 U/ml in these conditions which is appropriate for use in AFM sample preparation.

From turbidimetric analysis, a final thrombin concentration of 0.25 U/ml seemed apt for AFM analysis, as this produced a lag time of 90 sec before OD began to rise from baseline. From this information, to visualise fibrin fibril formation, fibrinogen (0.17 mg/ml) was pre-mixed with 2.5 mM CaCl_2 to a final volume of 10 μl in a 1.5 ml tube. 5 μl thrombin was added (final concentration 0.25 U/ml) and the reaction allowed to progress for 30, 60 and 90 sec. At each time point, the reaction was stopped by dilution by addition of 1530 μl PB. This was applied to a mica pre-treated the NiCl_2 using the same method as for fibrinogen monomers.

2.9.1.2 Imaging

The AFM is essentially a feedback system. It is composed of a sharp tip at the end of a cantilever, a laser, a photodiode detection system and the feedback mechanism. The sample surface can be scanned by the tip in various 'modes'. The system relies on the forces between the tip and the sample (e.g. Van de Waals, dipole-

dipole and electrostatic forces) affecting the deflection of the cantilever, which change with distance between the two. This deflection is detected by a laser which is directed onto the top of the tip and reflected into a photodiode. As the cantilever and tip move, so will the reflected laser on the photodiode as depicted in Figure 2-6.

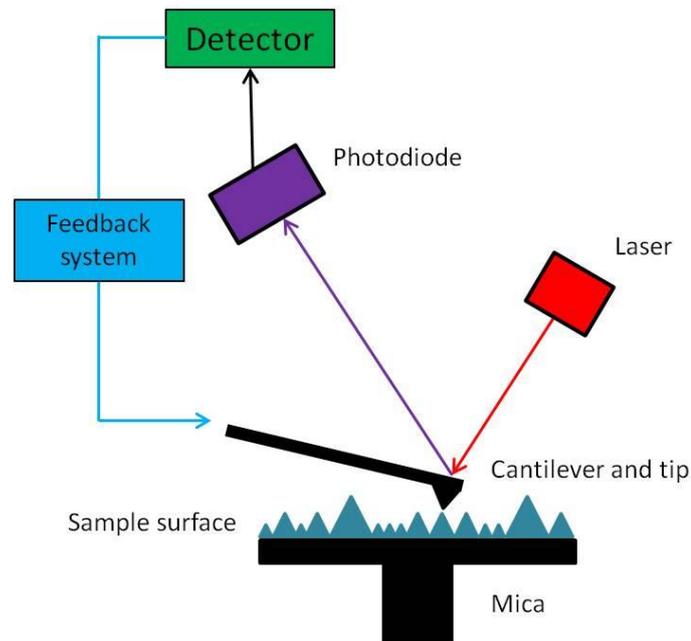


Figure 2-6. Diagrammatical representation of the AFM detection and feedback mechanism

A laser is reflected off the top of the tip at the end of a cantilever into a photodiode. When the sample is scanned, vertical movements of the tip/ cantilever due to the topography of the sample surface are thus detected as movement of the reflected laser.

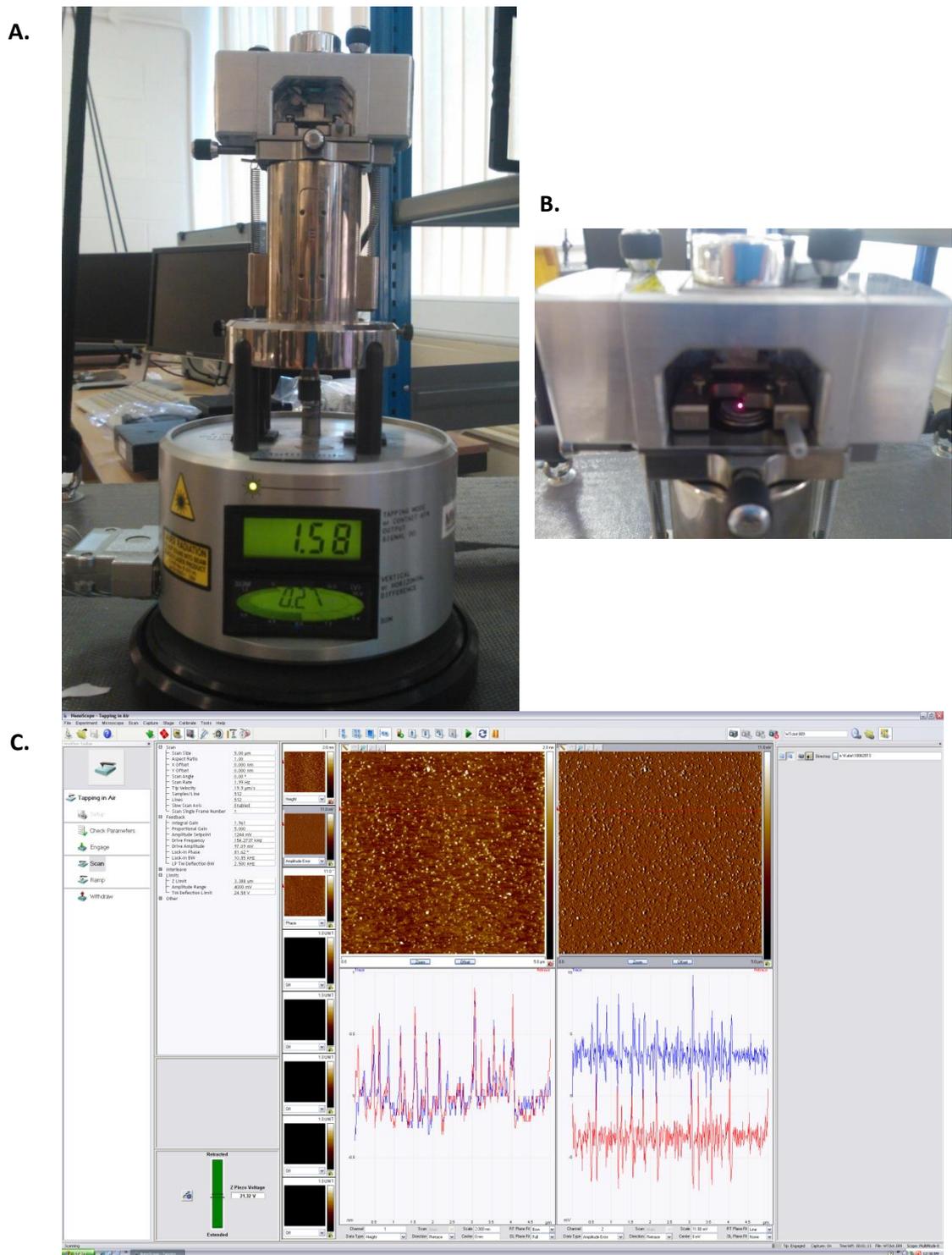


Figure 2-7. Photographs of AFM equipment

(A) Microscope assembled on elevated platform (B) Microscope 'reader' with tip inserted; detection laser can be seen reflected off the tip and the sample surface (C) Nanoscope software interface.

The AFM can be operated in a number of modes, divided into contact and non-contact modes.

2.9.1.2.1 Contact mode

In this mode the cantilever is in constant contact with the sample surface, across which it is dragged. The contours of the surface are measured directly from the deflection of the cantilever. This mode is appropriate only for very hard sample surfaces, as the cantilever can damage softer samples.

2.9.1.2.2 Non-contact mode

In this mode, the cantilever is oscillated above the surface of the sample. Interactions between the tip and the sample are capable of altering the oscillation amplitude. These changes in oscillation are detected and analysed to produce a topographical image of the surface. Since the tip does not contact the surface in this mode, the sample is not damaged. This makes this mode ideal for soft biological samples.

2.9.1.2.2.1 *Tapping mode*

Samples in this study were imaged using tapping mode AFM. This mode bypasses the problem often encountered in non-contact mode AFM in which the tip 'sticks' to the sample surface due to short range forces developed by a liquid meniscus forming on the sample surface in ambient conditions. Similarly to general non-contact mode, the cantilever is driven to oscillate up and down. However in this mode, the AFM adjusts the height of the cantilever in reaction to changes in forces between the tip and the surface when they approach each other, which would otherwise cause the amplitude of the oscillation to decrease. This feedback mechanism maintains set oscillation amplitude, producing an image by measuring the force of the intermittent contacts between the tip and surface. Image processing and analysis was performed using Nanoscope software (Veeco Instruments)

2.10 Mass Spectrometry

2.10.1 Principles of mass spectrometry

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

Since fibrinogen is large protein with three separate covalently, disulfide linked chains, it is important to reduce the protein before MS analysis as analysis of the whole protein would generate a single peak in a spectrum which would not give enough information about the individual protein chains. Following reduction, the α , β and γ chains were separated by SDS-PAGE and further fragmented by enzyme digestion with trypsin. Trypsin has a well-defined cleavage specificity, cleaving proteins on the C-terminal side of lysine and arginine residues, except when they are immediately followed by proline. Consequently, trypsin treatment of proteins yields peptides with an average length of about ten to fifteen amino acid residues, ideal for MS detection.

Having fragmented the protein into peptides, these fragments are subject to tandem MS analysis (MS/MS) as illustrated in Figure 2-8. Firstly, the masses of the sample fragments are determined in a MS survey scan. The peptide ions of interest are then isolated via their mass-to-charge ratio value by filtering away other ion species with different m/z values. The selected peptide ion species are activated by collisions with an inert gas such as Argon that imparts internal energy into the ions and thereby induces further fragmentation. The fragment ions are projected through the mass analyser and the m/z values determined. The presence of PTMs in proteins affects the molecular mass of the modified amino acids, which are detected by a mass increment or deficit on the MS/MS spectra.

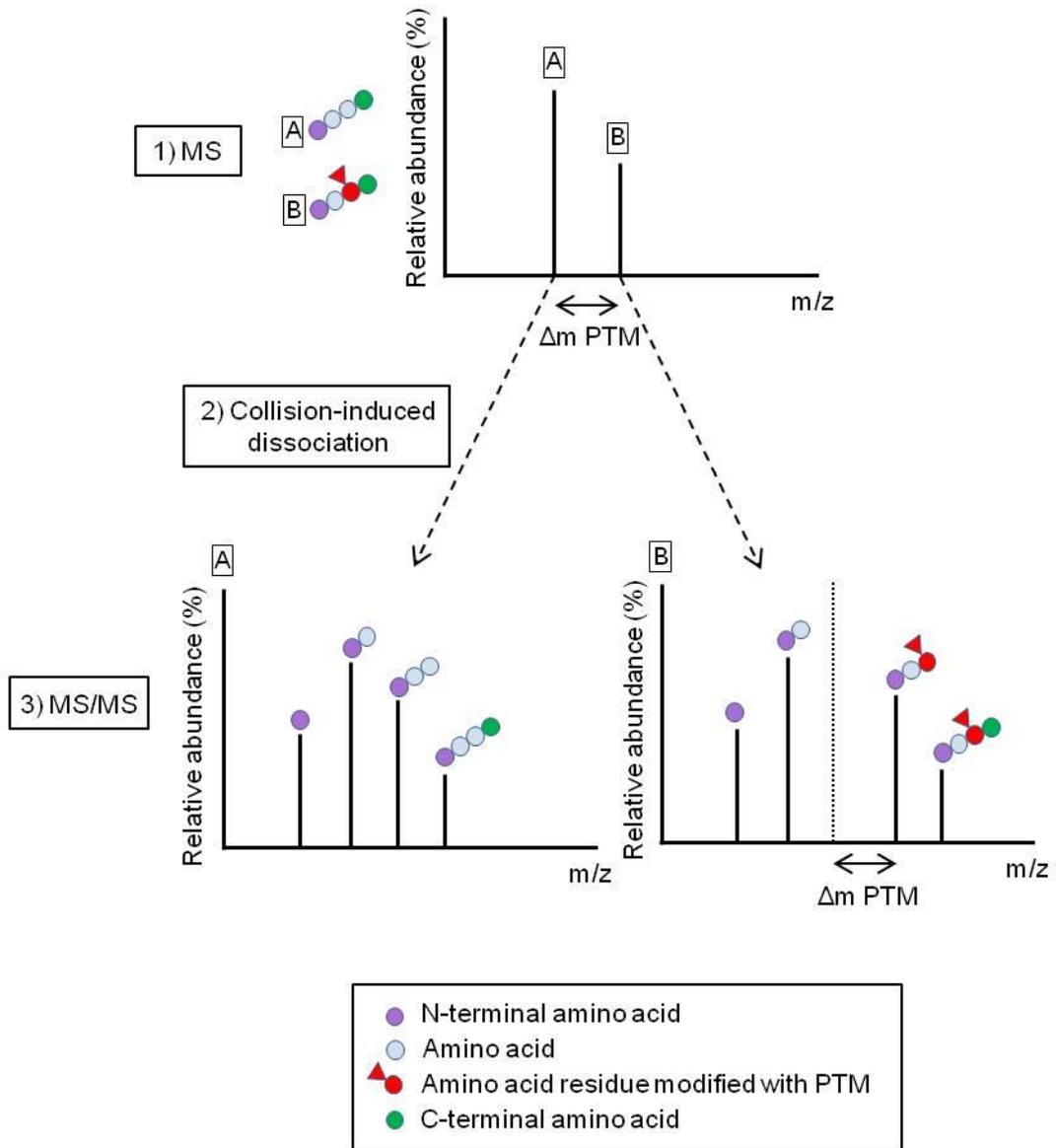


Figure 2-8. Diagrammatic representation of MS/MS for mapping of PTMs

The MS spectrum is acquired to determine the molecular mass of the peptides. Fragmentation of the peptide by collision induced dissociation produces a set of fragment ions that are then subjected to MS/MS analysis which produces a spectrum of relative abundance of ions as a function of their mass-to-charge ratio. The presence of a PTM will change the mass of the modified amino acid residue and of the peptide and cause a shift in the spectrum.

This technique is advantageous as it is highly sensitive, effective down to the femtomole scale thus requiring very little sample. It is able to detect molecular mass shifts corresponding to modifications at both the peptide and protein level; capable of distinguishing between the type of PTM and its location. However, this technique has several limitations; digestion, ionization and MS/MS activation can lead to dissociation

of the PTM from the molecule if the covalent bond between the amino acid and the PTM adjunct is chemically unstable. While certain PTMs will remain intact during MS and MS/MS experiments, some may not be detected if they dissociate in the preparation processes inherent to this technique. There is the added complication of isolating the site of modification in a peptide of a size suitable for mass spectrometric analysis, especially as the presence of PTMs can affect the cleavage efficiency of trypsin, generating unexpected or large peptide products. For these reasons, it is often useful to consider and explore several approaches for mapping of PTMs in proteomics¹²⁴. Consequently an electroelution technique was attempted in order to separate the chains without the need to excise them from a gel and digest with trypsin, this is advantageous because it has the potential to separate the fibrinogen chains to take intact mass measurement to determine modification profile.

2.10.2 Gel processing and tryptic digestion

A sample of each fibrinogen was run by SDS-PAGE and stained as described in section 2.1.3.6. Gel bands were excised and chopped into small pieces (~1 mm³), covered with 30% ethanol in a 1.5 ml microcentrifuge tube and heated to 70 °C for 30 min with shaking. The supernatant was removed and replaced with fresh ethanol solution and the digest heated to 70 °C for 30 min. This was repeated until all coomassie stain was removed from the gel. The destain solution was replaced with 50 µl 25 mM ammonium bicarbonate and was vortexed for one hr. The wash solution was discarded and the gel slices covered with 25 mM ammonium bicarbonate/ 50% acetonitrile and vortexed for ten min. The gel slices were then covered with 100% acetonitrile and left for five min with vortexing before the supernatant was discarded and replaced with a fresh aliquot of acetonitrile. Acetonitrile was removed and the gel pieces were completely dried under vacuum centrifugation for 30 min. Once dry, the gel slices were cooled on ice. The gel slices were then covered with ice cold trypsin solution (20 ng/µl in 25 mM ammonium bicarbonate) and left on ice for 30 min to rehydrate. Excess trypsin solution was removed and the gel slices were covered with a minimal amount of 25 mM ammonium bicarbonate. After briefly vortexing and centrifuging, the gel slices were incubated at 37 °C with shaking for 18 hr. The resulting digest was vortexed, centrifuged and 50 µl water was added. Following vortexing for 10 min, the supernatant was recovered and added to an eppendorf containing 5 µl acetonitrile/ water/ formic acid (60/ 35/ 5; v/v). 50 µl acetonitrile/ water/ formic acid (60/ 35/ 5; v/v) was added to the gel slices and vortexed for an additional 10 min. The supernatant was pooled with the previous wash and one additional wash of the gel slices was performed. The pool of three washes was dried by vacuum centrifugation.

The peptides were reconstituted in 20 µl acetonitrile/ water/ formic acid (2/ 97.9/ 0.1; v/v).

2.10.3 Liquid chromatography-mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS/MS) analysis of the peptide mixtures was performed on an Ultimate 3000 nano LC system (Dionex, Amsterdam, Netherlands). 2 µl of each sample in water was loaded onto a C18 guard column and washed with 2% acetonitrile/ 0.1% formic acid for 5 min at 25 µl/min. After valve switching, the peptides were then separated on a PepMap C18, 100 µm i.d.x15 cm analytical column (Dionex, Amsterdam, NL) by gradient elution of 2-60% solvent B (0.05% formic acid in 20% water/ 80% acetonitrile) in solvent A (0.05% formic acid in 98% water/ 2% acetonitrile) over 60 min at 0.3 µl/min. The column eluant was directly interfaced to a quadrupole-ion mobility-orthogonal time of flight mass spectrometer (Synapt HDMS, Waters UK, Manchester) via a Z-spray nanoflow electrospray source. The MS was operated in positive TOF mode using a capillary voltage of 3.2 kV, cone voltage of 25 V, backing pressure of 2.47 mbar and a trap bias of 4 V. The source temperature was 80 °C. Argon was used as the buffer gas at a pressure of 5.0×10^{-4} mbar in the trap and transfer regions. Mass calibration was performed by a separate injection of sodium iodide at a concentration of 2 µg/µl. GluFib was infused as a lock mass calibrant with a one sec lock spray scan taken every 30 sec during acquisition. Ten scans were averaged to determine the lock mass correction factor. Data acquisition was using data dependent analysis with a one sec MS over an m/z of 350-3000 being followed by three 1 sec MS/MS taken of the three most intense ions in the MS spectrum. Collision energy applied was dependent upon charge state and mass of the ion selected. Dynamic exclusion of 60 sec was used. Data processing was performed using the MassLynx v4.1 suite of software supplied with the mass spectrometer. Peptide MS/MS data were processed with ProteinLynx Global Server (Waters) and searched against UniProtKB/SwissProt database (release 2011/12).

2.10.4 Electroelution

50 µl protein sample (0.5 µg/µl) was diluted with 30 µl 5x acetate sample buffer (Expedeon Ltd, Harston, UK), 8 µl 1 M DTT and 62 µl deionised water. The sample mixture was heated at 50 °C for 10 min. The sample was cooled to room temperature and applied to the loading reservoir of a 10% Tris-Acetate GELFREE cartridge. The collection and electrode reservoirs contained HEPES running buffer (Expedeon, Harston, UK). The proteins were separated on the tube gel in the cartridge and eluted into the collection reservoir. Following GELFREE separation, the protein resolution was visualised by running a 1D PAGE 10% Tris-Glycine gel. Appropriate fractions were

chosen for detergent removal and MS analysis of the intact chains. All mass spectrometry laboratory work and data analysis was performed by James Ault of the Faculty of Biological Sciences, University of Leeds.

2.11 Statistical analysis

Analysis of the data was done using SPSS program version 16 and statistical significance accepted at $p < 0.05$. Between group comparisons of normally distributed variables were carried out using independent samples student t-test. Unless otherwise specified, data is presented as mean \pm standard error of the mean (SEM). Unless otherwise specified, a student t-test was used to assess statistical significance. The denotation of statistical significance is as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$. More specific statistical analyses are detailed in corresponding sections.

**Chapter 3. B β Arg448Lys Variants of Fibrinogen: Effects on
Fibrin Clot Structure & Fibrinolysis in Type 2 Diabetes**

Introduction

Despite advances in medical therapy, diabetes is a major health problem and cardiovascular complications remain the main cause of mortality in this population. The condition currently affects 347 million people worldwide and is projected to be the 7th leading cause of death by 2030 (www.who.int). The rapidly increasing global prevalence of diabetes is a significant cause for concern as the risk of mortality in diabetes subjects due to atherothrombotic complications is equal to that of nondiabetic individuals with a history of ischemic heart disease^{120, 125, 126}. In addition to increased risk of first atherothrombotic event, prognosis following vascular ischemia has barely improved, in contrast to individuals without diabetes^{1, 109, 110, 120, 125-127}. Current evidence indicates the adverse clinical outcome in diabetes following vascular ischemia is due to more extensive vascular pathology, increased thrombosis potential and high prevalence of heart failure, which is partly related to atherothrombotic complications^{120, 126}. The thrombotic milieu in diabetes plays a key role in increased cardiovascular risk in this population. Diabetes is associated with qualitative and quantitative changes in procoagulant, antifibrinolytic and inflammatory proteins (e.g. platelets, tissue factor, thrombin, PAI-1 and fibrinogen) leading to fibrin clots with tight network structure and resistance to lysis, predisposing to a thrombotic environment^{109, 110, 125}.

Fibrinogen B β Arg448Lys is one of the most common genetic polymorphisms, affecting around 25% of the white population, and is associated with both increased vascular risk and hypofibrinolysis^{1, 120, 126}. When compared to B β 448Arg, clots made from B β 448Lys variant fibrinogen have a compact structure of thin fibres and small pores, with increased stiffness and greater resistance to lysis⁹⁹⁻¹⁰². It is worth bearing in mind that this polymorphism is associated with elevated levels of fibrinogen in plasma due to its strong linkage disequilibrium with two other polymorphisms (B β Gly455Ala and B β Cys148Thr), lying in areas affecting gene transcription, thereby determining fibrinogen levels⁹⁹. The differences in fibrin structure of clots made from B β 448Lys and B β 448Arg variants of fibrinogen have been demonstrated using a recombinant system¹⁰², therefore directly implicating the polymorphism in the changes observed. This simple substitution of one polar residue for another can lead to the loss of multiple possible hydrogen bonds formed with the arginine residue which may play a role in conformation of the α C-terminal and thus affect lateral aggregation of fibrils, consistent with the formation of thinner fibres with B β 448Lys variant.

Previous work indicates that both diabetes and B β Arg448Lys variants of fibrinogen modulate cardiovascular risk and induce thrombotic changes in fibrin networks. Despite the relatively high prevalence of both conditions and their role in atherothrombotic conditions, the effect of B β Arg448Lys on clot structure in diabetes remains unknown. Therefore, the primary aim of this chapter was to identify any

additional effect of B β Arg448Lys on fibrin network in a condition with complex pathogenesis, such as T2DM. Secondary aims of the work include analysis of the role of polymorphism on previous and existing vascular pathology in this population. This may help to discover a high risk group for thrombotic events and help encourage more aggressive preventative and treatment strategies in this subset of patients.

3.1 Methods

3.1.1 Study population and examination

The study protocol of the Edinburgh Type 2 Diabetes Study has been described¹²⁸ and I summarise the parameters studied in relation to my work. All the clinical data have been collected by our collaborators, Dr. J Price and Dr. M Strachan in Edinburgh. A total of 1066 patients with T2DM (age, 60–75 yr) were randomly recruited; they are representative of those invited to participate (n=5454)¹²⁹. Of these, 822 plasma samples were available for genotyping and clot structure analysis.

3.1.2 Diagnosis of Type 2 Diabetes

Patients were recruited from the Lothian Diabetes Register (LDR), which includes those patients whose diagnosis of diabetes has been confirmed according to WHO criteria. Confirmation of diagnosis of type 2 diabetes was carried out by review of recorded data of potential participants; this was accepted as any individual treated with oral anti-diabetic agents and/ or insulin, or dietary modification with glycated haemoglobin (HbA1c) >6.5%. Patients were excluded if confirmation of a clinical diagnosis of type 2 diabetes was not possible by hospital or GP record review.

3.1.3 Diagnosis of previous CVD

Participants completed a questionnaire including questions on diabetes history and treatment, CVD and other co-morbidities (angina, CHD/ MI, stroke, peripheral arterial disease, carotid stenosis, hypertension and hypercholesterolaemia), medications and smoking habits. Data from hospital records and primary and secondary care data on the LDR were also collected to supplement self-reported history of CVD and diabetic complications as well as biochemical and clinical variables such as HbA1c, BP, serum cholesterol and glucose levels.

3.1.4 Diagnosis of current CVD risk factors and vascular disease

Participants also underwent physical examination for current CVD risk and vascular disease.

3.1.4.1 Blood pressure

Systolic and diastolic brachial blood pressures were measured in the right arm using a standard stethoscope and aneroid sphygmomanometer.

3.1.4.2 BMI

Standing height was measured without shoes using a wall-mounted vertical rule, and weight assessed without outdoor clothing or shoes using electronic scales. These measures were used to calculate BMI.

$$\text{BMI} = \text{Mass (kg)} / (\text{Height (m)})^2$$

3.1.4.3 Ankle brachial index

To assess ankle brachial index (ABI) right and left brachial as well as posterior tibial and dorsalis pedis systolic pressures were recorded after 5 min rest using an aneroid sphygmomanometer and Doppler probe. The lowest ankle pressure was divided by the highest brachial pressure to calculate ABI.

3.1.4.4 Intima media thickness

Bilateral carotid intima-media thickness (IMT) was assessed using an ultrasound imaging system to measure the far wall of the artery, 1-2 cm below the carotid bifurcation in an area free of plaque.

3.1.5 Collection of blood samples

Blood samples were taken into citrated tubes without a tourniquet after a 4 hr fast, usually at midday. The first 5 ml were used for clinical tests (DNA extraction, measurement of fibrinogen, glucose, total cholesterol, low and high density lipoprotein cholesterol, HbA1c and total protein levels, full blood count and estimated glomerular filtration rate (eGFR)) and platelet poor plasma separated from subsequent samples.

3.1.6 Genotyping for B β Arg448Lys

DNA was purified from blood samples and genotyping for B β Arg448Lys variants of fibrinogen was performed as previously described¹³⁰. Briefly, genomic DNA was isolated from whole blood by standard procedures and genotyping was carried out by KBioscience (Herts, UK) using their in-house chemistry of Competitive Allele Specific polymerase chain reaction (PCR).

3.1.6.1 Assessment of fibrinogen plasma levels

Fibrinogen levels were measured using the Clauss method⁹⁰ as described in section 2.2. This was done in Edinburgh and I was not involved in conducting the laboratory analysis.

3.1.7 Purification of fibrinogen

Pooled plasma samples were chosen for each variant (Arg/Arg, Arg/Lys and Lys/Lys; n=12 samples for each group) which were matched for sex and HbA1C levels. Fibrinogen was purified by affinity chromatography using a calcium dependent IF-1 monoclonal antibody (Kamiya Biochemical, Seattle, WA, USA) and an automated chromatography system (Biocad sprint, Applied Biosystems, Warrington, UK), as described in section 2.1.3.

3.1.8 Turbidity and Lysis

Clot turbidity and lysis measurements were conducted for plasma samples and for purified fibrinogen as previously described in section 2.4.

3.1.9 Confocal and electron microscopy

These were conducted as previously described in section 2.6 and 2.7.

3.1.10 Statistical analysis

Exploratory analysis was done using SPSS program version 16 (SPSS Inc., Chicago, IL). Between-group comparisons of normally distributed variables were carried out using t-test, non-normally distributed variables were log transformed before a t test was carried out. The R environment for statistical computing was used to fit logistic regression models to identify independent associations between B β Arg448Lys polymorphism and vascular disease. The effect of predictors in the regression model was characterised using 95% confidence intervals for the odds ratio coefficients. Statistical advice was provided by Dr. Paul Baxter.

3.2 Results

3.2.1 Clinical characteristics of patients

Clinical characteristics of patients are described in Table 3-1. B β 448Lys variant was present in 31.5% of subjects with similar frequency in each gender (30.6% in male, 32.3% in females, $p=0.604$). Cardiometabolic risk factors, including blood pressure, BMI, waist circumference, waist/hip ratio, HbA1c and lipid profile were similar in the two variants of fibrinogen. There was no difference in eGFR between the groups. Also, there was no difference in treatment comparing individuals with B β 448Arg and B β 448Lys variants of fibrinogen.

Table 3-1. Clinical characteristics, clot structure parameters and protein levels in carriers of B β Arg448Lys variants of fibrinogen

Variable	B β 448Arg	B β 448Lys	P-value
N	564	258	
Age, yr (range)	68.3 (60-75)	67.6 (60-75)	0.03
Duration of T2DM, yr (\pm SEM)	8.05(\pm 0.28)	8.19(\pm 0.39)	0.78
Current smokers, n (%)	71 (12.6)	36 (14.0)	
Vascular parameters			
Systolic BP, mmHg (\pm SEM)	133.6(\pm 0.7)	132.1(\pm 1.0)	0.21
Diastolic BP, mmHg (\pm SEM)	69.0 \pm (0.4)	68.5 \pm (0.5)	0.43
Anthropometrics			
Body mass index, kg/m ² (\pm SEM)	31.0 \pm (0.2)	31.9(\pm 0.4)	0.28
Waist circumference, cm (\pm SEM)	106.3(\pm 0.6)	107.7(\pm 0.8)	0.15
Waist/hip ratio (\pm SEM)	0.96 \pm (0.004)	0.96 \pm (0.006)	0.77
Metabolic and renal			
HbA1c, %/ mmol/mol, (\pm SEM)	7.4(\pm 0.05)/ (57 \pm 0.5)	7.4 \pm (0.07)/ 57(\pm 0.8)	0.90
Plasma glucose, mmol/l (\pm SEM)	7.48(\pm 0.09)	7.62(\pm 0.12)	0.34
Total cholesterol, mmol/l (\pm SEM)	4.3(\pm 0.04)	4.3(\pm 0.05)	0.76
HDL cholesterol, mmol/l (\pm SEM)	1.3(\pm 0.01)	1.3(\pm 0.02)	0.37
eGFR, ml/min/m ² (\pm SEM)	64.4(\pm 0.61)	63.6(\pm 0.9)	0.44
Treatment, n (%)			
Metformin	344 (61.0)	142 (55.0)	0.48
Aspirin	364 (64.5)	171 (66.3)	0.82
Insulin	57 (10.1)	33 (12.8)	0.31
Thiazolidinediones	93 (16.5)	45 (17.4)	0.78
ACEi & ARB	383 (67.9)	174 (67.4)	0.95
Statins	477 (84.6)	211 (81.8)	0.76
Sulfonylurea	180 (31.9)	69 (26.7)	0.27
Nitrates	99 (17.6)	44 (17.1)	0.88
Clot structure parameters			
Lag phase, sec (\pm SEM)	528.3(\pm 5.3)	524.8(\pm 7.3)	0.70
Maximum absorbance, au (\pm SEM)	0.35(\pm 0.004)	0.37(\pm 0.006)	0.02
Lysis time, sec (\pm SEM)	719(\pm 15)	761(\pm 20)	0.003
Plasma protein levels			
Fibrinogen, mg/ml (\pm SEM)	3.59(\pm 0.03)	3.70(\pm 0.04)	0.03

3.2.2 Effect of B β 448 variant on clot characteristics of T2DM patients

Clot MA in carriers of B β 448Lys was 5.7% higher than B β 448Arg, LT 5.8% longer and lysis area 8.2% greater. The effect of the B β Arg448Lys polymorphism on LT was still significant after adjusting for observed differences in fibrinogen levels between the variants. There was no significant difference in lag time between B β 448Arg and B β 448Lys plasma. Results are summarised in Figure 3-1.

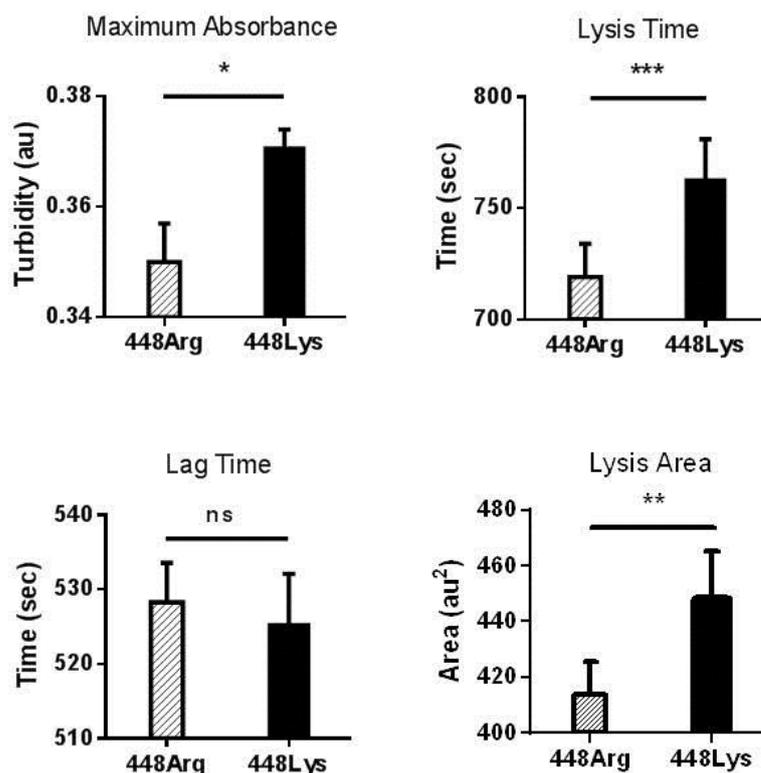


Figure 3-1. Plasma clot parameters in T2DM patients with B β 448Arg and B β 448Lys fibrinogen as measured by turbidimetric assay

Maximum absorbance was increased with B β 448Lys compared with B β 448Arg genotype suggesting the formation of denser clots. Lysis time was longer in patients with B β 448Lys variant, indicating compromised fibrinolysis. Lag time showed no difference comparing the two genetic variants of fibrinogen. Lysis area was larger with B β 448Lys compared with B β 448Arg variant. Lysis time and lysis area were log transformed before a t test was carried out.

3.2.3 Effect of gender on clot structure of T2DM patients

It has been previously demonstrated that clot maximum absorbance in female subjects with diabetes is higher than males and LT longer¹²². In the cohort used in this study, similar changes were seen; clot MA in female subjects with diabetes was 11.8% higher than males and LT 16.1% longer. There was no significant difference found in

lag time between males and females in the previous cohort or the one used in this study. Though not reported in the previous cohort, in this cohort lysis area was 23.6% greater in females than males. Consequently, the association between the B β Arg448Lys polymorphism and clot structure in the present cohort was analysed separately by gender.

Clot MA in carriers of B β 448Lys was numerically higher than B β 448Arg in both males (2.9%) and females (5.4%) though this failed to reach significance in both cases. LT in carriers of B β 448Lys was marginally longer than B β 448Arg in males (4.1%), with a bigger difference observed in females (6.6%). There was no significant difference in lag time in B β 448Lys subjects when compared to B β 448Arg in either males or females. Lysis area in carriers of B β 448Lys was similar to B β 448Arg in males, whereas a 13.1% increase was detected in females. When adjusted for fibrinogen levels, only the difference between LT in females remained significant.

Changes in MA, LT and lysis area were significant between males and females when both fibrinogen B β Arg448Lys variants were analysed separately. MA was greater, LT longer and lysis area greater in females when compared to males with both B β 448Arg and B β 448Lys. Differences in lag time were not significant between males and females when split by B β 448 variant.

Overall, females with B β 448Lys variant of fibrinogen had the most thrombotic clot phenotype with an associated resistance to fibrinolysis, whereas males with B β 448Arg had the most benign clot structure. Results summarised in Figure 3-2.

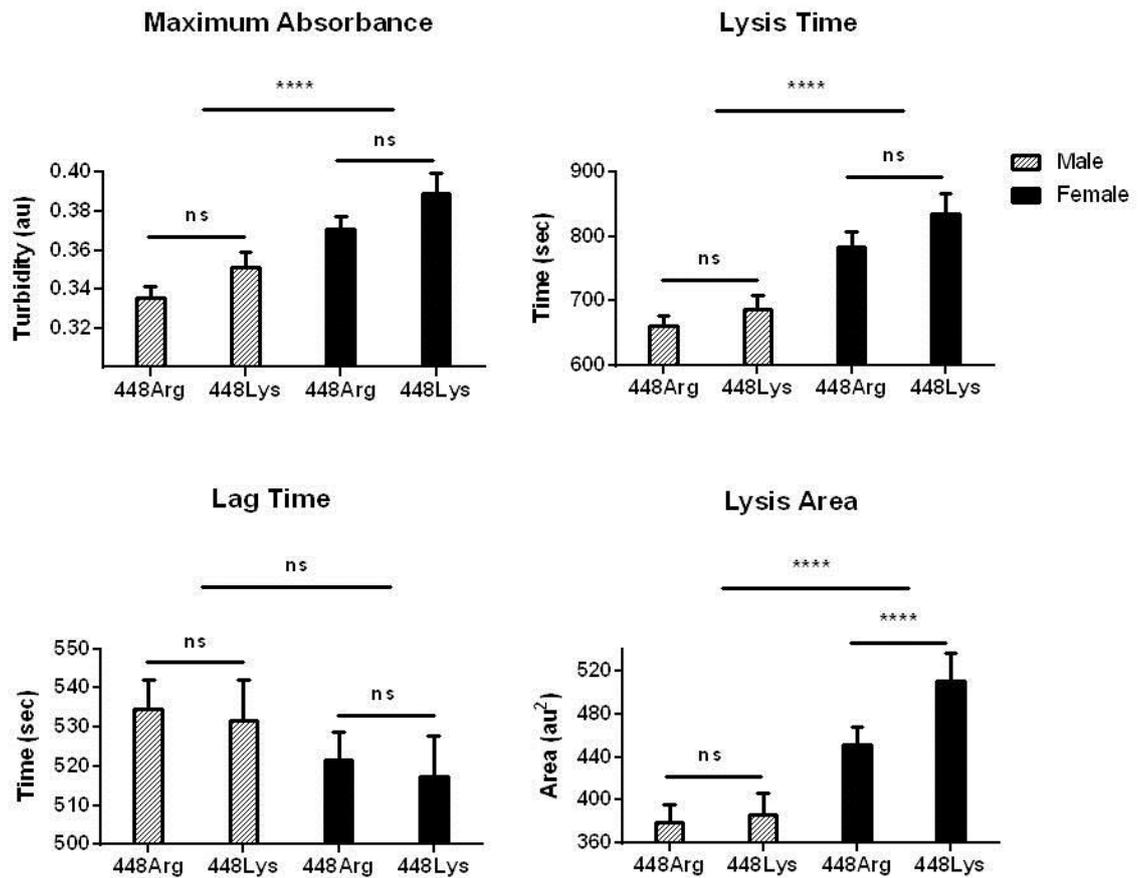


Figure 3-2. Effect of gender on plasma clot parameters in T2DM patients with B β 448Arg and B β 448Lys fibrinogen as measured by turbidimetric assay
 Maximum absorbance; no significant difference when analysed by gender. Lysis time; significant increase with B β 448Lys variant in females. Lag time; no difference with B β 448 variant in either males or females. Lysis area; significant increase with B β 448Lys variant in females but not males. Lysis time and lysis area were log transformed before a t test was carried out. Values shown not adjusted for fibrinogen levels.

3.2.4 Effect of B β 448 variant on fibrinogen levels of T2DM patients

Fibrinogen levels were 3% higher in B β 448Lys variant when compared to B β 448Arg over the whole group, however when split by gender, this difference was only significant in women. Across the whole group, fibrinogen levels were 7.7% higher in women compared with men (Figure 3-3).

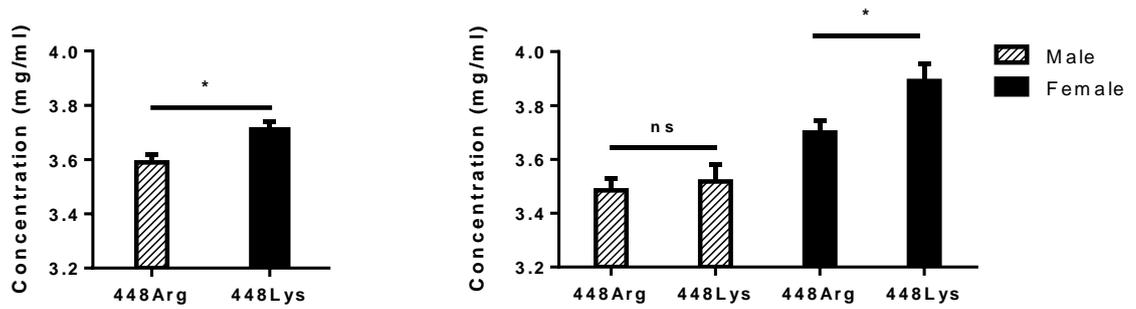


Figure 3-3. Effect of B β 448 fibrinogen variant on mean fibrinogen levels of T2DM patients

Fibrinogen levels were significantly higher in patients with the B β 448Lys variant. When split for gender, this significance was only relevant in women.

3.2.5 Effect of B β 448 allele clot structure of T2DM patient plasma pools

Density of clots did not differ from plasma pools of Arg/Arg, Arg/Lys and Lys/Lys patients by LSCM and there was no significant difference in turbidity between each of the pools. Fibre thickness decreased with increasing copies of the B β 448Lys allele (35.5% with Arg/Lys and 51.6% with Lys/Lys) (Figure 3-4).

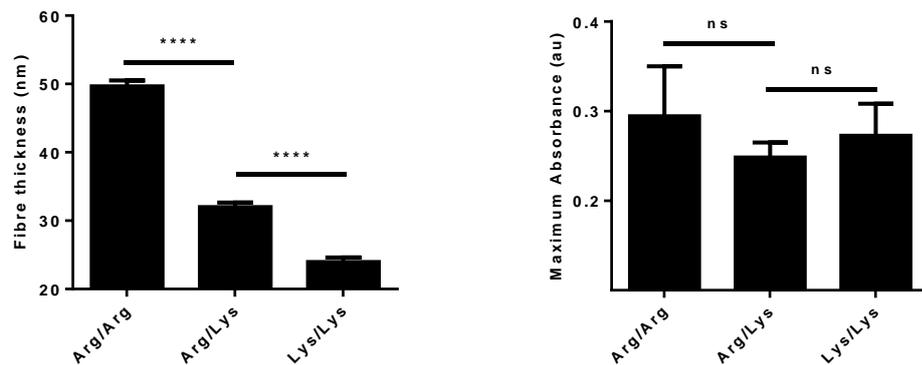
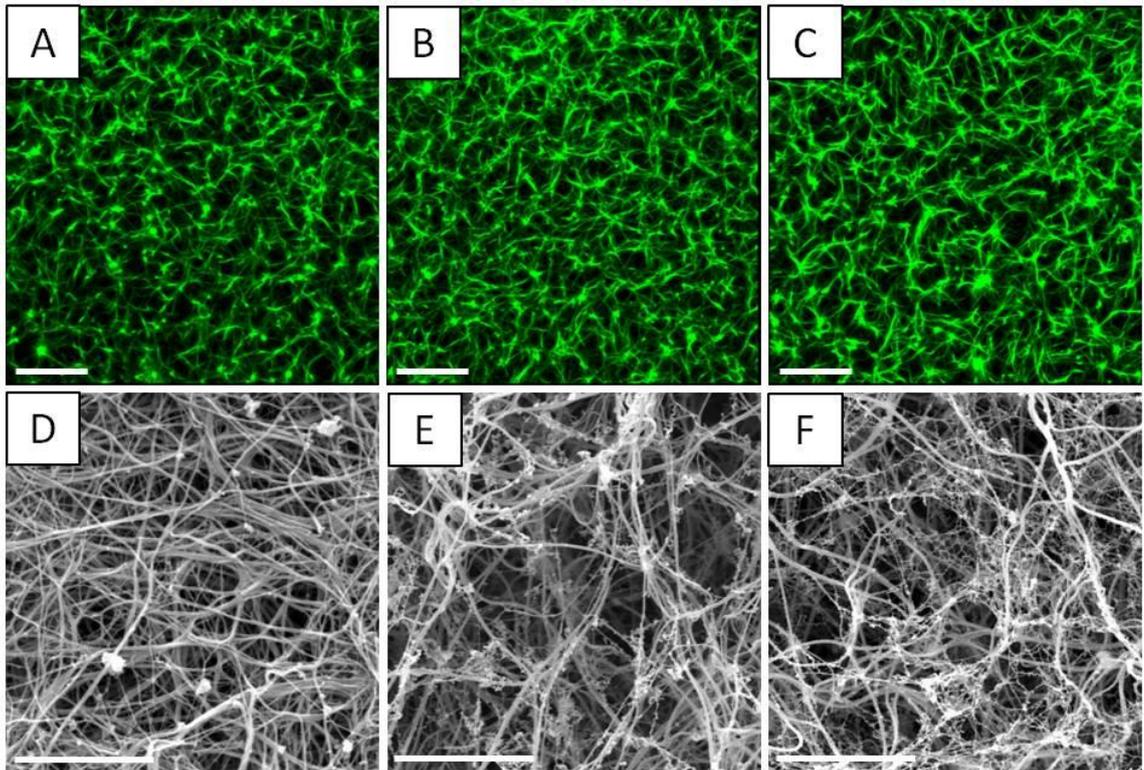


Figure 3-4. LSCM and EM images of clots made from patient plasma pools, EM fibre thickness and MA

LSCM: (A) Arg/Arg (B) Arg/Lys (C) Lys/Lys. Scale bars represent 45 μm EM: (D) Arg/Arg (E) Arg/Lys (F) Lys/Lys. Scale bars represent 3 μm . Clots did not differ from Lys/Lys to Arg/Arg in LSCM or turbidimetric analysis, but EM showed fibres to get thinner with increasing copies of the $B\beta 448\text{Lys}$ allele.

3.2.6 Effects of B β 448 allele on fibrinogen purified from T2DM patients

The difference in clot structure and fibrinolysis comparing the two variants of fibrinogen was still evident after correcting for fibrinogen levels. However, in order to confirm that differences in clot structure are not related to quantitative changes in fibrinogen or an effect of other plasma proteins, I purified fibrinogen from 3 pools of individuals with Arg/Arg, Arg/Lys and Lys/Lys genotype.

3.2.6.1 Integrity of purified patient plasma fibrinogen

All fibrinogen samples were clean and not degraded (Figure 3-5).

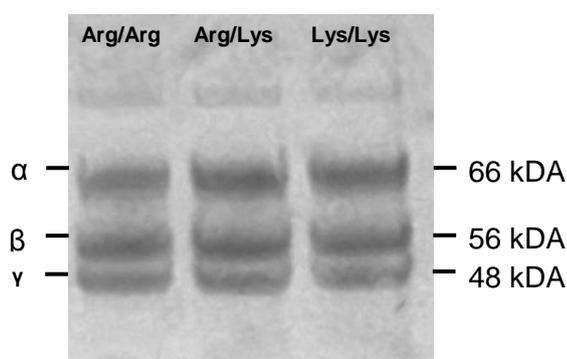


Figure 3-5. Purified fibrinogen resolved on an 4-12% SDS PAGE gel

The gel shows all 3 different fibrinogen chains intact confirming integrity and purity of the fibrinogen purified.

3.2.6.2 Effect of B β 448 allele on clot structure and lysis of purified patient plasma fibrinogen

Fibrin network made from purified Arg/Arg fibrinogen was 79% less dense than that of Arg/Lys and 76% less dense than Lys/Lys as assessed by the in-house macro. There was no significant difference in clot density between Arg/Lys and Lys/Lys fibrinogen. Fibre thickness of clots made from Arg/Lys fibrinogen was 17.9% less than those of Arg/Arg. Lys/Lys fibrin fibres were also 34.9% thinner than Arg/Lys. Clot MA of Arg/Arg fibrinogen was 11.2% greater than Arg/Lys and 20.2% greater than Lys/Lys fibrinogen. Arg/Lys final turbidity was also 8.1% greater than that of Lys/Lys fibrinogen though this did not reach significance (Figure 3-7).

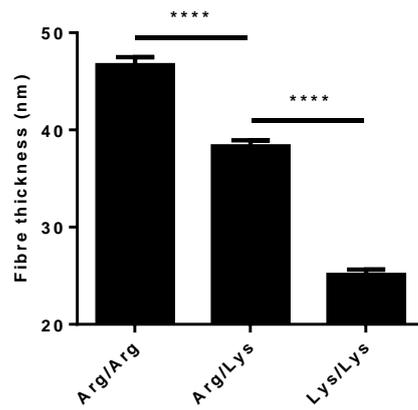
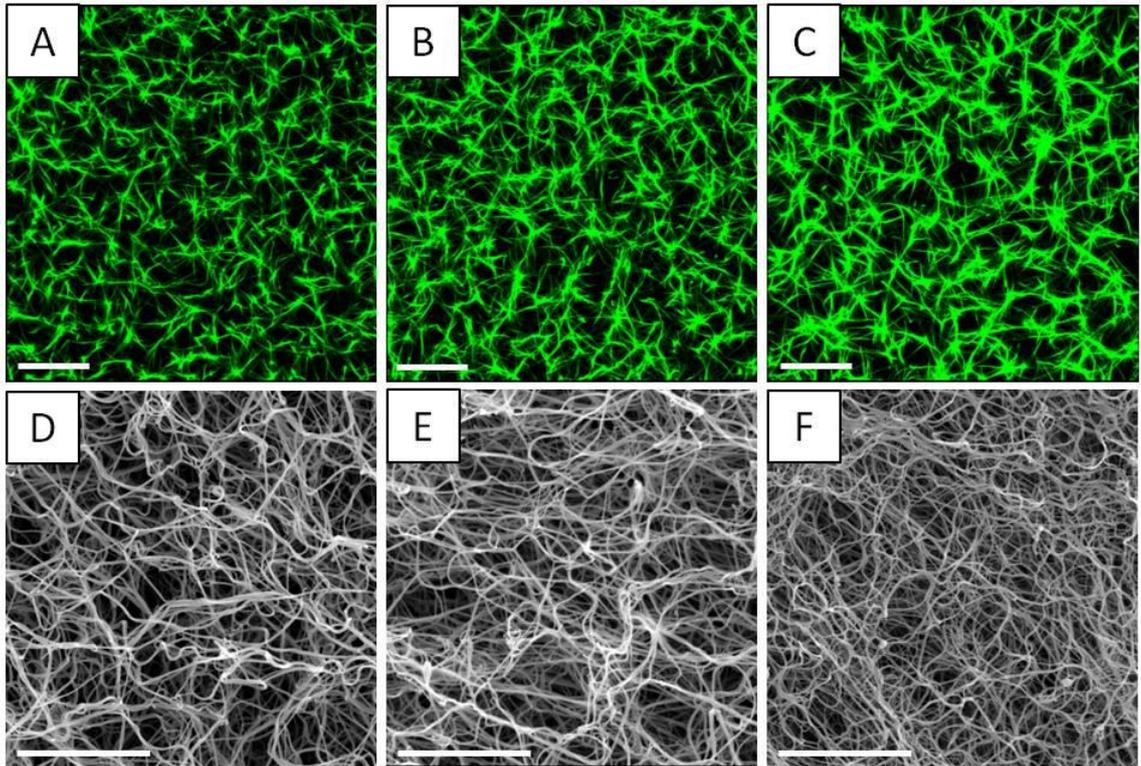


Figure 3-6. LSCM and EM images of clots made from purified patient fibrinogen and EM fibre thickness

LSCM: (A) Arg/Arg (B) Arg/Lys (C) Lys/Lys. Scale bars represent 45 μm . EM: (D) Arg/Arg (E) Arg/Lys (F) Lys/Lys. Scale bars represent 3 μm . Clot density increased while fibre thickness decreased with increasing copies of the B β 448Lys allele.

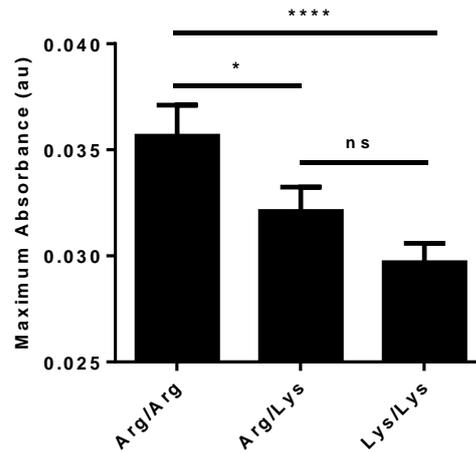


Figure 3-7. Maximum absorbance of clots made from purified patient fibrinogen
 Maximum absorbance decreased in a stepwise manner with increasing copies of the $B\beta 448Lys$ allele.

Lysis time was 5.5% greater in Arg/Lys than Arg/Arg fibrinogen and 13.7% greater in Lys/Lys than Arg/Lys, though these differences were not significant. A significant difference was found between Arg/Arg and Lys/Lys fibrinogen, where Lys/Lys fibrinogen had a 20% greater LT than Arg/Arg (Figure 3-8), directly implicating the polymorphism in altered clot lysis in diabetes.

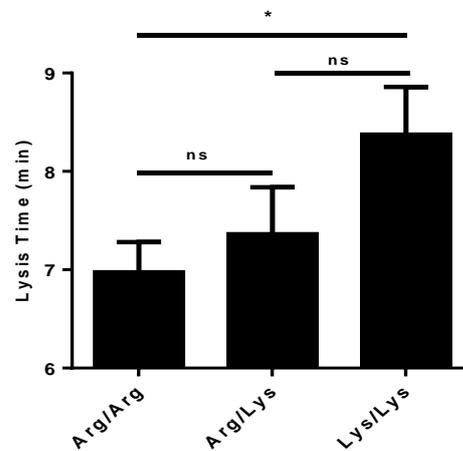


Figure 3-8. The effect $B\beta 448$ fibrinogen allele on LT of clots made from purified patient plasma fibrinogen

Lysis time increased with increasing copies of the $B\beta 448Lys$ allele. Data were log transformed before a *t* test was carried out

3.2.6.3 Differences in MA and fibre thickness

In a plasma system, as EM fibre thickness decreases, turbidimetric MA concomitantly increases. However, in a purified system, as EM fibre thickness decreases, as does turbidimetric MA as shown in Table 3-2.

Table 3-2. Changes in MA and fibre thickness in clots with the B β 448Lys allele when compared with the B β 448Arg allele

	Plasma	Plasma Pool	Purified
Fibre thickness (EM)	↓	↓	↓
MA (turbidimetric)	↑	=	↓

3.2.7 Clinical Data

3.2.7.1 Effect of B β 448 variant on age of T2DM patients

Mean age of subjects with B β 448Lys was 1.2% lower compared with B β 448Arg across the whole group. This age difference was only significant in women (1.3%) and not men (0.6%). Results are summarised in Figure 3-9. There was no significant difference in age between males and females across the whole group.

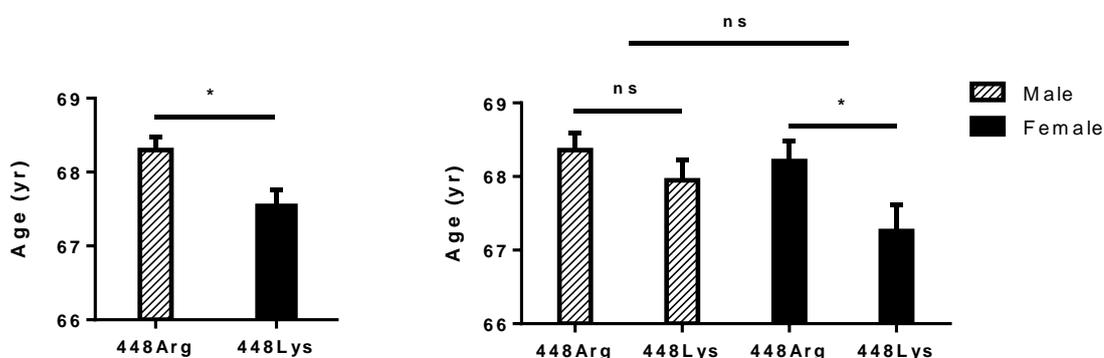


Figure 3-9. Effect of B β 448 fibrinogen variant on mean age of T2DM patients

Patients with the B β 448Lys variant were significantly younger than those with the B β 448Arg variant. When split for gender, this significance was only relevant in women.

3.2.7.2 Effect of B β 448 variant on history of risk of cardiovascular disease

In the whole group, the B β 448Lys variant was associated with previous cerebrovascular incidents and transient ischaemic attacks [OR 1.87 (1.13, 3.11), $p=0.015$]. This value was evident even after adjusting for traditional risk factors, including total cholesterol (TC), high density lipoprotein cholesterol (HDL), HbA1c,

blood pressure and smoking [OR 1.98 (1.16, 3.37), $p=0.016$]. In contrast, the B β 448Lys was not associated with previous history of cardiac ischaemia.

When the group was split by gender, the predictive value of B β 448Lys variant for stroke/transient ischaemic attack (TIA) was only significant in females and not males, whether adjusted [OR 4.18 (1.56, 11.22), $p=0.003$ female], [OR 1.43 (0.74, 2.76), $p=0.27$ males] or unadjusted [OR 3.69 (1.49, 9.13), $p=0.0045$ female] [OR 1.38 (0.73, 2.62), $p=0.32$, male]. Data summarised in Figure 3-10. No association between B β Arg448Lys polymorphism and a history of coronary artery disease or myocardial infarction was detected in the whole population or when men and women were analysed separately.

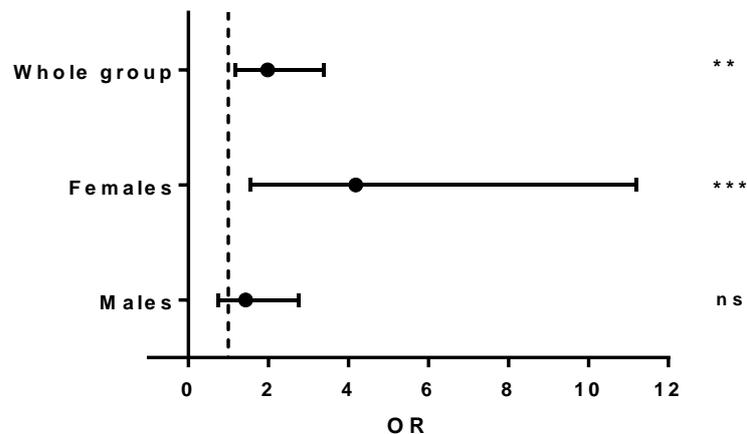


Figure 3-10. Predictive value of B β 448Lys fibrinogen variant for stroke/TIA

B β 448Lys fibrinogen predicted previous stroke and TIA, when split for gender this predictive value was only significant in women. Data displayed as odds ratio (OR), error bars denote \pm confidence interval. Model adjusted for TC, HbA1c, sBP, dBP, smoking and HDL. The R environment for statistical computing was used to fit logistic regression models to identify independent associations between B β Arg448Lys polymorphism and vascular disease. The effect of predictors in the regression model was characterised using 95% confidence intervals for the odds ratio coefficients.

3.2.7.3 Effect of B β 448 variant on current vascular pathology

Carotid intima media thickness (IMT) was similar in carriers of B β 448Arg and B β 448Lys variants and no difference was detected for ankle-brachial index (ABI). When analysed by gender, IMT was similar in carriers of B β 448Arg and B β 448Lys in males and females. ABI was similar in carriers of B β 448Arg and B β 448Lys in males but was 6.3% higher in B β 448Lys variant than B β 448Arg in females.

3.3 Discussion

Diabetes, a prothrombotic condition, is associated with compact fibrin networks and resistance to fibrinolysis, which contribute to the increased risk of vascular events in this population. This work is the first to investigate the functional and clinical role of B β Arg448Lys variants of fibrinogen in individuals with diabetes and we demonstrate an additional effect of this polymorphism on clot structure and atherothrombotic risk.

There are a number of novel observations emerging from this work in relation to individuals with type 2 diabetes: i) Carriers of B β Lys448 variant of fibrinogen display compact plasma clots and impaired fibrinolysis, changes that are independent of fibrinogen plasma levels, ii) B β Lys448 is associated with thinner fibrin fibres, leading to impaired fibrinolysis, iii) maximum absorbance of plasma clots is not a simple reflection of fibre thickness, iv) Carriers of B β Lys448 variant are younger, particularly women, suggesting early mortality in this group, v) B β Lys448 variant may constitute a risk factor for cerebrovascular and peripheral vascular disease in women but not men with diabetes.

When plasma samples were studied, clot density was higher, lysis time longer and lysis area greater in individuals with B β 448Lys compared with B β 448Arg variant. Although fibrinogen levels were higher in carriers of B β 448Lys, correction for protein levels still showed a difference in clot structure parameters, suggesting a direct effect of the polymorphism on fibrin network structure and fibrinolysis. Confocal and electron microscopy data from plasma pools of Arg/Arg, Arg/Lys and Lys/Lys backed up these findings, showing increasing density of the clot and decreasing fibre diameter with increasing copies of the B β 448Lys allele. Plasma clots from female patients also had a higher maximum absorbance, longer lysis time and greater lysis area than males, identifying them as carrier of a particularly thrombotic phenotype. When this gender difference was investigated further, LT and lysis area were significantly greater in females with the B β 448Lys compared with the B β 448Arg variant. This difference was not seen in males in either LT or lysis area, implicating gender as a predictor for the effect of the B β 448 variant. Maximum absorbance was greater in carriers of the B β 448Lys variant in both males and females, though in neither case did this reach significance though differences cannot be discounted due to the relatively small study size.

As previously demonstrated, circulating plasma fibrinogen levels were significantly higher in patients with the B β 448Lys variant. Interestingly, the association between B β 448Lys and plasma fibrinogen levels, presumably due to LD with polymorphisms that determine protein levels, was only evident in women. This suggests that B β Gly455Ala and B β Cys148Thr variants of fibrinogen have little effect on plasma protein levels in men with diabetes and environmental factors have a more

important role in the population studied. It has previously been shown that female gender, diabetes and B β 448Lys variant are associated with hyperfibrinogenaemia^{73, 125, 131}. It is interesting that our data show these conditions to have an additive effect on fibrinogen levels, specifically in diabetic women carriers of the B β 448Lys variant. Since high concentrations of fibrinogen are associated with dense, tight clots made of thin fibrin fibres³ this may go some way to explaining the differences seen in clot structure and lysis in diabetic females with the B β 448Lys variant.

Although correction for fibrinogen levels did not eliminate the differences in clot structure/lysis comparing the two variants of fibrinogen, I have undertaken experiments in a purified system and conclusively demonstrated that clot maximum absorbance decreases with increasing number of copies of the B β 448Lys allele. This further supports the role of this polymorphism in altered clot structure in individuals with diabetes. These turbidimetric data were reinforced by confocal and electron microscopy, showing increasing density of the fibrin clot and decreasing fibre thickness with increasing copies of the B β 448Lys allele. Collectively, these results confirm the observed changes in clot structure with plasma samples are independent of fibrinogen levels and that the polymorphism studied has an additive effect on clot structure in individuals with diabetes.

The turbidimetric and EM data (see Table 3-2) provide some interesting data regarding what MA in turbidimetric assays is actually a measure of, and how care should be taken when interpreting data using different systems. Fibre thickness consistently decreases in each of the preparations with increasing copies of the B β 448Lys allele. However, in plasma pool data, no difference in MA could be seen, despite decreases in fibre thickness with the B β 448Lys allele. In the plasma system MA was higher with the B β 448Lys variant, while in the purified system MA was decreased, despite consistency in the decreasing fibre thickness in both preparations. These data suggest that plasma MA is a measure of both fibre thickness and clot density while in a purified system MA tends to reflect fibre thickness alone.

Despite similarities in clot structure using purified variant fibrinogen in this study to that of recombinant B β 448 variant fibrinogen, my work shows differences in clot lysis outside the plasma environment that are not observed in the recombinant system. In our previous study using recombinant protein, a difference in clot lysis between the two variants was only evident in plasma environment. In contrast, our current study shows differences in clot lysis made from the different variants of fibrinogen in the absence of plasma proteins. Other data (Chapter 4) using variant recombinant protein has shown α 2-AP to prolong lysis of clots made from recombinant B β 448Lys fibrinogen significantly more than those of B β 448Arg fibrinogen, despite their equivalent lysis times in the absence of plasma proteins. This implicates the interaction of clot structure

and plasma proteins in the difference in lysis times seen in non-diabetic patients with the variant fibrin. There are a number of possible explanations for the discrepancies between these data. The current work used purified fibrinogen, which may have been contaminated with small quantities of plasma proteins not detected on the SDS-PAGE gel. Another explanation is related to diabetes inducing post-translational modifications in fibrinogen that affect clot structure and fibrinolysis. A differential glycation, or other modification such as oxidation, may have occurred in the two variants, consequently resulting in altered clot lysis, particularly as lysine residues are amenable to such modifications. Though these suggested changes were not detected by MS of the recombinant B β Arg448Lys variants (Chapter 4), different mechanisms could influence PTMs both *in vivo* and in diabetes. This, alongside the potential flaws of the MS technique used mean these modifications cannot be ruled out in this study. Alternatively, subtle differences in the methodology or reagents used may have accounted for these findings, but this was ruled out by conducting repeated studies on recombinant proteins, which reproduced our previous results.

Individuals with B β 448Lys variant were significantly younger than those with the B β 448Arg variant. This indicates this fibrinogen mutation may be associated with premature mortality in this population, possibly related to increased thrombosis risk through the formation of more compact clots with compromised fibrinolysis. Such an observation has not been documented before and a longitudinal prospective study is needed to clarify whether this is indeed the case. Interestingly, this difference in age was only observed in women, suggesting a gender difference. The possible reduction in life expectancy of B β 448Lys variant may be related to denser fibrin clots and impaired fibrinolysis, which we have shown in this work, predisposing to early cardiovascular events.

A predictive value of B β 448Lys for stroke and transient ischaemic attacks was identified in the whole group, which was still evident after adjusting for traditional risk factors. Again our data suggest a gender difference in the interaction between this polymorphism and cerebrovascular disease, a finding that has been documented in the non-diabetic population¹⁰¹, and therefore requires further investigation. However, B β 448Lys showed no predictive value for cardiac ischemia or MI, in contrast to studies on individuals without diabetes^{99, 100}. It is possible that the association between the polymorphism and coronary artery disease is weaker in individuals with diabetes due to the heterogeneous nature of this condition, although an effect in a subgroup of patients, yet to be identified, cannot be ruled out. Alternatively, the cross-sectional nature of the study may have failed to detect an association between this polymorphism and CAD and therefore future prospective studies are warranted to investigate this in more detail.

This study identifies the B β 448Lys variant as having a functional role as a predictor for TIA and stroke in diabetes, independent of the traditional risk factors associated with these conditions. This shows diabetic women with B β 448Lys to be a particularly high risk group for ischemic events. This may be explained by their particularly high fibrinogen levels and the B β 448Lys variant combining to predispose to clots with particularly thin fibres and tight network structure which have been shown to increase risk of atherothrombotic events.

The data on current vascular pathology suggests that, although there was no difference across the whole group between B β Arg448Lys variants, changes in ABI are associated with this polymorphism in females. ABI was lower in the presence of B β 448Lys variant when compared to B β 448Arg in females. As a low ABI is an indicator of peripheral vascular disease, and thus increased vascular risk, this could be another mechanism by which B β 448Lys variant governs vascular risk in women with T2DM, though further work is needed to clarify if this is a direct effect. In contrast, B β Arg448Lys variant had no effect on vascular pathology of men in this cohort.

As mentioned, diabetes, female gender and the B β Arg448Lys polymorphism predispose to hyperfibrinogenaemia. Elevated plasma fibrinogen concentration alone is an independent risk factor for cerebrovascular and peripheral arterial disease development and MI. This synergistic increase in cardiovascular risk with increasing fibrinogen concentration is due to a rise in fibrinogen and fibrin incorporation into arteriosclerotic plaques as well as increased blood viscosity and platelet aggregability at higher fibrinogen concentration⁹⁹. This may go some way to explaining the increased risk of TIA and stroke in diabetic women with the B β 448Lys variant, a patient group with particularly high fibrinogen levels. This, alongside the fact that even after adjustment for fibrinogen levels, women with the B β 448Lys variant have an increased risk of stroke and TIA in this population, suggests there is a direct effect of the polymorphism on this risk, independent of fibrinogen levels.

In summary, the current work demonstrates that the single gene polymorphism B β Arg448Lys has an additive and functional effect over that of diabetes on fibrin clot structure, fibrinolysis, age, fibrinogen levels and predisposition to stroke and TIA. This indicates an important role for genetic factors in determining thrombosis risk even in a complex multifactorial condition such as type 2 diabetes. Furthermore, female carriers of B β 448Lys variant are younger and appear to be predisposed to cerebrovascular disease, suggesting a direct adverse clinical effect for this polymorphism in diabetes that is gender specific. Given the observational nature of our study, however, we should be cautious in our interpretations and future long-term longitudinal studies are now warranted to fully establish the role of this polymorphism in predisposition to cardiovascular disease in individuals with diabetes.

**Chapter 4. Molecular Mechanisms for the Differences in Clot
Structure and Lysis in B β Arg448Lys Variants of Fibrinogen**

Introduction

The blood clot is composed of a mesh of fibrin fibres with cellular elements embedded in this network. The structure of the fibrin clot can determine predisposition to atherothrombotic conditions, as compact clots composed of thin fibres and small pores are associated with premature and more severe CVD. The fibrinogen B β Arg448Lys polymorphism occurs in 15-25% of the white population and is associated with changes in fibrin clot formation, structure and lysis, implicating it in increased risk of CAD, triple vessel pathology, venous thromboembolic disease, hypofibrinolysis and embolisation⁹⁹⁻¹⁰¹. When compared with B β 448Arg, clots formed from B β 448Lys variant have a more compact structure composed of thin fibres and small pores with increased stiffness. Fibrinolysis rates are significantly slower in the B β 448Lys variant when compared to B β 448Arg in the plasma environment. However, in a purified system, no difference between lysis rates of clots of the two variants is seen¹⁰². This latter observation challenges the accepted concept that fibrin network structure directly determines lysis efficiency²³.

The mechanisms behind the observed changes in fibrin structure and fibrinolysis rates of B β 448Arg and B β 448Lys variants need further exploration, though many hypotheses for the influence of the mutation have been suggested. The proximity of the B β Arg448Lys to 3 important sites in the β -chain that influence clot structure, formation and lysis (a β -chain polymerisation site, an interaction site for the C-terminal of the α -chain and a calcium binding site on the β -chain) may implicate these areas in the observed changes. Impairment in lateral aggregation, altered FXIII cross-linking and differences in interactions with plasma fibrinolytic proteins may have a role. Loss of hydrogen bonds due to the polymorphism in an area of the β -chain (B β 448-B β 315 and B β 398-A α 157) thought to be involved in lateral aggregation and stabilisation of the coiled-coil could also affect the conformation of the α C domain. The mutation is in strong LD with two other mutations, the B β Gly445Ala and the B β Cys148Thr which cause higher plasma fibrinogen levels in the B β 448Lys variant¹⁰¹. Whilst this may have an effect in vivo, it does not explain the differences in fibrin network structure using in vitro studies controlling for fibrinogen levels.

The aims of this chapter are to investigate the mechanisms for altered clot structure and resistance to fibrinolysis, in a plasma environment, of clots made from B β 448Lys variant of fibrinogen. Therefore, I analysed the effects of the B β Arg448Lys variants of fibrinogen on i) FXIII activation and cross-linking to understand differences in clot stiffness and lysis, ii) binding and crosslinking of the main anti-fibrinolytic protein, α 2-AP to investigate the variable lysis in plasma, iii) plasmin generation comparing the two variants in the presence and absence of α 2-AP, iv) early protofibril formation to understand the mechanisms for the differences in fibre thickness and v) potential

differences in post-translational protein modifications to further elucidate the reasons for changes in structure.

Collectively, these studies will strengthen understanding of molecular mechanisms determining fibrin network structure and the effects of B β Arg448Lys. Moreover, this work will help to elucidate the role of the α C-domain, an area of the fibrinogen molecule which remains fairly unexplored. This in turn will pave the way to develop more effective preventative and management strategies for individuals at risk of atherothrombotic conditions.

4.1 Methods

4.1.1 Protein production

A CHO cell line with the B β Arg448Lys polymorphism was already available in the lab¹⁰². These were grown and protein harvested and precipitated as described in section 0.

4.1.2 Protein purification

Fibrinogen was purified by affinity chromatography using a calcium dependent IF-1 monoclonal antibody (Kamiya Biochemical, Seattle, WA, USA) and an automated chromatography system (Biocad sprint, Applied Biosystems, Warrington, UK) as previously described in section 2.1.3.

4.1.3 Analysis of clot formation and Lysis

Clot turbidity and lysis measurements were conducted as previously described in section 2.4.

4.1.4 Confocal microscopy

To visualise α 2-AP in the clot, it was labelled with Alexa Fluor 488 (green) (Invitrogen). Given the use of Alexa fibrinogen 488 by the group, initial attempts were made to label α 2-AP using red Alexa Fluor 594 labelling kits (Invitrogen), but it was not possible to clearly see the protein in this preparation. Therefore, Alexa Fluor 488 was used instead, which is the brightest and most stable of the conjugates together with commercially available 594 Fibrinogen Conjugate (Invitrogen), throughout the experiments. Confocal clot preparation and visualisation were conducted as described in section 2.6.

4.1.5 Fluorescent labelling of α 2-AP

Calbiochem α 2-AP was labelled using the Alexa Fluor 488 Protein Labelling Kit (Invitrogen) according to kit guidelines. Briefly, 200 μ g α 2-AP was dissolved in 200 μ l PB and 20 μ l 1 M bicarbonate added to raise the pH of the reaction mixture to the optimum for tetrafluorophenyl (TFP) ester reactions. This mixture was added to a vial of reaction dye and stirred for 1 hr at room temperature. A provided purification column was assembled by addition of purification resin, which separates free dye from labelled proteins. The sample was then loaded onto the column and allowed to enter. Elution buffer was then slowly added until the protein was eluted. Progress of the protein through the column was checked by periodically illuminating the column with a

handheld UV lamp. Two coloured bands were present, the first band being the labelled protein and the second, slower moving band, the unincorporated dye. The first band was collected and stored at -20 °C.

4.1.6 Electron microscopy

Electron microscopy was conducted as previously described in section 2.7.

4.1.7 FXIII crosslinking gel

For each variant, 10 µl of 0.8 mg/ml fibrinogen was added to 7 tubes labelled 0, 5, 30, 60, 120, 180 and 180(-FXIII) min and put in a 37 °C DB-5A Dri-block heating block (Techne, Cambridge, UK). 5 µl NuPAGE loading dye and 2.5 µl reducing buffer were added to time point 0. To each tube, 10 µl AM (± 260 nM FXIII A_2B_2 , 0.25 U/ml thrombin, 10 mM CaCl₂) was added and a timer started. Time point 0 tubes were immediately transferred into a 95 °C heating block and incubated for 10 min. This was repeated for other time points as appropriate and samples kept on ice until ready to be run on a 4-12% NuPAGE Novex Bis-Tris gel as described in section 2.1.3.6.

4.1.8 FXIII- $\alpha 2$ -AP crosslinking gel and blot

For each variant, 20 µl of 0.8 mg/ml fibrinogen was added to 9 tubes labelled 0, 5, 10, 20, 30, 45 60, 120 and 180 min and put in a 37 °C heating block. 10 µl of NuPAGE loading dye and 5 µl reducing buffer were added to time point 0. 10 µl $\alpha 2$ -AP (final concentration 3.3 µg/ml) was added to each time point followed by 10 µl AM (260 nM FXIII A_2B_2 , 0.25 U/ml thrombin, 10 mM CaCl₂) and a timer started. Time point 0 tubes were immediately transferred into a 95 °C heating block and incubated for 20 min. This was repeated for other time points as appropriate and samples kept on ice until ready to be run on a 4-12% NuPAGE Novex Bis-Tris gel for SDS-PAGE or western blot analysis. Each sample was halved and run on one of two gels, one was stained using coomassie blue as described in section 2.1.3.7 and one was blotted for $\alpha 2$ -AP as described in section 2.5.

4.1.9 FXIII activity assay

4.1.9.1 Biotin incorporation

A Nunc-Immuno MicroWell 96 well MaxiSorp flat bottom plate was coated with 100 µl 40 µg/ml fibrinogen in TBS and incubated for 40 min at room temperature. The plate was then blocked with 300 µl blocking buffer (1% bovine serum albumin (BSA)/TBS) for 90 min at 37°C. The plate was washed 3 times with TBS Tween buffer (0.1%

Tween20/ TBS) and 10 μ l 22 μ g/ml FXIII_{A₂B₂} (or TBS for control) added to each well as appropriate. 90 μ l reaction mix (100 μ M DTT (Sigma), 0.27 μ M Biotin (Thermo Scientific), 1 U/ml thrombin, 1 mM CaCl₂ in TBS) was added to each well at 10 sec intervals. The reaction was stopped at intended time points (0, 5, 10, 15, 20, 25 min) by adding 200 μ l of 200 mM EDTA. After the last time point, the plate was washed 3 times. Subsequently, 100 μ l 1 mg/ml streptavidin alkaline phosphatase (Sigma) in 1% BSA/ TBS/ 0.1% Tween20 was added to each well and incubated at 37 °C for 1 hr. The plate was washed 3 times and 100 μ l substrate solution (1 mg/ml p-nitrophenol phosphate (Sigma), 1 M Diethanolamine (Sigma), 0.1 M MgCl₂) added to each well and the OD allowed to develop to ~0.5au. 100 μ l stop solution (4 M NaOH) was added and the plate read at 405 nm on a MRX Microplate Reader (Dynex Technologies, Ashford, Middlesex, UK).

4.1.9.2 α 2-antiplasmin incorporation

A Nunc-Immuno MicroWell 96 well MaxiSorp flat bottom plate was coated with 100 μ l of 0.24 μ M fibrinogen and incubated for 40 min at room temperature. The plate was washed 3 times with TBS pH 7.4 and blocked with 300 μ l blocking buffer (TBS/ 3% BSA) overnight at 4 °C with 1 hr in the morning at 37 °C. The plate was washed 3 times with washing buffer (TBS/ 0.05% Tween); 100 μ l fibrinogen AM (1 U/ml thrombin, 5 mM CaCl₂ in TBS) was added to each well and incubated for 45 min at room temperature. The plate was washed 3 times with high salt washing buffer (40 mM Tris, 1 M NaCl, 0.05% Tween20) and 3 times with washing buffer, 200 μ l stop solution (0.2 M EDTA) was then added to each well representing time point zero. 10 μ l 22 μ g/ml FXIII was added to each well as appropriate (TBS as a control), followed by the addition of 90 μ l FXIII reaction mix (100 μ M DTT, 10 μ g/ml α 2-AP, 1 U/ml thrombin, 5 mM CaCl₂) to each well at 10 sec intervals. The reaction was stopped at intended time points (0, 10, 20, 30, 40, 50 min) by adding 200 μ l of 200 mM EDTA. The plate was washed 3 times with washing buffer, 100 μ l antibody solution (2 μ g/ml Goat antihuman α 2-AP peroxidase conjugate IgG (ERL) in TBS/ 0.1% Tween/ 3% BSA) was added to each well and incubated at room temperature for 1 hr. The plate was washed 3 times with washing buffer before adding 100 μ l OPD substrate (0.6 mg/ml OPD/ 0.41% H₂O₂) to each well at 10 sec intervals and OD allowed to develop to ~0.2 au. 100 μ l stop solution (0.5 M H₂SO₄) was added to each well and the plate read at 490 nm on a MRX Microplate Reader (Dynex Technologies, Ashford, Middlesex, UK).

4.1.10 S2251- plasmin generation assay

To wells of a Grenier 96 well clear polystyrene flat bottom microplate, 100 μ l 0.5 mg/ml fibrinogen, 0.0125 mg/ml plasminogen, \pm 5.5 μ g/ml FXIII, \pm 3.3 μ g/ml α 2-AP in buffer B (40 mM Tris-HCl, 0.01% Tween20, 75 mM NaCl) was added as appropriate. Clotting was initiated by addition of 50 μ l AM containing 1.5 U/ml thrombin, 0.27 M CaCl_2 in buffer B and clotting followed by measuring absorbance at 280 nm every 12 sec on ELx-808 IU ultramicroplate reader (BIO-TEK Instruments INC, USA) for 15 min to ensure full clot formation. 50 μ l LM containing 0.75 mM S2251 substrate and 0.05 μ g/ml tPA in buffer C (40 mM Tris-HCl, 0.01% Tween20, 75 mM NaCl, 1 mg/ml BSA) was added to the formed clot and OD monitored at 405 nm every 30 sec for 300 min at 37 $^{\circ}$ C, shaking 2 sec before every reading on ELx-808 IU ultramicroplate reader (BIO-TEK Instruments INC, USA).

4.2 Results

4.2.1 Purity, integrity and function of recombinant protein

Both B β 448Arg and B β 448Lys recombinant fibrinogen were clean, pure and not degraded once purified, dialysed and concentrated as shown by SDS-PAGE gel (Figure 4-1A). Maximum turbidity of B β 448Lys fibrinogen was 83.4% less than that of B β 448Arg fibrinogen (Figure 4-1B), indicating the formation of thinner fibres with this variant and confirming previous data.

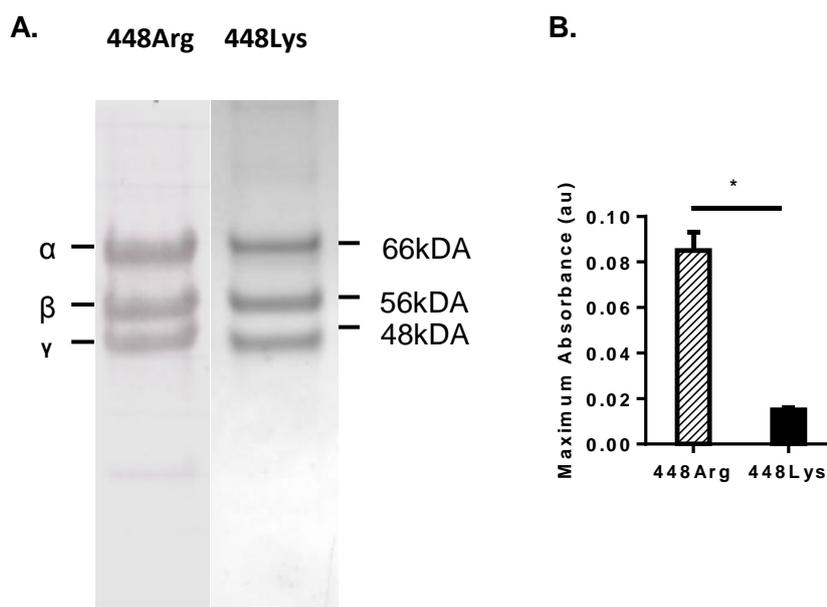


Figure 4-1. Recombinant fibrinogen purity, integrity and function

(A) Variant recombinant fibrinogen resolved on a 4-12% SDS PAGE gel (B) Maximum absorbance of clots formed from recombinant variant fibrinogen as measured by turbidimetric assay; significant decrease with B β 448Lys allele pointing towards thinner fibres. Experiments were repeated at least on three occasions.

The difference in fibrin fibres was further confirmed by EM. Fibres were 57.6% thinner in B β 448Lys than B β 448Arg as assessed by EM. Clot structure also appeared more compact in B β 448Lys as assessed by both EM and LSCM (Figure 4-2). When confocal clot density was assessed using the in-house macro it was confirmed that density of fibrin fibres per unit² was 357% higher in the B β 448Lys variant than B β 448Arg.

4.2.2 Effects of B β 448 variant on clot structure

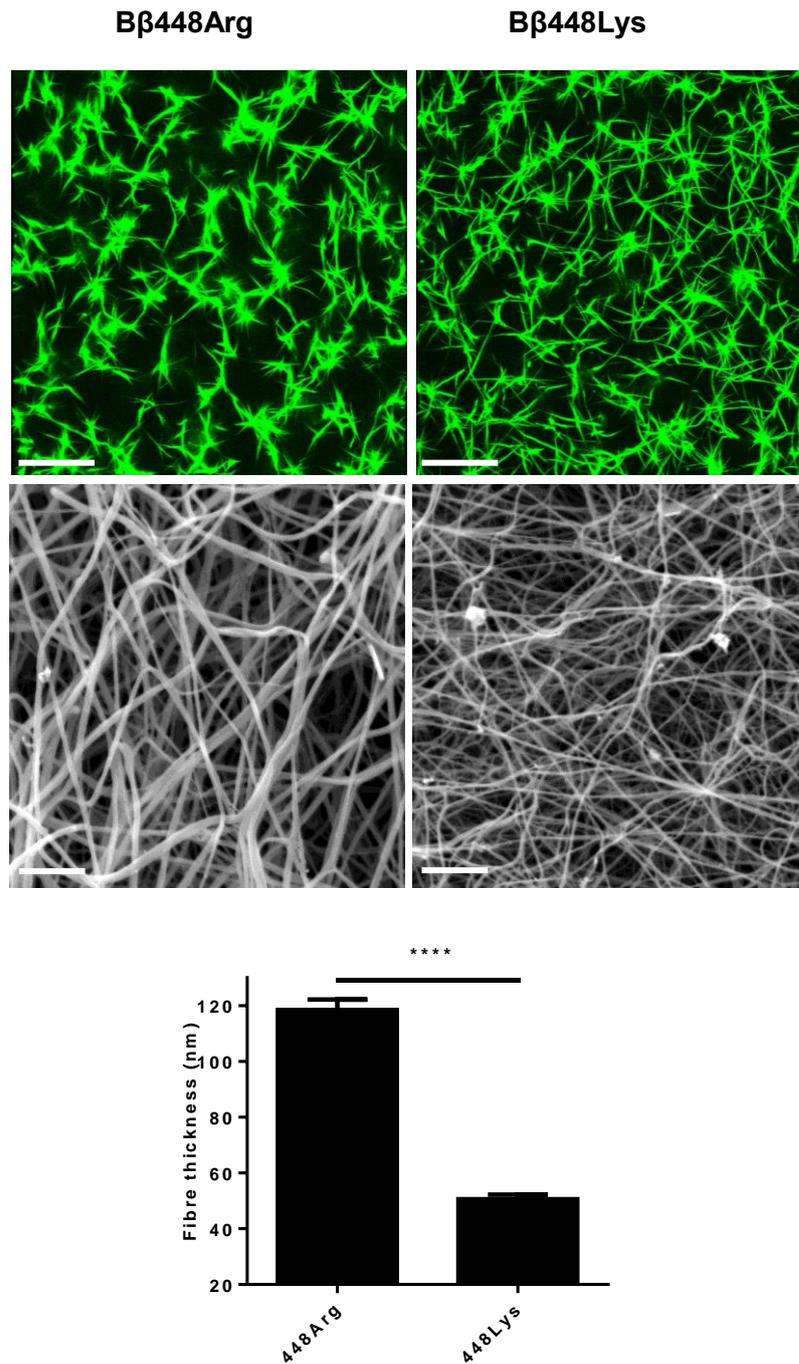


Figure 4-2. LSCM and EM images of clots made from B β 448 variant recombinant fibrinogen and EM fibre thickness

Clots made from B β 448Lys fibrinogen were made of thinner fibres with denser network architecture. 10 fibres were measured from 3 areas of 3 different EM clots of each variant. Scale bars represent LSCM: 45 μm , EM; 1 μm .

4.2.3 Effects of B β 448 variant on fibrin clot lysis

Our lab has previously demonstrated that fibrinolysis rates between recombinant B β Arg448Lys fibrinogen variants are comparable in a purified system. However, significant differences in lysis rates between the two variants were seen when recombinant protein was added to fibrinogen-depleted plasma. In this preparation, lysis time increased for both variants as expected, however this increase was much greater for the B β 448Lys variant, confirming an independent effect of this polymorphism on lysis rate in the plasma environment¹⁰². Given that α 2-AP is the main antifibrinolytic factor and the difference in plasma clot lysis comparing B β Arg448Lys variants of fibrinogen, I tested whether α 2-AP is the plasma protein responsible for this difference. Results presented in Figure 4-3.

As documented previously, there was no significant difference in LT between the variants in the absence of plasma proteins. FXIII did not prolong LT in either variant in the absence of other plasma proteins. In contrast, α 2-AP prolonged LT in both variants in the absence of FXIII. This effect was significantly greater in B β 448Lys than in B β 448Arg (50.0% in B β 448Arg and 91.5% in B β 448Lys). FXIII increased LT in the presence of α 2-AP in both variants, though this increase was only marginal and non-significant with B β 448Arg (14.0%), whereas a more impressive increase was noted with B β 448Lys variant (65.6%). Results are summarised in Figure 4-3.

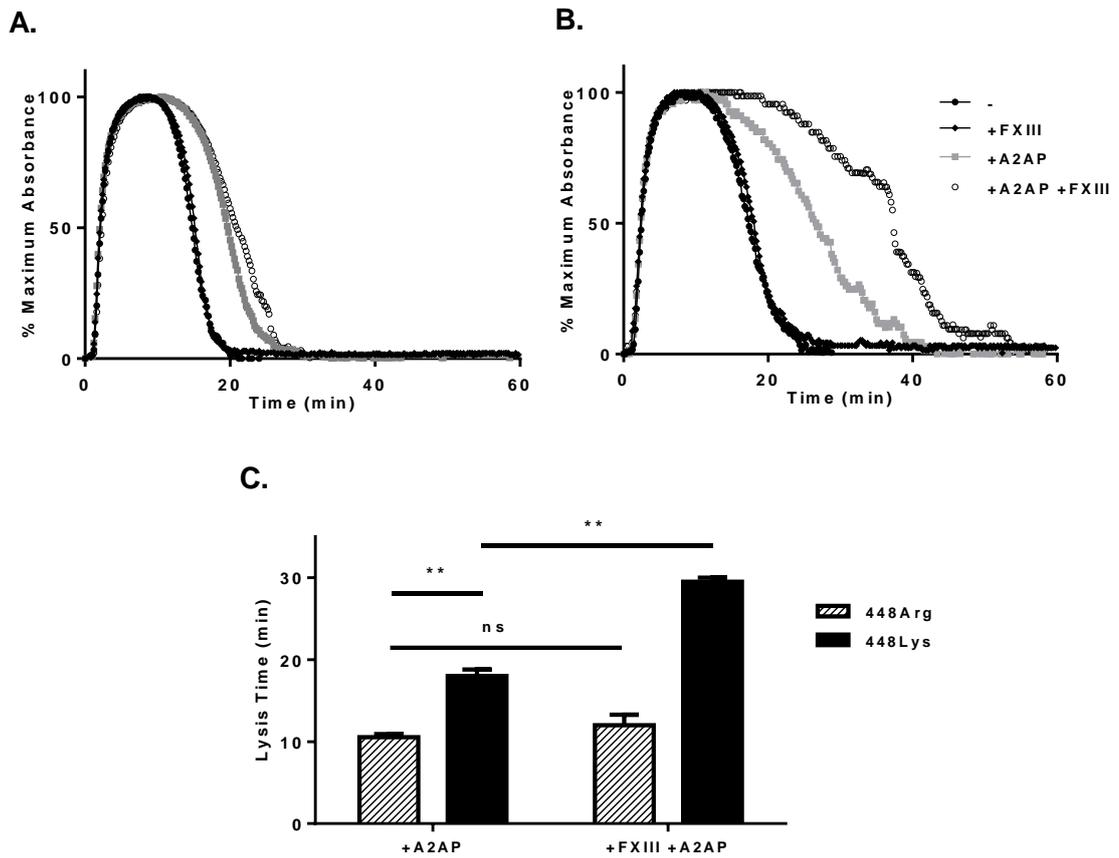


Figure 4-3. Clot lysis parameters of clots formed from recombinant Bβ448Arg and Bβ448Lys fibrinogen as measured by turbidimetric assay

Turbidity and lysis traces from (A) Bβ448Arg and (B) Bβ448Lys recombinant fibrinogen in clots made from recombinant fibrinogen alone, in the presence of FXIII, α2-AP or both FXIII and α2-AP. (C) LT of clots ± FXIII (11 µg/ml) + α2-AP (3.3 µg/ml). A total of 4 independent experiments were conducted in duplicate.

4.2.4 Effect of B β 448 variant on fibrin FXIII crosslinking

FXIII crosslinking activity did not differ between the variants significantly at any time point in the biotin assay (Figure 4-4A). FXIII crosslinking did not differ between the variants significantly at any time point in the α 2-AP assay (Figure 4-4B).

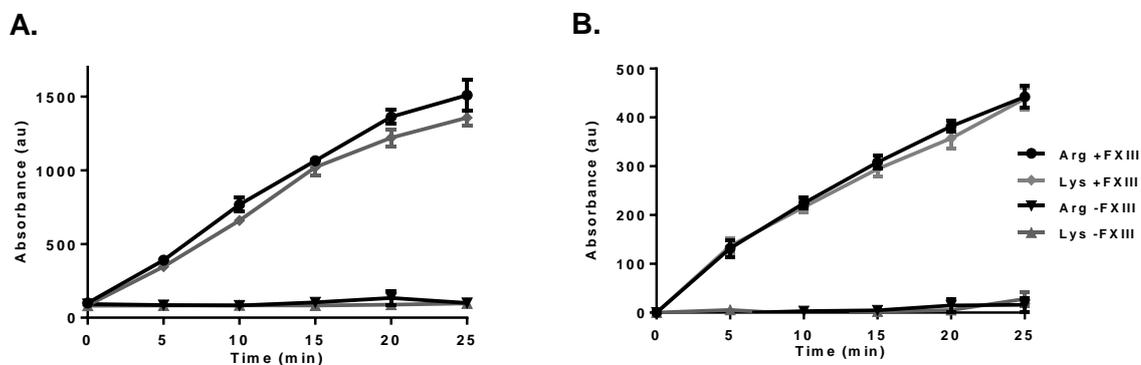


Figure 4-4. FXIII substrate crosslinking to recombinant B β 448Arg and B β 448Lys fibrin

FXIII catalysed crosslinking of (A) biotin (B) α 2-AP to recombinant B β Arg448Lys variants. 3 independent experiments were conducted in triplicate.

There was no clear difference in the FXIII crosslinking of fibrin chains between the variants in the absence of α 2-AP (Figure 4-5A) or in its presence (Figure 4-5B) as assessed by FXIII crosslinking gel/ blot.

In Figure 4-5, the species that can be identified are labelled and are as follows, in order of ascending molecular weight. A species of a lower molecular weight (50 kDa) than that expected for α 2-AP. We suggest it is either a cleaved form of α 2-AP or a contaminant of the calbiochem α 2-AP used. It is unlikely this and the 67 kDa band represent the Met- and Asn- forms of α 2-AP, as Lee and colleagues⁶⁸ showed these two variants to run to the same molecular weight band on SDS-PAGE gels. To identify exactly what this band is, a sample could be sent for mass spectrometry analysis. However, I have run a gel of each component of the crosslinking reaction on a gel and blotted for α 2-AP (Figure 4-6). This shows α 2-AP is only picked up by the α 2-AP antibody in the well in which it was applied with no contamination present in any other well. The <67 kDa molecular weight band is present in this α 2-AP preparation, and thus we can conclude it is part of the calbiochem α 2-AP and not a product of the crosslinking reaction. Interestingly all proteins except fibrinogen are at a concentration in the FXIII crosslinking reaction at which they are not picked up by coomassie staining on the SDS-PAGE gel, even α 2-AP. γ -chain monomers can be seen at 48 kDa, these become less dense then disappear as time progresses and they become crosslinked, forming γ -dimers, as well as polymers with α -chains. These disappear first as formation

of γ -dimers is the first event in FXIII crosslinking. β -chain monomers can be seen at 56 kDa, these remain the same density at each time point as they are not crosslinked by FXIII. α -chain monomers can be seen at 67 kDa, this band becomes less dense as time progresses as α -chain polymers form and α_2 -AP becomes crosslinked to fibrin chains. Free α_2 -AP can also be seen at 67 kDa not crosslinked to any fibrin chain. Free FXIII can be seen at 75 kDa and γ - γ dimers, the first product of FXIII crosslinking of fibrin chains, at \sim 100 kDa. Species of molecular weights $>$ 100 kDa are α -chain dimers and polymers, before α_2 -AP crosslinking occurs and α -chain polymers with α_2 -AP and increasingly high molecular weight polymers of α -chain with α_2 -AP crosslinked. These are presumed to fade as time increases past \sim 60 min not because they are no longer present, but as the molecular weight of the aggregates becomes so great, they can no longer enter the gel.

There was no clear difference in FXIII crosslinking of α_2 -AP to the fibrinogen variants in gel, blot, or incorporation assays.

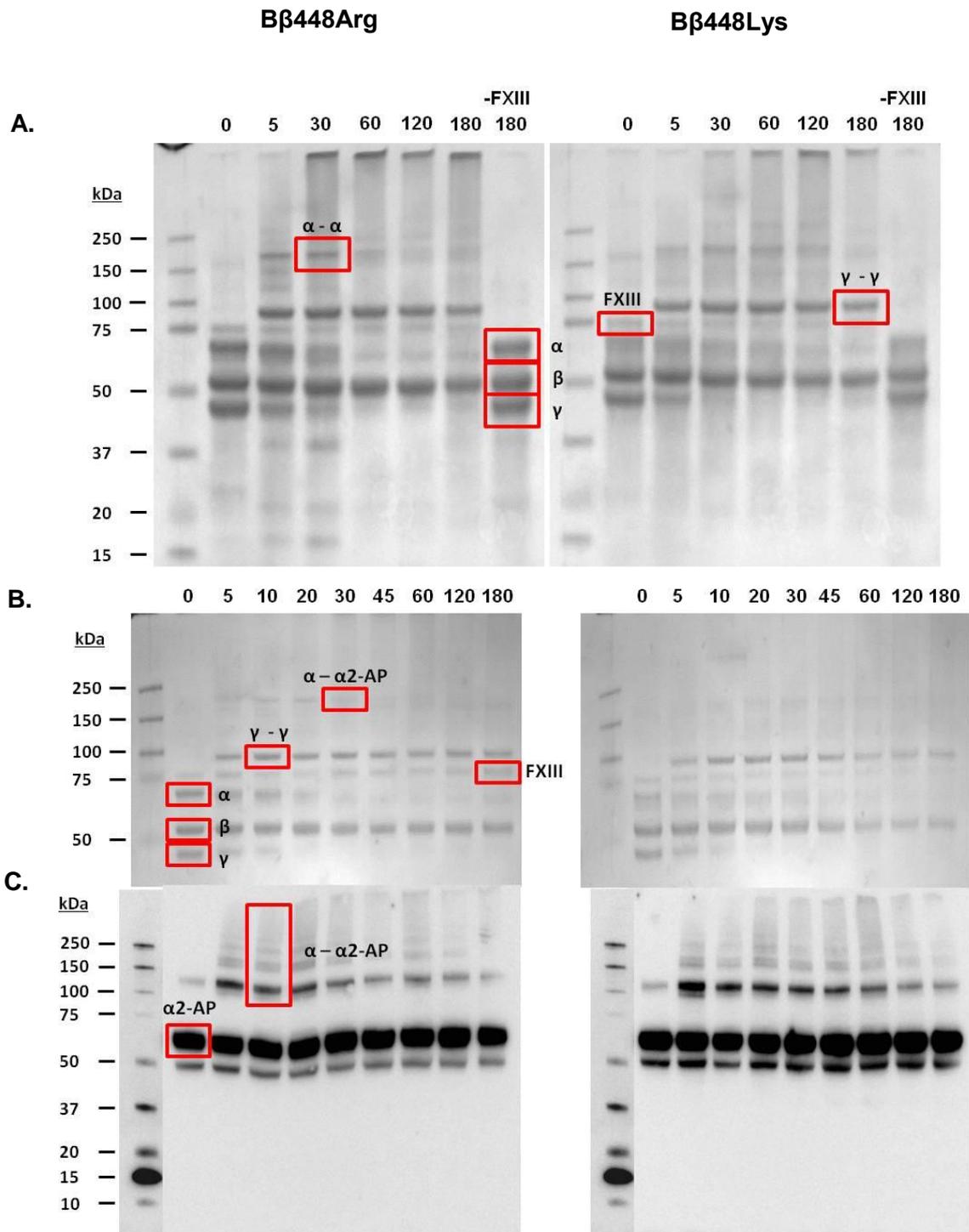


Figure 4-5. FXIII crosslinking of recombinant B β 448Arg and B β 448Lys fibrinogen (A) FXIII crosslinking of fibrin resolved on 4-12% SDS-PAGE reducing gels (B) FXIII crosslinking of B β Arg448Lys fibrinogen resolved on 4-12% SDS-PAGE reducing gels in the presence of $\alpha 2\text{-AP}$ and (C) corresponding blots for $\alpha 2\text{-AP}$. Numbers indicate min after initiation of clotting. 3 independent experiments were conducted.

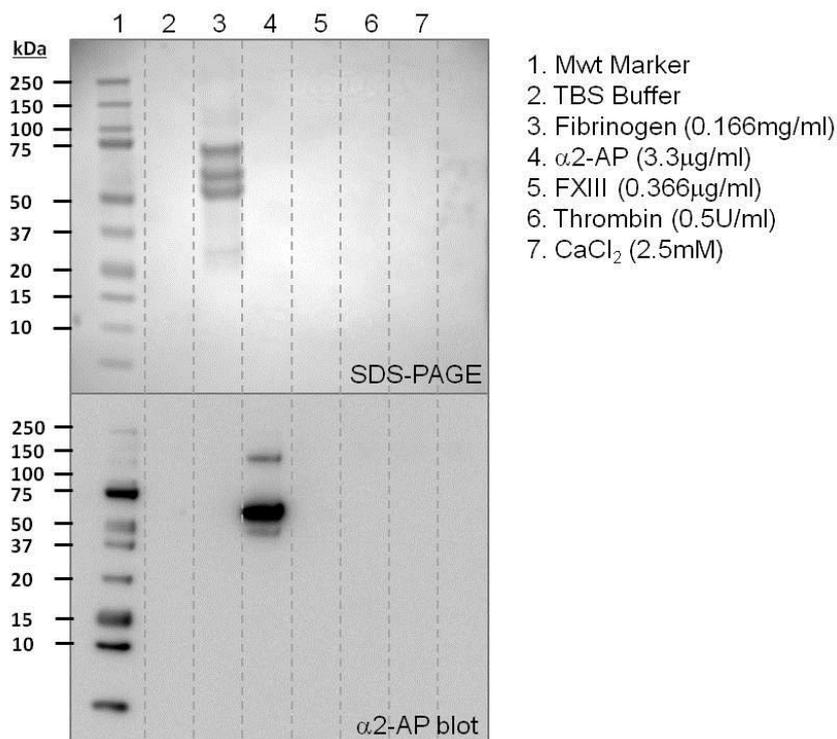


Figure 4-6. SDS-PAGE gel and α 2-AP blot of constituents of FXIII- α 2-AP crosslinking gel

The α 2-AP antibody only picks up α 2-AP in the lane in which it was run, no contamination present in any other well. The lower molecular weight band is present in this α 2-AP preparation. All proteins except fibrinogen are at a concentration in the FXIII crosslinking reaction at which they are not picked up by coomassie staining on the gel.

4.2.5 Confocal microscopy to visualise α 2-AP incorporation

Labelled red, α 2-AP could not be detected in confocal preparations: There were only 1.26 moles of dye per mole protein as determined by calculations set out in the manual when the Dylight 584 labelling kit was used despite repeated labelling attempts. After labelling, the protein stock was at 80 μ g/ml. The optimum number of moles of dye per mole protein for this labelling reaction is 4 so this may account for the lack of fluorescence detected. There were 4.15 moles of dye per mole of protein as worked out by calculations set out in the manual when the Alexa Fluor 488 labelling kit was used. This is within the optimum stated of 4-9 moles of dye per mole of protein. The stock produced was 40 μ g/ml, so the yield was low, but labelling efficiency adequate for these preparations. Results presented in Figure 4-7.

Labelled α 2-AP could only be detected when crosslinked to the B β 448Arg fibrin clot where it co-localised to fibrin. Little to no α 2-AP could be detected in the equivalent B β 448Lys fibrin clots (Figure 4-8).

When B β 448Arg clots were lysed, there was a slight build-up of the α 2-AP along the lysis front which was co-localised to the fibrin. When B β 448Lys clots were lysed, the α 2-AP seemed to ‘appear’ along the lysis front. Similarly, it was co-localised to the fibrin as shown by the yellow structures in the bottom panel. Results presented in Figure 4-9.

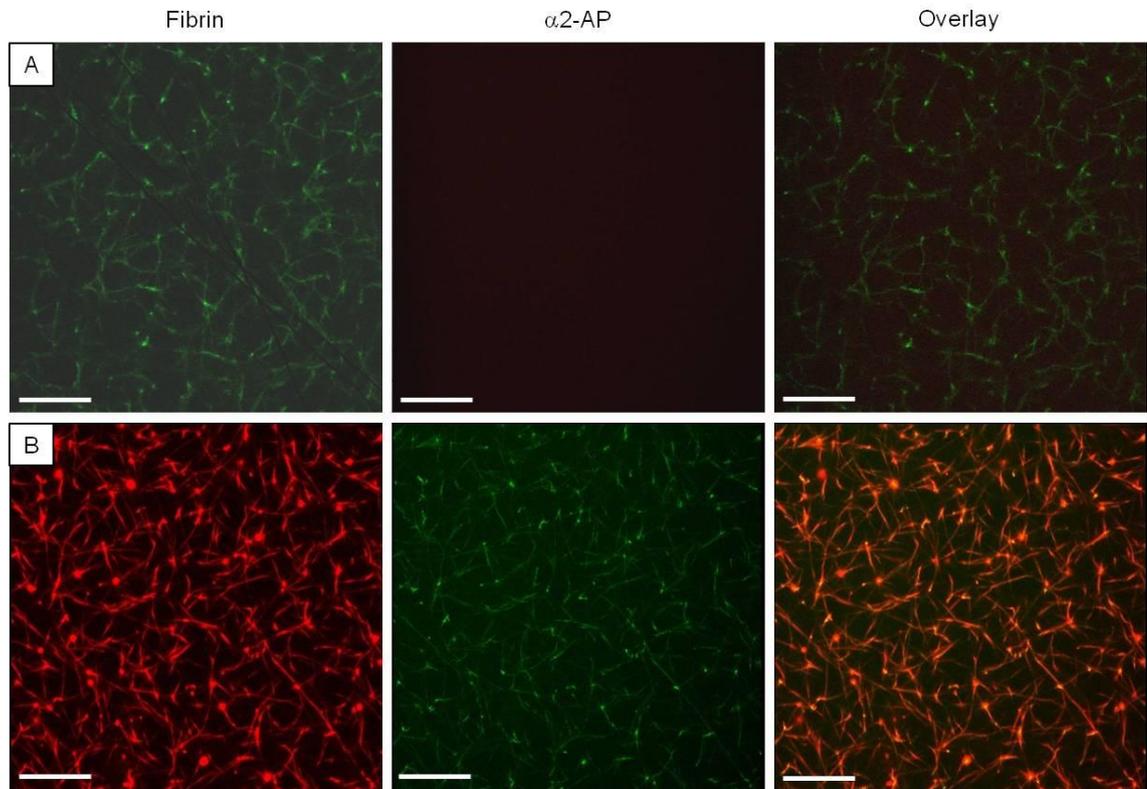


Figure 4-7. LSCM images of fibrin, α 2-AP and their co-localisation

(A) Green fibrin, red α 2-AP (B) Red fibrin, green α 2-AP. α 2-AP could not be detected when it was labelled red and was only visible when labelled green. Scale bars represent 45 μ m.

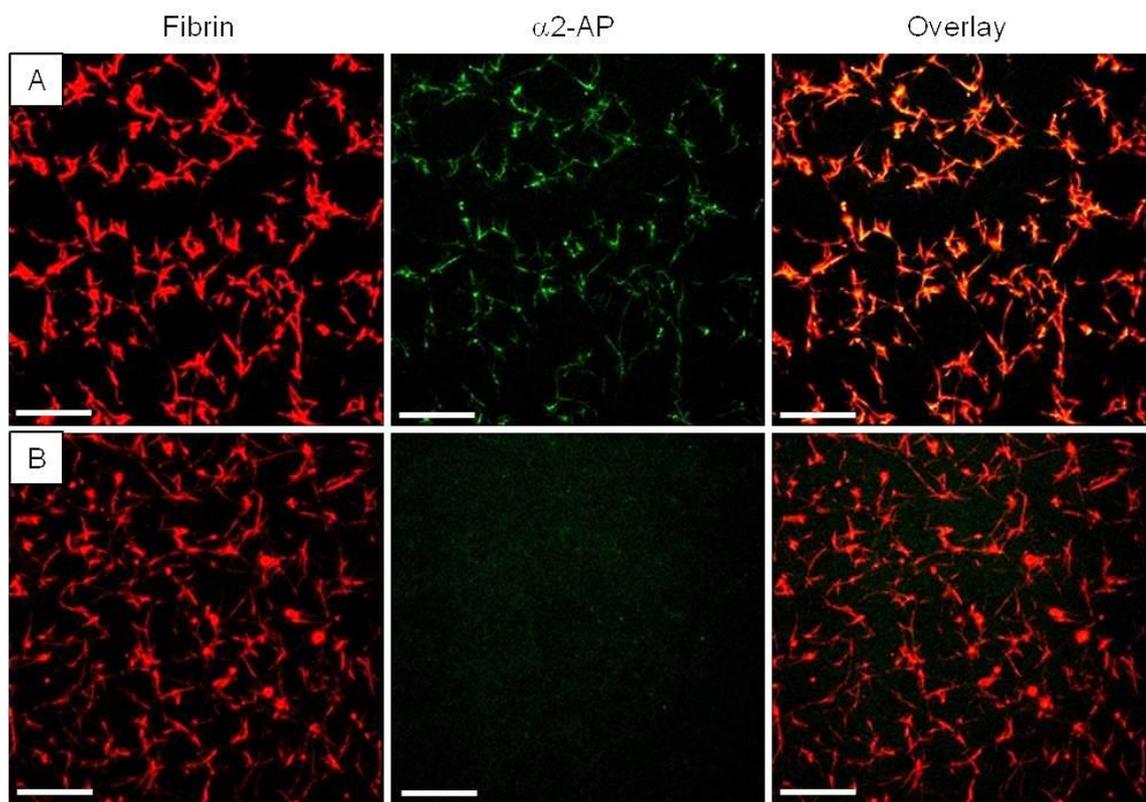
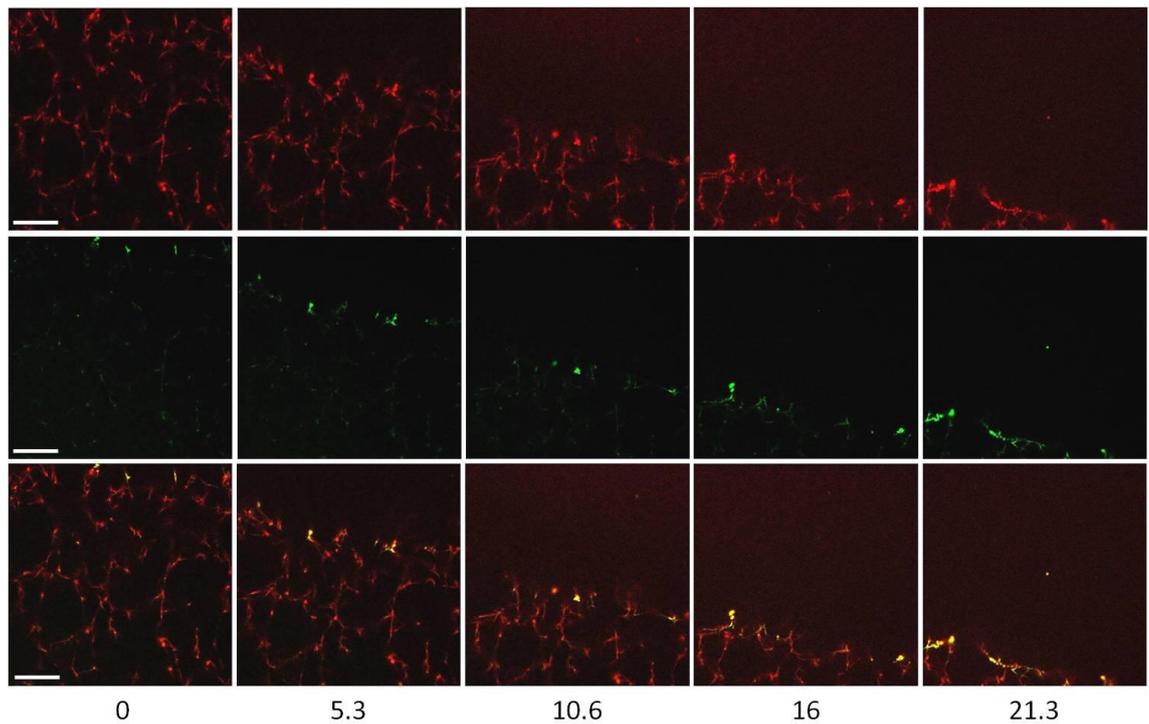


Figure 4-8. LSCM images of clots made from recombinant B β 448 variant fibrinogen in the presence of FXIII and α 2-AP

(A) B β 448Arg fibrin (B) B β 448Lys fibrin. 3 independent experiments were conducted in duplicate. Scale bars represent 45 μ m

B β 448Arg



B β 448Lys

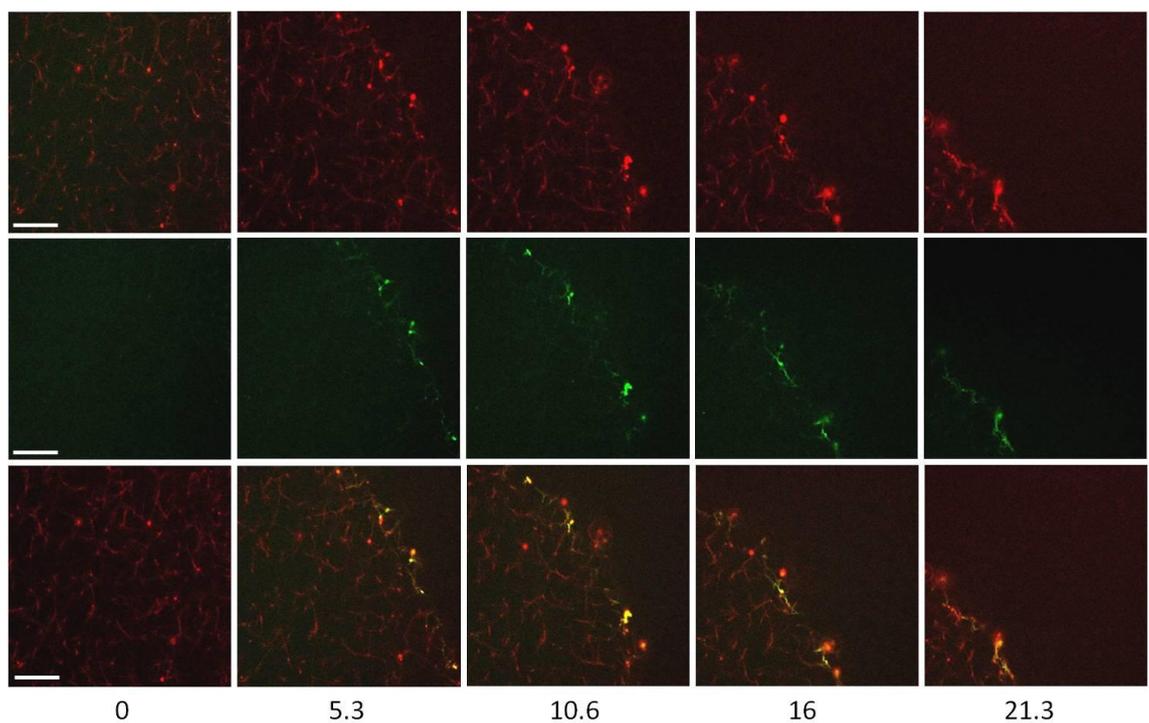
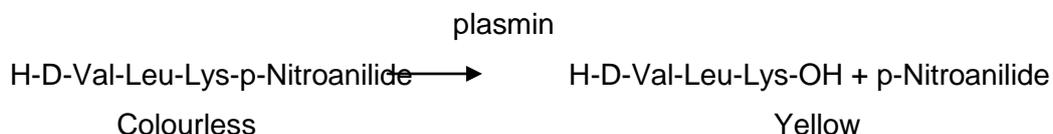


Figure 4-9. LSCM images lysis of clots made from recombinant B β 448 variant fibrinogen

Top panel; Red fibrin. Middle panel; green α 2-AP. Bottom panel; overlay. 3 independent experiments were conducted in triplicate. Min after beginning of time series indicated below panels. Scale bars represent 45 μ m.

4.2.6 Effect of B β 448 variant on plasmin generation

S-2251 peptide cleavage assay was used to assess plasmin activity between the variants in the various turbidimetric conditions. This assay relies on the cleavage of the S-2251 substrate by plasmin as below:



The rate of p-Nitroanilide formation is thus equivalent to the enzymatic activity of plasmin and can be monitored by an increase in absorbance at 405nm. This assay thus measures both the conversion of plasminogen to plasmin, and the consequent activity of the plasmin enzyme. Given that differences in lysis comparing the two variants are only detected in the presence α 2-AP, experiments were conducted with and without this protein.

Five min after initiation of lysis, plasmin generation was significantly greater in the presence of B β 448Lys fibrin than B β 448Arg, in the absence of FXIII (216.7%) as well as in its presence (661.5%). In the presence of α 2-AP, this difference was abolished. Plasmin generation was not significantly different comparing B β 448Arg and B β 448Lys preparations in the presence of α 2-AP alone or in the presence of α 2-AP and FXIII. α 2-AP significantly reduced plasmin activity in B β 448Lys variant both in the absence and presence of FXIII (72.4% and 90.9%), but made no difference to plasmin activity in B β 448Arg.

After 10 min, plasmin activity in the absence of α 2-AP was still greater in B β 448Lys than B β 448Arg (116% without FXIII and 112% with FXIII), however, only a trend was observed. As time progressed, plasmin activity in the absence of α 2-AP between the variants became equivalent (as shown in Figure 4-10; 20, 30 and 60 min panels). At 10 min, α 2-AP had no effect on plasmin generation by B β 448Arg variant in the absence of FXIII or in its presence. However, α 2-AP significantly reduced plasmin activity in the B β 448Lys preparation both in the absence of FXIII (70.0%) and in its presence (77.1%). There was no significant effect of FXIII on plasmin activity in the presence of α 2-AP in either variant.

At 15 min, α 2-AP significantly reduced plasmin generation of the B β 448Arg variant both in the absence (46.7%) and presence (55.0%) of FXIII. As time progressed, α 2-AP continued to cause a significant reduction in plasmin activity with both fibrinogen variants in the presence of FXIII.

At each time point, plasmin generation between the variants was equivalent in the presence of α 2-AP alone, and in the presence of FXIII and α 2-AP. FXIII had no significant effect on plasmin generation in the presence of α 2-AP until 50 min, when it

increased plasmin generation in the B β 448Lys variant (47%), but not in the B β 448Arg variant. At 60 min, the addition of FXIII significantly increased plasmin activity in the presence of α 2-AP in both B β 448Arg (32.5%) and B β 448Lys fibrinogen (55.0%). Data summarised in Figure 4-10.

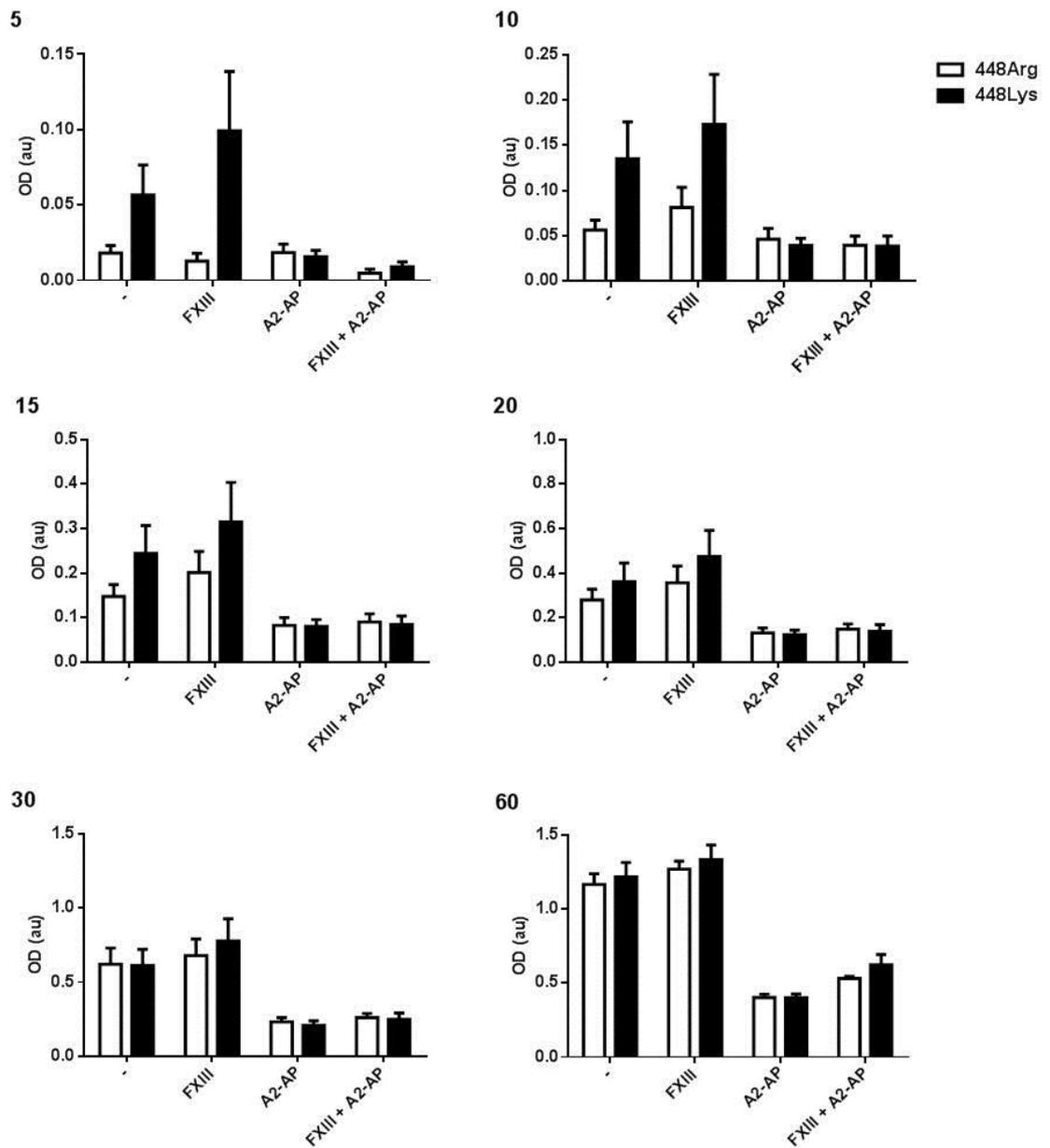


Figure 4-10. Conversion of S2251 by plasmin in the presence of clots made of $B\beta$ 448Arg/Lys fibrinogen

Conversion of S2251 substrate by plasmin as monitored by optical density in the presence of clots made of $B\beta$ Arg448Lys fibrinogen \pm FXIII \pm a2-AP. Panels show OD 5, 10, 15, 20, 30 and 60 min after initiation of lysis.

4.2.7 Atomic Force Microscopy

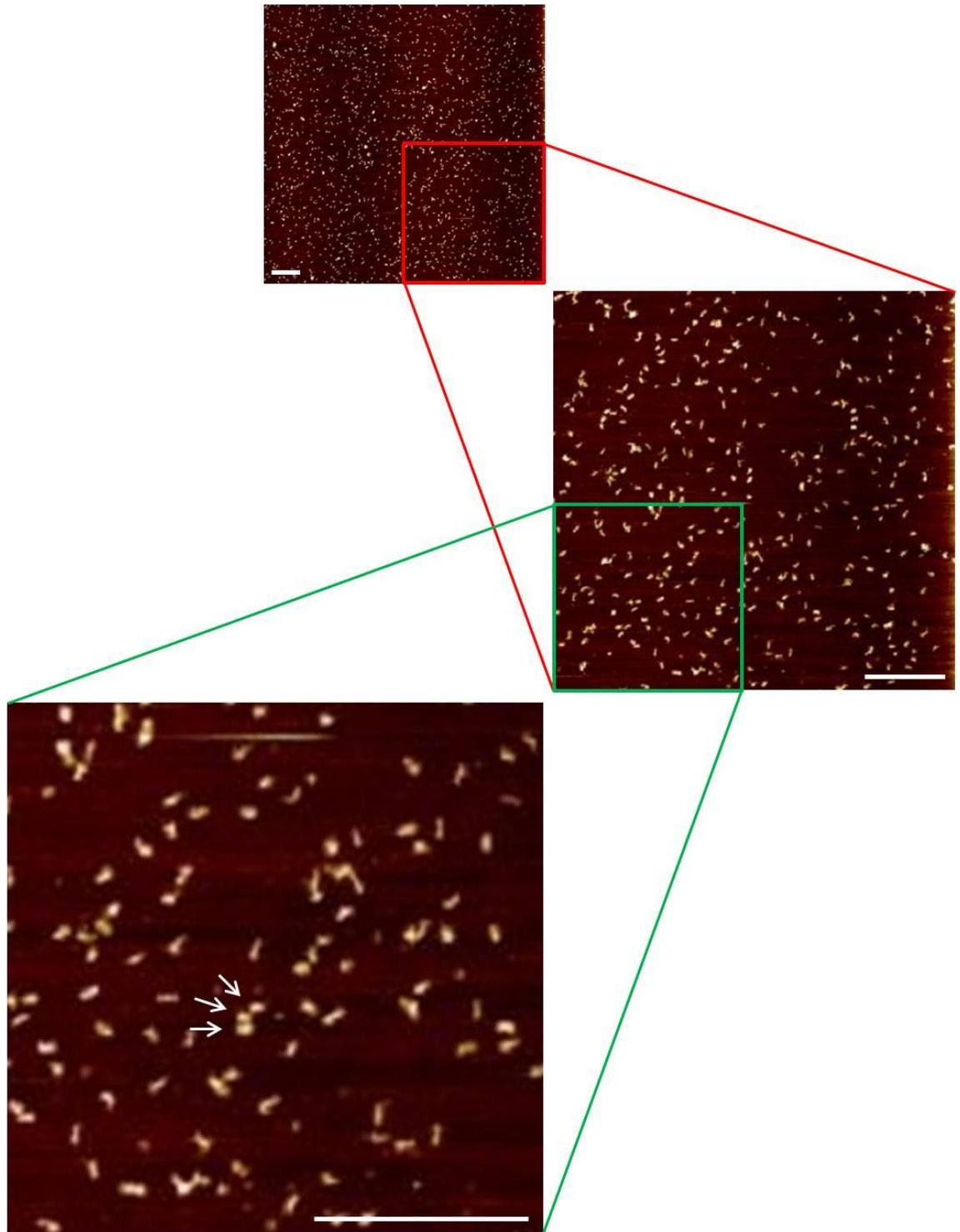


Figure 4-11. Individual fibrinogen molecules resolved by AFM

An area of $5 \times 5 \mu\text{m}$ of sample was scanned (top panel), which has been zoomed in on $\times 4$ (middle panel) and $\times 16$ (bottom panel) to resolve individual fibrinogen molecules. The 3 domain structure of the fibrinogen molecule can be seen (indicated by the 3 white arrows). Scale bars represent $0.5 \mu\text{m}$.

Images of single molecules were able to be obtained as in Figure 4-11. Each of the 3 three domains (D-E-D) could be resolved. When clotting was attempted, no fibres were visualised, only large aggregates could be seen despite repeated attempts with various sample preparations and coating techniques (as shown in Figure 4-12C).

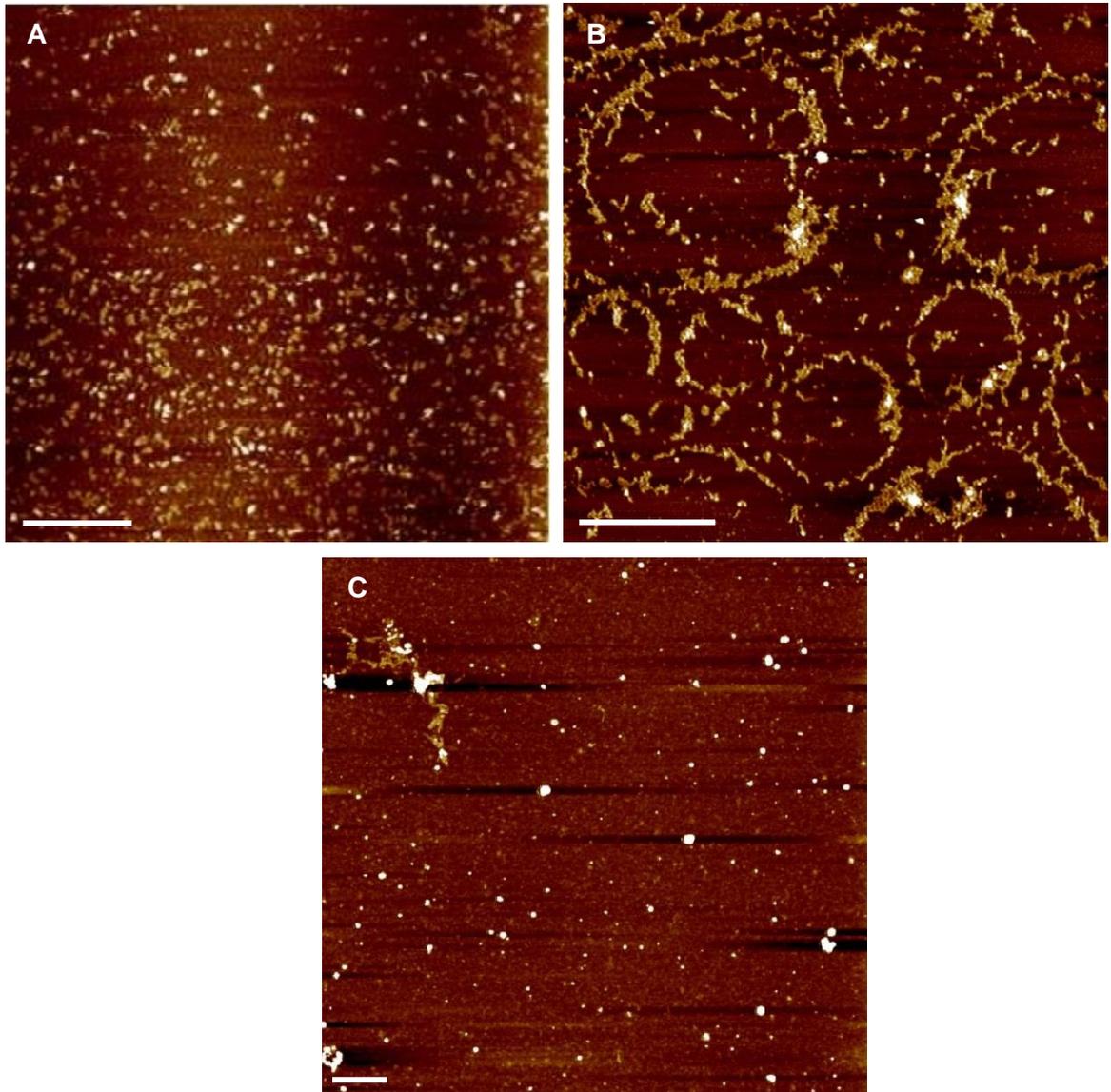


Figure 4-12. Images of fibrinogen showing various problems encountered using AFM

(A) Fibrinogen on mica surface showing uneven distribution of fibrinogen molecules over a $2.5 \times 2.5 \mu\text{m}$ area of mica. (B) Aggregation of fibrinogen molecules on mica surface due to uneven drying process. (C) Typical result of initiation of clotting, large aggregates form 'lumps' on the mica surface and disrupt the rest of the image, no fibres are clearly seen. Scale bars represent $0.5 \mu\text{m}$.

4.2.8 Mass Spectrometry

4.2.8.1 Fibrinogen α -chain

In both B β Arg448Lys variants, there was methionine oxidation at A α 254 and 259 and proline hydroxylation at A α 566. Proline hydroxylation was evident in both variants at A α 289/302 though the position remains unconfirmed. Sequence coverage was poor for both B β Arg448Lys variants, at 20% for B β 448Arg and 17% for B β 448Lys.

4.2.8.2 Fibrinogen β chain

In both B β Arg448Lys variants, there was proline hydroxylation at B β 61 and methionine oxidation at B β 148, 254, 344 and 456. There was evidence of methionine oxidation at B β 477 in the B β 448Lys variant, however we must exercise caution in this observation as this can also be caused as an artefact of the gel processing and digestion of the proteins. Sequence coverage was 55% and 50% for B β 448Arg and B β 448Lys respectively, and in both cases this covered the B β 448 site at which no PTMs were detected in either variant.

4.2.8.3 Fibrinogen γ chain

In both B β Arg448Lys variants, there was tryptophan oxidation at γ 279, proline hydroxylation at γ 102 and methionine oxidation at γ 290. Twelve potential glycosylated peptides were identified as a -162 Da signal shift, characteristic of glycation, detected in the MS signal of each B β Arg448Lys variant (Figure 4-13), but these were not matched to particular peptides so details of their possible localisation are not available. All signals observed were common to both B β Arg448Lys variants. Sequence coverage was 45% and 53% for B β 448Arg and B β 448Lys respectively.

Table 4-1. Summary of modifications on each fibrinogen chain as identified by MS analysis

Chain	Modifications
Alpha	A α 254 & 259 - methionine oxidation A α 566 & A α 289/302 – proline hydroxylation
Beta	B β 61 – proline hydroxylation B β 148, 254, 344 & 456 – methionine oxidation B β 477 (B β 448Lys variant only) – methionine oxidation
Gamma	γ 279 – tryptophan oxidation γ 201 – proline hydroxylation γ 290 – methionine oxidation

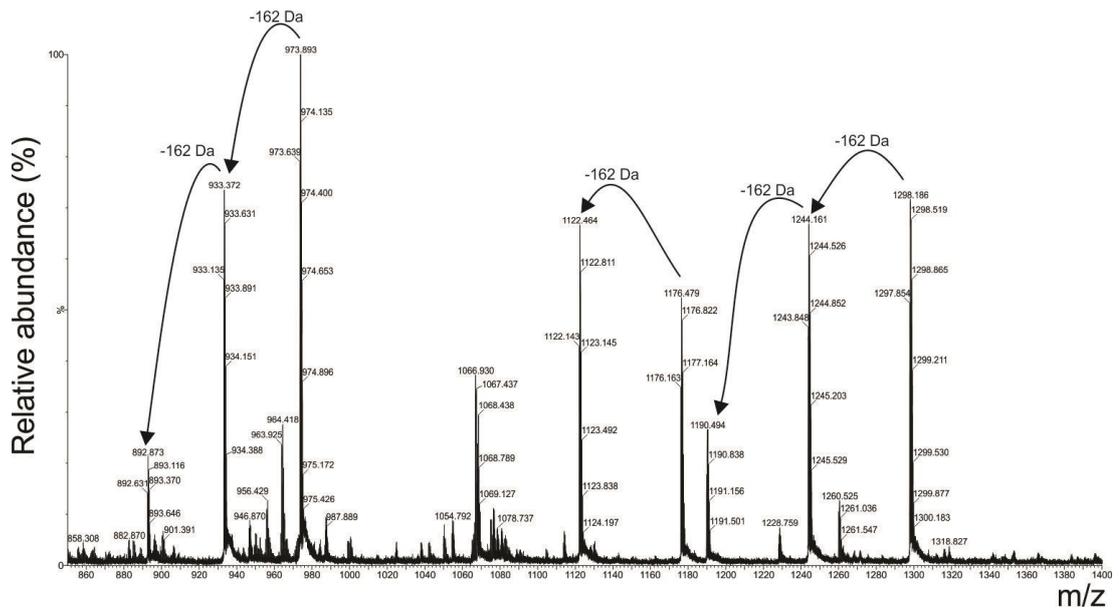


Figure 4-13. Example MS spectrum

A typical MS spectrum plotting mass/charge ratio against relative abundance. The characteristic 162 Da mass shift indicating glycation is shown.

4.3 Discussion

Using recombinant fibrinogen, significant differences in clot structure between B β 448Arg and B β 448Lys variants were found in turbidimetric, LSCM and EM preparations. B β 448Lys fibrin has nearly 6 fold lower turbidity than B β 448Arg, suggesting thinner fibres, which was confirmed by EM analysis. LSCM also revealed the structure of clots made from B β 448Lys fibrinogen to be more compact than those of B β 448Arg. This structure has previously been linked to more thrombogenic clots^{91, 92} suggesting B β 448Lys variant may predispose to thrombotic disorders.

The thinner fibres of clots made of B β 448Lys fibrin suggests lateral aggregation is affected in this variant. Molecular modelling has shed light on some of the possible mechanisms behind this difference in structure. The analysis suggests the B β Arg448Lys substitution could lead to weakening or loss of hydrogen bonds close to the b-site, and in the coiled-coil structure, as well as causing increased mobility of residues in the B-b interaction site. These changes likely affect fibrin polymerisation and final clot ultra structure as all are in regions proposed to be involved in lateral aggregation, via B-b and α C domain interactions¹⁰².

To further investigate the mechanisms behind the thinner fibres of the B β 448Lys fibrin clots, I attempted AFM analysis of early protofibril formation. The aim of this technique was to visualise the individual fibrinogen molecules coming together to assemble protofibrils, identifying differences between the B β Arg448Lys variants.

Despite success in imaging individual fibrinogen molecules using AFM, a number of difficulties were encountered when using this technique, rendering it inappropriate for use for the intended analysis. There is a great deal of sample variability across any given mica surface; the amount of material which adheres to the mica is uneven despite application of a uniform sample (as shown in Figure 4-12A). This has been suggested to be caused by treatment with NiCl₂, which may result in a non-uniform charge across the mica. This in turn leads to patches that are more attractant to negative charge (and thus fibrinogen molecules) than others. The fibrinogen is also liable to aggregate on the surface which can lead to the tip becoming dirty or breaking with resultant drastic drop in image quality. Added to this, the way in which the mica is dried with nitrogen before and after sample application also affects the outcome (as shown in Figure 4-12B).

When clotting was initiated, rather than being able to see fibrils forming, the fibrinogen molecules seemed to clump together to form large aggregates in more globular structures than fibre-like structures (as shown in Figure 4-12C). These problems may have arisen due to the materials I was using. All previous published data using this technique^{132, 133} have been obtained using commercially bought fibrinogen, whereas this study has used recombinant protein. This commercial material may be

less fragile, more robust, or contain contaminants such as FXIII which could lead to the difference in fibrin clot formation. Given the time constraint, I have been unable to develop this technique further and this approach was therefore abandoned.

When lysis of clots formed from the B β Arg448Lys variants was assessed, a difference in clot lysis between the two variants was only evident in the presence of anti-fibrinolytic proteins, with equivalent lysis times seen between the variants in their absence. This suggests that differences in clot structure alone do not account for differences in lysis between the variants. Alternatively, Arg to Lys substitution may result in different effects on the fibrinolytic system, accounting for similar lysis time despite the observed changes in clot structure. It has been suggested previously that this similarity in lysis in the absence of other plasma proteins could be explained by the increased number of thin fibres lysing at a quicker rate at the level of individual fibres, thus 'evening out' the disparity in fibre number¹⁰².

However, our plasmin generation data suggests this phenomenon may be yet more complex, going further to explain the lysis rates of the B β Arg448Lys variants. Despite the differences in clot structure, equivalent lysis rates between the variants may be, at least partially, explained by the initial difference in plasmin generation/activity identified in this assay. This suggests differences in plasmin generation between the variants 'equals out' the lysis time between the B β Arg448Lys variants in the absence of other plasma proteins, causing them to lyse at equivalent rates despite their difference in structure. This implies the B β 448Lys clot structure of thin fibres is more resistant to plasmin activity, but since there is more generated in the absence of other plasma proteins, this structure lyses at a similar rate to B β 448Arg fibrin clots.

The mechanism by which more plasmin is generated in the B β 448Lys clot has not been elucidated and needs further investigation, but it may be due to the increased density of fibres providing more binding sites for plasminogen, where it is most rapidly converted to plasmin by tPA due to colocalisation on the fibrin surface. It has indeed been shown that binding of tPA to fibrin enhances the activation of plasminogen by tPA, increasing its local concentration^{5, 24}. The increased fibrin density of the B β 448Lys variant could thus work as a greater surface area to co-localise tPA and plasminogen on the its surface, working to enhance plasmin generation.

The mechanism by which B β 448Lys fibrin structure is inherently more resistant to plasmin is not clear and requires further investigation. It is encouraging that our work supports that published previously, showing dense clots made of thin fibres to be more resistant to lysis in the plasma environment than those made of loosely woven thick fibrin fibres²³. However, the mechanisms behind this are not completely in line with those published previously. In support of our work, earlier studies suggest that at the

level of individual fibrin fibres, thick fibres are more resistant to lysis, but that a difference in distribution and movement of fibrinolytic components through the fibrin network results in equivalent lysis rates. They show loosely woven clots made of thick fibres with decreased fibrin density allow fibrinolytic components such as tPA to bind more broadly across the clot and move quicker through the fibrin network²³. This gives weight to our theory that B β 448Arg clots are less resistant to plasminogen activity: Due to the increased porosity of the thick fibred clot, fibrinolytic proteins move quicker through the structure, binding more broadly across the clot and increasing lysis rates. However, previous work also suggests plasminogen binding and activation may be inhibited in clots made of fine fibres²⁴ an idea which contradicts the results of our study which suggest increased plasminogen generation in the presence of clots made of thin fibres. This warrants further investigation of the mechanisms behind differences in lysis between clots of differing structure.

When studied in a system more akin to that *in-vivo*, our data show significantly greater prolongation of lysis in the presence of α 2-AP of clots made from recombinant B β 448Lys fibrinogen than those of B β 448Arg fibrinogen, implicating the interaction of clot structure and plasma proteins in the difference in lysis times between the variant fibrins. In line with our data, it has been shown previously that when the B β Arg448Lys recombinant variants were added to fibrinogen-depleted plasma, a comparable large difference in fibrinolysis rates between the variants was observed¹⁰². It was therefore concluded that lysis rates are not directly associated with clot structure, rather the interaction between structure and other plasma proteins. Differential incorporation of α 2-AP and plasminogen into the clot and consequent conversion of plasminogen to plasmin could be explained by differences in crosslinking and binding, quantitatively or qualitatively, of plasma proteins due to the variation in clot permeability and compactness between B β Arg448Lys fibrin variants. My data go further to identify α 2-AP as one of the main factors responsible for this prolongation in lysis, as well as the disparity in lysis rates between the B β Arg448Lys variants in the plasma environment. The observation that FXIII plays a role in α 2-APs inhibitory effect on fibrinolysis in our study indicates that cross-linking of the protein plays a key role in α 2-AP induced changes in lysis. On the other hand, the inhibition of fibrinolysis in B β 448Lys compared to the B β 448Arg in the absence of FXIII suggests that binding also plays a role. Accordingly, we investigated FXIII crosslinking of B β Arg448Lys fibrin as well as α 2-AP to the fibrin variants.

Perhaps surprisingly, given the stark differences in the clot structure between the variants and the role FXIII plays in formation of the clot, FXIII crosslinking of the fibrin chains in the absence of other factors did not differ between the variants. This suggests FXIII does not interact with the variant fibrinogens differently and does not play a direct

role in the difference in structure between B β 448Arg and B β 448Lys fibrinogen. Furthermore, our plasmin generation data suggests the difference in lysis between the B β Arg448Lys variants is not related to differential plasmin generation. We suggest that in the presence of α 2-AP there are equivalent amounts of plasmin between the variants causing the B β 448Lys variant to lyse slower due to the fibrin structure being inherently more resistant to lysis, the potential mechanisms behind which are discussed above. Therefore, it appears that α 2-AP 'evens out' the initial difference in plasmin generation between the variants in the absence of other plasma proteins, having a greater effect on the initially enhanced plasmin generation in the B β 448Lys variant. This greater effect of α 2-AP may mirror that of plasminogen and tPA discussed above; the increased surface area of the B β 448Lys fibrin clot could provide more binding sites for α 2-AP, allowing for it to exert a greater effect on both the fibrin's resistance to lysis, and the conversion of plasminogen to plasmin by tPA. One difficulty in interpreting these results fully is the inability of this assay to distinguish between plasmin generation and plasmin activity so it cannot be determined definitively how the fibrin variants are affecting the fibrinolytic activity. Investigating plasminogen conversion to plasmin using immunoblotting, as recently described⁸⁰, represents one technique to differentiate between plasminogen conversion and plasmin activity, which remains an area for future research. The differences in lysis between the two variants is yet more complex as this theory of differential plasmin generation/ activity does not account for the increased lysis time in the presence of FXIII when α 2-AP is present, as a result this merits future investigation of the complex interactions between B β Arg448Lys, clot structure, plasmin generation, FXIII cross-linking, α 2-AP and lysis rates.

Again unexpectedly, despite the differences in the effect of α 2-AP between the variants, the rate and amount of incorporation of the protein by FXIII into the fibrin network did not differ between B β 448Arg448Lys variants in the biotin/ α 2-AP incorporation or western blot assays. This suggests the protein is incorporated to the same degree between the B β Arg448Lys variants. Differences in lysis could thus be due to a different structural arrangement or interaction of the α 2-AP between the two B β 448 variants. This theory was further explored by LCSM analysis.

The LCSM study of B β 448 fibrinogen variants with fluorescently labelled α 2-AP has produced further unexpected data and has provided further insight into the difference in interaction between the two proteins. It is interesting that the α 2-AP can be seen in static clots made from B β 448Arg only and not B β 448Lys variant. However, it is clear that there is α 2-AP present in the B β 448Lys preparations as it 'appears', as green fluorescence, along the lysis front when the clot is lysed. There are two suggested explanations for this; the α 2-AP is incorporated into the static clot through an unusual arrangement with the fibrin fibres that makes this undetectable until the clot

undergoes lysis, which exposes the protein. Alternatively, free $\alpha 2$ -AP in the clot only binds to the B β 448Lys fibrin upon initiation of clot lysis. It seems more likely, given the equivalent incorporation of $\alpha 2$ -AP between the variants in other assays such as biotin/ $\alpha 2$ -AP crosslinking and western blot analysis, that the $\alpha 2$ -AP is incorporated into the B β 448Lys LSCM clots, but somehow differently to that in the B β 448Arg clots, which could result in it not being detected in the static clot preparation. It was suggested that the $\alpha 2$ -AP could be binding/crosslinking with such high affinity to the B β 448Lys fibrin that the 594 Alexa signal of the fibrin was quenching the 488 Alexa $\alpha 2$ -AP fluorescence. This was ruled out by preparing clots from both B β Arg448Lys variants with only 488 Alexa $\alpha 2$ -AP, and no labelled fibrinogen. In these preparations, the 488 Alexa $\alpha 2$ -AP could still only be seen in the B β 448Arg preparations, mimicking the structure of the fibrin network, presumably due to it being bound to the non-fluorescent fibrin structure. However, 488 Alexa $\alpha 2$ -AP fluorescence could still not be detected in the B β 448Lys preparations. These findings warrant further investigation of the interaction between $\alpha 2$ -AP and the B β Arg448Lys variants of fibrinogen to characterise their binding and crosslinking activity. However we postulate that $\alpha 2$ -AP may be incorporated more tightly into the fibrin fibres of the B β 448Lys variant such that it is not detected in LSCM because it is embedded deeper in the fibre. Conversely the $\alpha 2$ -AP in B β 448Arg fibrin preparations may be visible due to the more loosely woven fibres allowing the $\alpha 2$ -AP molecules to be seen on the outside of the fibres and thus detected by LSCM. Another theory suggests the different way in which $\alpha 2$ -AP is incorporated into B β 448Lys fibrin prevents excitation of the 488 fluochrome, meaning the protein cannot be seen in the clot but can when the clot is lysed.

Since glycation, oxidation, phosphorylation, homocysteinylation and nitration of fibrinogen cause stark differences in clot structure and lead to clots which are more resistant to lysis (described in section 1.10.2.2.1), it was reasonable to suggest these changes may cause the differences seen in the recombinant B β Arg448Lys variants, especially as lysine residues are particularly amenable to glycation and oxidation. MS analysis revealed evidence of glycation, oxidation, and hydroxylation across all three of the fibrinogen chains; though no differences in modifications between B β Arg448Lys variant samples were observed. However, the possibility of differences in PTMs cannot be ruled out due to low sequence coverage, especially of the alpha chain, meaning some PTMs may have been missed by this analysis. Poor tandem MS fragmentation also meant that glycation sites could not be determined, leading to the need for further MS analysis. This poor coverage and fragmentation may be attributable to trypsin not being ideal for the digestion of fibrinogen, which has a large number of basic residues. The overall character of the peptides could also play a role as extreme hydrophilicity, poor ionisation and presence of modifications can affect the coverage of an MS

spectrum. These issues could be overcome by combining with alternative proteases for fragmentation, higher specification instrumentation and/or alternative fragmentation techniques. While electroelution was a promising technique for separating out the intact fibrinogen chains, further optimisation of sample preparation before loading onto the MS is needed to obtain intact mass of the individual chains. The use of electron transfer dissociation analysis, a method of fragmenting ions by transferring electrons to them, could also be valuable as it ensures sample fragmentation along the peptide backbone, thereby ensuring preservation of PTMs¹³⁴. It should be stressed, however, that no difference in PTMs was seen at B β Arg448Lys position comparing the two variants and therefore potential differences, if any, would be secondary to an indirect effect of the polymorphism on other sites of the fibrinogen molecule.

It is accepted that not only rate of plasmin generation but its movement through the clot is rate limiting for fibrinolytic activity and that it is not fibrin fibre diameter, but fibrin network structure, that governs the distribution of fibrinolytic proteins²³. Although our data lead to a similar conclusion, we suggest that fibrin network structure plays a much more important role than previously suggested. Clots formed from thin fibres with a compact structure are more resistant to lysis/ plasmin activity, most likely due to the decrease in porosity and permeability of these clots to fibrinolytic components. The observation that plasminogen activity is increased in the presence of clots made of thin fibres requires further investigation, especially given the discrepancy with a previous study which suggests plasminogen binding and activation may be inhibited in clots made of fine fibres, contradicting our data²⁴.

Together the data presenting in this chapter suggest that α 2-AP is crosslinked to B β Arg448Lys fibrin variants to a similar extent. However, the structural conformation of these binding and crosslinking interactions is markedly different, such that an initial increase in plasmin activity in the absence of α 2-AP, results in similar lysis rates of the two variants due to the inherent increased resistance to lysis of the B β 448Lys clot. However, in the presence of α 2-AP, this is no longer the case and hence the exaggerated prolongation of clot lysis time with B β 448Lys variant of fibrinogen. It follows that α 2-AP has a more marked effect on plasmin generation in the B β 448Lys clot, indicating an interaction between genetic variants of fibrinogen and the fibrinolytic system.

In summary, we have found that differences in lysis between recombinant B β Arg448Lys variants in the plasma environment are attributable to the interplay between porosity and permeability of the clot, the fibrin- α 2-AP interaction and plasmin generation. This leads to the need for future work to clarify the mechanisms behind differential plasmin generation and interaction of α 2-AP in fibrin clots of differing structure. These data indicate that the previously proposed direct association between

clot structure and lysis²³ is perhaps a simplistic approach and in depth studies are needed to understand the relationship between clot structure and activation of the fibrinolytic system, which may have future therapeutic implications.

**Chapter 5. Fibrinogen Birmingham II - A Novel Variant
Associated with an Unusual Clinical Presentation and Altered
Fibrin Network Characteristics**

Introduction

Dysfibrinogenemia, a group of conditions in which an abnormality in the fibrinogen molecule is inherited, results in defective fibrin clot formation, with a clinical presentation that may be life-threatening¹⁰³. These conditions provide a useful tool to investigate the molecular mechanisms for the effect of small changes in the fibrinogen gene on clot structure and susceptibility to disease.

Nonsense/missense mutations, insertions/deletions and chain truncations in the fibrinogen genes have all been identified as responsible for various dysfibrinogenemias resulting in modifications of fibrinopeptide release, fibrin polymerisation, fibrin crosslinking and/or fibrinolysis. The position in which the mutation lies governs whether the dysfibrinogenemia will present as a bleeding or prothrombotic disease. The genetic changes often lead to the development of hypofibrinogenemia due to their effect on intracellular transport mechanisms and consequent accumulation of fibrinogen in the endoplasmic reticulum¹⁰⁴. In more severe cases, a complete lack of expression of the mutant molecules in plasma can occur due to the inability of the mutant chain to be assembled with the other fibrinogen chains. The severity of the condition can depend on whether the variant is homo- or heterozygous. Very few homozygote dysfibrinogenemias have been identified, and these result in a homodimeric fibrinogen molecule with two mutant chains. The more common heterozygous forms can result in one of two homodimers (either two normal or two abnormal chains) or a heterodimer with one normal chain and one abnormal chain. Heterozygotes that have 50% normal fibrinogen molecules are usually asymptomatic as this amount of normal fibrinogen molecules is able to maintain normal blood coagulation. Symptoms appear when the dysfibrinogen interferes with functional components of the fibrinogen molecule³⁵.

A rare novel fibrinogen variant has been detected in a 34 year old woman, which is associated with hypodysfibrinogenemia¹⁰⁸. Clinically, the individual presented with a bleeding tendency associated with paradoxical venous thrombotic events. A familial history of hypofibrinogenemia, with a bleeding tendency was recorded but there were no cases of thrombosis. The complex clinical picture was associated with a compound heterozygosity for Trp334Cys and Asn335Tyr mutations in the α -chain of fibrinogen. Two mutations in the polypeptide have been identified at positions 1002 and 1003 causing a guanine and an adenine respectively to be both replaced by thiamine. The amino acid residues, affected by the candidate mutations, are both highly conserved residues in the α C domain of fibrinogen, which has a role in crosslinking reactions during fibrin polymerisation.

The primary aim of this chapter was to fully characterise the effect of Fibrinogen Birmingham II (BII) on clot structure and lysis and elucidate whether changes in the fibrin network, if any, are responsible for the clinical picture. Secondary aims of the

work include investigating the molecular mechanisms behind the identified changes in the clotting of this variant using both purified and recombinant variant fibrinogen.

5.1 Methods

5.1.1 Sequencing/ genotyping

DNA was prepared using Qiagen extraction kit. Genetic analysis was carried out by PCR and nucleotide sequencing of the entire coding regions/splice junctions of FGA, FGB and FGG genes. Sequencing was carried out using BigDye terminators v1.1 on an ABI 3130 genetic analyser. TA cloning was carried out to investigate inheritance pattern. This was done using a TOPO vector (Invitrogen) / TOP10 E. coli and XL1 Blue cells. DNA was prepared using Qiagen miniprep kit, and EcoR1 digest products sequenced as above. Sequencing was carried out by our collaborator, Dr. Marian Hill in Nottingham.

5.1.2 SIFT analysis (Sorting Intolerant From Tolerant)

SIFT is a program which predicts whether an amino acid substitution affects protein function and hence, potentially alters phenotype (<http://www.blocks.fhcrc.org/sift/SIFT.html>). Unlike Polyphen, the SIFT algorithm relies solely on sequence, sequence homology and the physical properties of amino acids to predict the effect of the amino acid substitution. To predict whether an amino acid substitution in a protein will affect protein function, SIFT considers the position at which the change occurs and the type of amino acid change. The program presumes that important amino acids are conserved so changes at well-conserved positions are predicted as deleterious. If a position in an alignment contains a hydrophobic residue then SIFT assumes that this position can only contain amino acids with hydrophobic character. At this position, changes to other hydrophobic amino acids are usually predicted to be tolerated but changes to other residues, such as charged or polar, will be predicted to affect protein function¹³⁵. This analysis was performed by our collaborator, Dr. M Hill.

5.1.3 Polyphen analysis (polymorphism phenotyping)

Polymorphism Phenotyping (www.bork.embl-heidelberg.de/polyphen) is a tool which predicts possible impact of an amino acid substitution on the structure and function of a human protein. A sequence-based characterisation of the mutation site is performed to identify any functionally important regions (e.g. an active/ binding/ trans-membrane region). Mutations at these sites are assessed to evaluate their effect. Mapping of amino acid replacement to the known 3D structure reveals whether the replacement is likely to destroy the hydrophobic core of a protein, electrostatic interactions, interactions with ligands or other important features of a protein such as

secondary structure, solvent accessible surface area, inter-chain contacts or contacts with functional sites. Accordingly, the mutation is appraised by empirically determined cut-offs used to predict if the substitution is 'probably damaging', 'possibly damaging' or 'benign'¹³⁶. This analysis was performed by our collaborator, Dr. M Hill.

5.1.4 Analysis of clot formation and Lysis

The patient gave informed consent for her blood sample to be studied further to elucidate the mechanisms for the clinical presentation. Blood samples were taken into citrated tubes, plasma spun down within 2 hr of collection and samples were stored in -80 °C until analysis. Clot turbidity and lysis measurements were conducted as previously described in section 2.4.

5.1.5 Protein purification

Fibrinogen was purified by affinity chromatography using a calcium dependent IF-1 monoclonal antibody (Kamiya Biochemical, Seattle, WA, USA) and an automated chromatography system (Biocad sprint, Applied Biosystems, Warrington, UK) as previously described in section 2.1.3.

5.1.6 Recombinant protein engineering

5.1.6.1 Vector construction

Vectors were constructed and recombinant protein expressed as detailed below. A vector including the fibrinogen A α chain was altered using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA);

5.1.6.1.1 Primer design

Mutagenic primers, sourced from Invitrogen (Poole, United Kingdom) were designed that contained the changed bases needed to code for the new amino acid, in this case the two bases changed were next to each other at position 1002 and 1003 on the fibrinogen α -chain. These mutations present as G>T and A>T substitutions and cause amino acid changes Trp334Cys and Asn335Tyr respectively. The primers were designed using the sequence for the fibrinogen alpha-chain gene from the National Centre for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/nucleotide/>) and the web-based QuikChange Primer Design Program on the agilent website (<https://www.genomics.agilent.com/CollectionSubpage.aspx>). This program ensured the primers extended 10 to 15 bases on either side of the altered base, had an overall length of between 25 and 45 bases and a melting temperature of 78 °C. It was ensured

both of the designed mutagenic primers contained the desired mutation and annealed to the same sequence on opposite strands of the plasmid with a minimum GC content of 40% and terminated in C or G bases. The primers were PAGE purified. The primers generated were checked to see if they attached to any other, undesired, part of the plasmid using Clustal software on the European Bioinformatics Institute webpage (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The output generated allowed determination of how many times the primer sequence corresponded to the plasmid sequence. Alignment of the primers on the sequence more than once meant they could not be used. The primers generated for mutation of the fibrinogen α chain (mutated bases in bold) were:

Forward 5' GAAGTGCTGGAAGCTG**TT**ACTCTGGGAGCTCTGG 3' and

Reverse 5' CTTACGACCTTCGAC**AAT**GAGACCCTCGAGACC 3'

5.1.6.1.2 Genetic manipulation

A gene is made up of many different sections, transcribed into a single RNA molecule and consequently translated into a specific protein. Alongside the sequence that is directly translated into the resulting protein, there are many regulatory sequences that control the translation of the protein. These are depicted in Figure 5-1. The promoter (P), a region that binds RNA polymerase, and the specific transcription starting point (T_c) lies before the transcriptional unit in an area known as the upstream regulatory region which may also contain enhancers and operators, which are other controlling elements. At the other end of the transcriptional unit lies the transcription stop site (t_c). Between T_c and t_c is the region of DNA transcribed into RNA. The coding sequence lies within this transcribed region and is defined by a translation start (T_L) and stop (t_L) site. This sequence is made up of translated (exons) and non-translated (introns) regions. The introns are removed in the nucleus before the messenger RNA is translated and the resulting sequence translated into amino acids to form the mature protein.

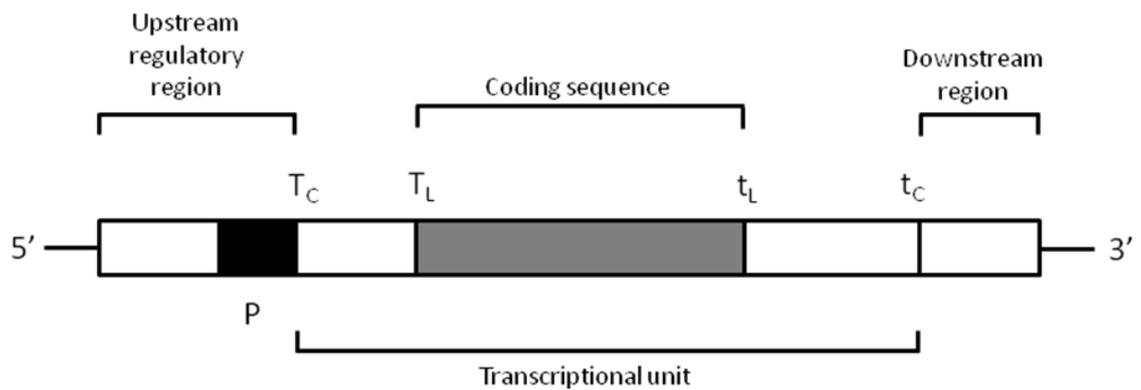


Figure 5-1. Diagrammatical representation of a gene

Upstream regulatory region containing the promoter, coding sequence contained within the transcriptional unit, downstream region, transcription start and stop sites and translation start and stop sites are shown.

To generate recombinant protein, recombinant DNA must be created, which is subsequently inserted into a plasmid. This can be achieved using restriction enzymes and DNA ligase to cut DNA, producing 'sticky ends' that can then be annealed together with other DNA fragments with complimentary cohesive ends by the same restriction enzymes.

When designing a plasmid, certain considerations should be kept in mind: They should be fairly small DNA molecules to allow isolation and handling. They must also contain several regions; an origin of replication, allowing DNA copying and preservation of the plasmid DNA in the cell population; a selectable marker, allowing the plasmid to be selected for, such as an antibiotic resistance gene or fluorescence tag; and at least one unique restriction endonuclease recognition site to allow insertion of recombinant DNA. Many plasmids possess a region known as the multiple cloning site (MCS) which contains many unique restriction endonuclease sites in a short DNA fragment, allowing a choice of sites for DNA insertion.

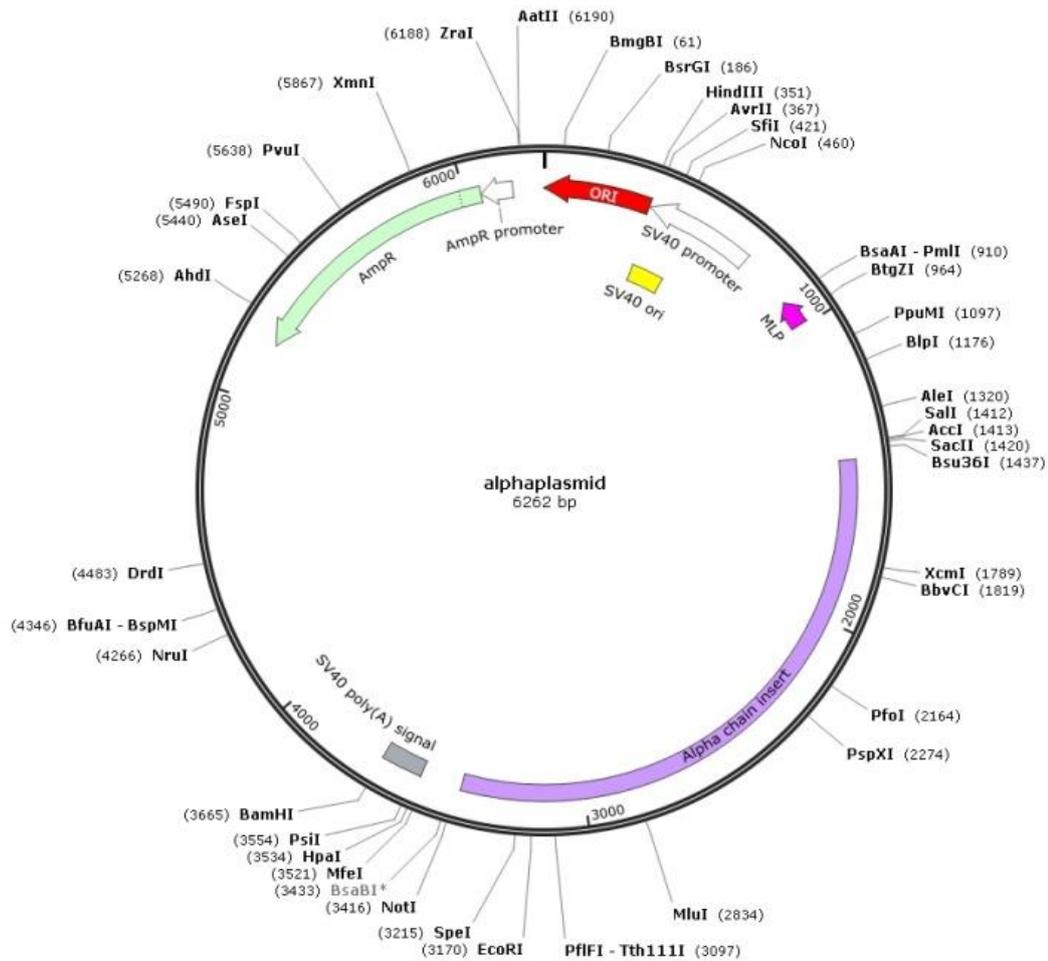


Figure 5-2. Map of the plasmid containing the fibrinogen α -chain plasmid

Alpha chain insert, major late promoter, origin of replication and ampicillin resistance gene are shown.

We used this plasmid (Figure 5-2) and changed the bases of the inserted coding sequence for the fibrinogen alpha chain to our purpose with our designed primers. This was done with the use of The QuikChange II site-directed mutagenesis kit (Agilent, CA, USA) which is able to make point mutations, replace amino acids, and delete or insert single or multiple adjacent amino acids.

5.1.6.1.3 Site directed mutagenesis

The process of site directed mutagenesis works in 5 steps. Firstly mutant strand synthesis takes place by thermal cycling to denature the DNA template then mutagenic primers containing the desired mutation are annealed to the DNA and extended with pfuUltra DNA polymerase. The template is Dpn I digested and finally the mutated molecule is transformed into competent cells for nick repair. Two complimentary

oligonucleotides containing the desired mutation, flanked by the unmodified nucleotide sequence of the fibrinogen alpha chain were synthesised as above.

A control reaction was prepared in a PCR tube containing reaction buffer, 0.2 ng/μl pWhitescript 4.5 kb control plasmid, 2.5 ng/μl oligonucleotide control primer #1 and #2, deoxyribonucleoside triphosphates (dNTPs) and 0.05 U/μl PfuUltra HF DNA polymerase. A series of 3 test reactions using various amounts of double stranded DNA (dsDNA) template were prepared containing reaction buffer, 5/ 20/ 50 ng dsDNA template, 2.5 ng/μl forward and reverse primer, dNTPs and 0.05 U/μl PfuUltra HF DNA polymerase. The reaction mixtures in the PCR tubes were mixed by pipetting up and down several times, sealed with a lid and placed in a PTC-200 Peltier Thermal Cycler (MJ Research, Quebec, Canada). Thermal cycling was then carried out: The reaction was held at 95 °C for 30 sec before 18 cycles of; 30 sec at 95 °C, 60 sec at 55 °C and 60 sec at 68 °C. The reaction was then held at 4 °C until sample collection.

5.1.6.1.4 Digestion of non-mutated DNA

After thermal cycling was complete, 1 μl of the Dpn I restriction enzyme (Agilent) (10 U/μl) was added to each amplification reaction and each reaction was then gently and thoroughly mixed by pipetting the solution up and down several times. The reaction mixtures were centrifuged for 1 min at 13000 rpm and immediately incubated at 37 °C for 1 hr to digest the parental (nonmutated) supercoiled dsDNA.

5.1.6.1.5 Transformation of mutated DNA into XL-blue cells

The nicked vector DNA containing the desired mutations was then transformed into XL1-Blue supercompetent cells (Agilent). XL1-Blue supercompetent cells were gently thawed on ice. For each control and sample reaction to be transformed, 50 μl of the supercompetent cells were aliquoted to a pre-chilled 15 ml Falcon tube and 1 μl of the Dpn I-treated DNA from each control and sample reaction transferred to separate aliquots of the supercompetent cells. The transformation reactions are swirled gently to mix and incubated on ice for 30 min. The reactions were then heat shocked for 45 sec at 42 °C and then placed on ice for 2 min. 0.5 ml of SOC broth (Sigma-Aldrich, MO, USA) preheated to 42 °C was added and the reactions incubated at 37 °C for 1 hr with shaking at 250 rpm. The transformation reactions were plated on agar/ampicillin plates (50 μg/ml ampicillin); the mix evenly distributed on the plate with the plating tool, sealed with film, turned upside down and incubated at 37 °C for >16 hr. For each transformation, 3-4 plates with different dilutions of the transformed cells were prepared. Volumes between 100 and 250 μl of each transformation reaction were used. The gene for ampicillin was introduced into the cells with the mutant vector to

ensure that only bacteria which were successfully transformed were able to grow in ampicillin-supplemented media.

5.1.6.1.6 Colony selection

After 16 hr, single bacterial colonies appeared on the agar/ ampicillin plates. The plates were removed from 37 °C and stored at 4 °C to halt colony growth. 10-15 single isolated colonies were picked per transformation. The colonies to be removed from the plate were circled on the bottom of the plate to prevent the same colony being selected twice if more cells had to be picked if no positive clone was found in the first batch.

For each colony, a 15 ml falcon tube containing 5 ml LB Broth (0.17 M NaCl, 0.063 M tryptone, yeast extract, pH 7) containing 50 µg/ml ampicillin was prepared. Each colony was picked with a sterile pipette tip and put in the LB broth. Tubes were loosely closed and incubated overnight at 37 °C with shaking (~12-16 hr). If the incubation period was too short, the yield of DNA was low, but if left too long, overgrowth killed the bacteria. The DNA from all colonies was isolated the next morning using a QIAprep Spin Miniprep Kit (Qiagen, CA, USA).

5.1.6.1.7 Isolation of mutated DNA

The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. The procedure consists of three basic steps: Bacteria are lysed under alkaline conditions, and the lysate is subsequently neutralised and adjusted to high-salt binding conditions. DNA is adsorbed to the QIAprep membrane, while RNA, cellular proteins and metabolites are not retained on the membrane. Finally endonucleases and salts are removed by a wash step and high quality plasmid DNA is eluted with water.

The bacterial cells from overnight growth were harvested by centrifugation at >8000 rpm (6800 g) in a MIKRO 2000 centrifuge (Hettich, Germany) for 3 min at room temperature. All supernatant were then removed. The pelleted bacterial cells were resuspended in 250 µl Buffer P1 and transferred to a microcentrifuge tube. The bacteria were resuspended completely by vortexing until no cell clumps remained. 250 µl of Buffer P2 was added and mixed thoroughly until the solution became viscous and slightly clear. The lysis reaction was not allowed to proceed for more than 5 min before 350 µl Buffer N3 was added and mixed until the solution became cloudy. The mix was centrifuged for 10 min at 13,000 rpm resulting in formation of a compact white pellet (all consequent centrifugation steps were carried out at 13,000 rpm). The supernatants were applied to the QIAprep spin column, the columns centrifuged for 60 sec and the flow-through discarded. The QIAprep spin columns were then washed by addition of

0.5 ml Buffer PB and centrifuging for 60 sec. Again, the flow-through was discarded. 0.75 ml Buffer PE was added to the columns to wash again and the columns centrifuged for a further 60 sec. Having discarded the flow-through, the QIAprep columns were centrifuged for an additional 60 sec to remove residual wash buffer. The QIAprep columns were placed in clean 1.5 ml microcentrifuge tubes and DNA eluted by addition of 50 μ l water to the centre of each QIAprep spin column, which were left to stand for 1 min, then centrifuged for 60 sec. Eluted DNA was stored at -20 °C.

5.1.6.1.8 Analysis of integrity of eluted DNA

A gel was run to check the DNA eluted from the miniprep was of sufficient quality, was the same length as the plasmid and had not been fragmented. For a 1.5% gel, 1.5 g metaphor agarose powder (Lonza, Basel, Switzerland) was added to 100 ml Tris-acetate-EDTA buffer (TAE; 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8) in a wide necked conical flask. The flask was then weighed before microwaving with intermittent shaking until the mixture was dissolved. The flask was weighed again and distilled water added back to the initial weight. A gel tray was set up so both ends were sealed and an adequate number of separators to form wells slotted in the setup. 10 μ l ethidium bromide (Sigma) was added to the gel mix which was slowly poured into the corner of the tray and left to set for 30 min. The ends of the tray and the separators were removed and the gel transferred to a chamber of TAE connected to a power supply. It was ensured the gel was placed in the correct orientation so the migration of the negative DNA towards the positive (red) electrode was in the correct direction. Enough TAE was added in order to cover the gel and fill the wells. For each miniprep elutant and the original plasmid, a 15 μ l sample of 1 ng/ μ l DNA with 5 x DNA loading buffer (Bioline, MA, USA) was prepared and loaded into a well of the set gel along with a lane of high molecular weight bioladder (5 μ l) (Bioline). The chamber was run at 100 V for 1 hr. The gel tray was removed from the chamber and drained of excess TAE. The tray was placed in the MutliImage™ Light Cabinet (Alpha Innotech, CA, USA) and the gel viewed using Chemiimager software (Alpha Innotech).

5.1.6.1.9 Analysis of DNA Sequence

When confirmation that the plasmid DNA from the miniprep was not fragmented and of adequate quality, samples were sent off for sequencing to the University of Dundee (<http://www.dnaseq.co.uk/products.html>). Initially only the length of plasmid containing the desired mutation was sequenced to confirm the presence of the mutation. This was done with the use of pre-designed primers from the lab that attach throughout the length of the fibrinogen α -chain. A forward and a reverse primer that

read the sequence around the mutation were identified from the position they attach on the α -chain sequence. The sequencing results were checked for the presence of the mutation by searching for the wild-type sequence 10-12 base pairs around the area mutated (in this case TGGAACTCTGGG), since there is a repeat of this sequence upstream, if one of this sequence was present then the mutation was present in the plasmid. If two of the wild-type sequences were present, the mutation was not present. To further check the presence of the mutation in those plasmids with only one wild-type sequence, the sequence of the two amino acids in which the mutation lie were searched for, this time with the mutated sequence (in this case TGTTAC, rather than the wild-type TGGAAC). A further check was run, searching for the sequence directly downstream of the desired mutation (AACTCTGGG) and the two base pairs upstream of this checked to be 'GA'.

5.1.6.1.10 Further production and purification of mutant DNA

Once the presence of the mutation in the α -chain plasmid had been confirmed by sequencing, a Qiagen Plasmid Maxi Prep of the mutated plasmids was performed to purify more DNA for the transfection into CHO cells which required more and highly pure DNA, requiring the removal of RNA, proteins, dyes and low-molecular weight impurities.

Before starting the maxiprep, the plasmid was transformed into competent DH5 α cells (Invitrogen). This was done in the same way as for the miniprep, but using the plasmid product of the miniprep, instead of PCR products, and DH5 α cells, instead of XL-blue supercompetent cells. Cells were grown and plated in the same way. After overnight incubation, the maxiprep protocol began.

Single colonies were picked from freshly streaked plates and added to 5 ml LB-ampicillin broth and incubated for 8 hr at 37 °C with shaking at 300 rpm. 200 μ l of this 'starter culture' was then added to 100 ml LB-ampicillin broth and grown for 16 hr at 37 °C with shaking at 300 rpm. The bacterial cells were harvested by centrifugation at 6000 g for 15 min at 4 °C. The supernatant was discarded and the pellet resuspended completely in 10 ml of Buffer P1. 10 ml Buffer P2 was added and the solutions mixed before incubation at room temperature for 5 min. During this incubation period, the cap was screwed onto the outlet nozzle of the QIAfilter cartridge which was then placed in a 50 ml falcon tube. 10 ml buffer P3 was added to the solution, mixed and poured into the barrel of the QIAfilter Cartridge and left for 10 min at room temperature. The cap of the QIAfilter cartridge was then removed and the plunger inserted into the top of the cartridge. The cell lysate was filtered into a 50 ml falcon containing 2.5 ml buffer ER. The tube was inverted to mix the solution and incubated on ice for 30 min. During this incubation, 10 ml buffer QBT was applied to a QIAGEN-tip 500 and allowed to flow

through the column. The incubated lysate was applied to the QIAGEN-tip and left to enter the column by gravity flow. The column was washed twice with 30 ml buffer QC, allowing movement through the column by gravity. The DNA was eluted with 15 ml buffer QN into a 30 ml falcon tube. DNA was precipitated by adding 10.5 ml isopropanol to the elutant and centrifuging at 15,000 g for 30 min at 4 °C. The supernatant was discarded and the DNA pellet washed with 5 ml endotoxin-free 70% ethanol. It was then centrifuged at 15,000 g for 10 min and the supernatant discarded. The pellet was air-dried for 5-10 min and the DNA redissolved in 200 µl endotoxin free buffer TE. The yield of DNA was determined by spectrophotometry and agarose gel analysis as described in section 5.1.6.1.8. The DNA was then sent to be sequenced again to check the mutation was still present and that no further mutations had occurred in the cell grow-up. This time the whole α -chain insert was sequenced using a whole set of forward and reverse primers for the α -chain. The DNA was frozen at -20 °C until transfection into CHO cells.

5.1.6.2 CHO cell engineering

To express mutated recombinant fibrinogen, the mutated plasmid had to be introduced into engineered CHO cells harbouring the genes for the B β - and γ - chain of fibrinogen. Three CHO cell lines are available, each of which has 2 out of the 3 fibrinogen genes in their genomic DNA. These cells cannot express fibrinogen because they lack the gene for the third chain. The CHO cells were co-transfected with pMSVHis, conferring resistance to L-histidine, when the third fibrinogen chain was transfected. During transfection, the plasmic DNAs were inserted into the genomic DNA, resulting in a permanent transfection. When grown in media with histadinol, colonies that survived had successfully taken up the selection marker and possibly the gene of interest.

5.1.6.2.1 Transfection of CHO cells

$\beta\gamma$ CHO cells were removed from liquid nitrogen and thawed in a water bath at 37 °C. 9 ml GM was added to 1 ml of thawed cells in a 50 ml falcon and spun at 1000 rpm for 10 min. Media was removed from the cell pellet and the pellet resuspended in fresh GM and the cells transferred into a 100 mm petri dish containing 10 ml GM. After a growth phase, when the cells were confluent, the cells were split 1:10 with trypsin/ EDTA and plated; 2 experiment plates with Geneticin 418 (G418) media (GM/ 0.4 mg/ml G418 (Invitrogen)), the remaining 8 plates with GM for freezing. The next morning media on experiment plates was changed to 10 ml fresh G418 media and incubated for 2 hr. One hr before transfection, the DNA was prepared. A total of 10-20

ng mutated plasmid DNA and 1 ng pMSVHis were added to 50 μ l of sterile filtered 2.5 M CaCl_2 in a 15 ml tube, supplemented by water to a final volume of 500 μ l. 500 μ l of sterile filtered 2xHeBS (115 mM NaCl, 1.2 mM CaCl_2 , 1.2 mM MgCl_2 , 2.4 mM K_2HPO_4 , 20 mM HEPES) was put in a 50 ml falcon and this solution bubbled with a mechanical pipettor while the DNA/ CaCl_2 solution was slowly added. The mix was then vortexed and rested at room temperature for 30 min. The mix of DNA/ CaCl_2 / HeBS was slowly added to the cultured cells and carefully but thoroughly mixed with the G418 media. The plates were left in the incubator for 4 hr at 37 °C. Subsequently, the transfection process was terminated with a glycerol shock of the cells. Media were aspirated with a vacuum from the cells and 2 ml sterile filtered 10% glycerol solution added and left for 3 min at room temperature. Glycerol was aspirated and the plate washed 3 times with 5 ml sterile filtered PBS. Cells were fed 10 ml G418 media and placed in the incubator for 2 days, followed by splitting and plating at different densities to allow the growth of colonies from single cells that could be picked (2x1:10, 2x1:5, 1x3:10). Plates were shaken softly but thoroughly to spread the cells evenly and placed in the incubator at 37 °C. After 24 hr the G418 media was exchanged with 10 ml selection media (GM/ 0.4 mg/ml G418/ 0.25 mM L-histidinol (Sigma)) and placed back in the incubator. To remove dead cells, culture media were changed to fresh selection media every 2 days.

Cell growth was periodically checked under the microscope, to enable identification of single, well defined CHO cell colonies. When this was achieved, 24 single colonies were selected under the microscope and circled on the bottom of each plate with permanent marker. The clones that were selected had to be well separated from other clones and be made of large cells that looked healthy. Wells of a 24 well plate were labelled with clone numbers (1-24) and to each well 0.5 ml selection media was added. The media on the plates with colonies was removed and autoclaved glass cloning cylinders placed with tweezers over the circled clones and pressed down to achieve good contact between the glass and the plastic plate. 50 μ l trypsin/ EDTA were added to each cloning cylinder and the cells incubated for 5-10 min. The cells in each cloning cylinder were suspended by pipetting up and down with a glass pipette and transferred to 1 of the 24 wells containing 4 ml selection media and mixed carefully. The 24 well plate was placed in the incubator overnight at 37 °C. Selection media were replaced the next day to remove traces of trypsin/ EDTA and dead cells and to equalise the amount of liquid in each well. The cells were then grown without media change until the cells in about 60-70% of the wells reached confluence (~1 week). At this point the fibrinogen content in each of the 24 wells was determined by ELISA. 350 μ l of media was removed from each well and 100 μ l added in triplicate to a 96 well plate and fibrinogen levels were measured as described in section 2.3. From ELISA, 5-8 clones producing high levels of fibrinogen were selected. For each selected clone, two 60 mm

dishes containing 4 ml selection media were prepared. The selected clones were split from the 24 well plate with 0.5 ml trypsin/ EDTA and evenly distributed between the two 60 mm dishes. Cells were left at 37 °C until they reached confluence (4-5 days). Having reached confluence, one dish of cells was frozen (as described in section 2.1.1.3), and the other plate washed 3x with SFM before incubation for 7 days with 4 ml SFM to check if cells still produced fibrinogen in this media. After this, an aliquot of each medium was subjected to a fibrinogen ELISA. The 2 or 3 clones producing the highest yield of fibrinogen were chosen for fibrinogen production in roller bottles as described in section 0

5.1.7 Analysis of fibrinogen-albumin binding

Albumin can bind fibrinogen and cause changes in clotting parameters such as limiting lateral fibril growth and decreasing final turbidity¹³⁷. Albumin binds preferentially to cysteine residues due to the residues high affinity binding .Therefore, it is reasonable to suggest that there may be increased amounts of albumin bound to Fibrinogen Birmingham II when compared to control due to Trp334Cys substitution, consequently affecting fibrin network structure. To investigate this possibility 2 identical SDS-PAGE gels were run as below

1	2	3	4	5	6	7	8	9
Molecular Weight Marker	BII Plasma (1:100)	Normal pool plasma (1:100)	BII purified fibrinogen (0.02 mg/ml)	Normal pool purified fibrinogen (0.02 mg/ml)	BII recombinant fibrinogen (0.02 mg/ml)	WT recombinant fibrinogen (0.02 mg/ml)	BSA Control (0.02 mg/ml)	Molecular Weight Marker

Each gel was transferred to blotting membrane and one blotted for fibrinogen and the other for albumin as described in section 2.5.

5.1.8 Confocal microscopy

Confocal microscopy was conducted as previously described in section 2.6.

5.1.9 Electron microscopy

Electron microscopy was conducted as previously described in section 2.7.

5.2 Results

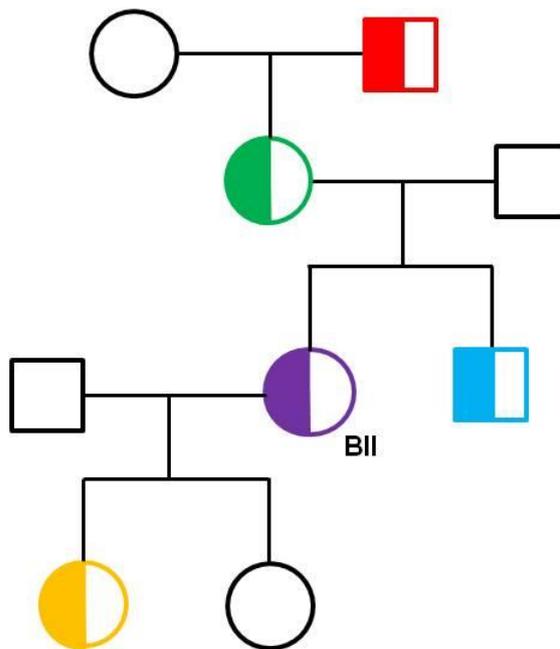
5.2.1 Patient characteristics

This novel fibrinogen variant was detected in a 34 year old woman and was associated with hypodysfibrinogenemia. Her clinical symptoms included a strong history of thrombosis; requiring insertion of an inferior vena cava (IVC) filter, in addition to a bleeding diathesis. She had suffered two deep vein thrombi ((DVT); one 6 weeks post-partum and one while taking the contraceptive pill), two cases of thrombophlebitis (vein inflammation related to a thrombus), bleeding after vein ligation and when an IVC filter was inserted and an IVC thrombosis. Two additional unconfirmed DVTs were reported and the patient is on long term anticoagulation therapy. She showed no risk factors for arterial thrombosis and successfully came to term on two live births with no history of miscarriage.

Fibrinogen BII plasma was associated with lower fibrinogen levels (1.1 mg/ml) when compared with control (Normal range: 1.5-4.5 mg/ml). Prothombin time was increased (23 sec) compared to control (normal range 9-13 sec) and activated partial thromboplastin time was marginally increased (40 sec) compared to control (normal range 26-35 sec). Thrombin time was also prolonged (22 sec) compared to control (normal range 12-16 sec). Taken together, this indicates a defect of the fibrinogen, due to quantitative and/or qualitative change in the protein.

5.2.2 Family history

There was a family history of hypodysfibrinogenemia associated with a range of clinical symptoms, which included thrombosis and/or bleeding, and asymptomatic individuals. The patients' mother bled after childbirth and post-surgery, her maternal grandfather had a DVT, her brother has dysfibrinogenemia but is asymptomatic and she has one affected and one unaffected daughter.



- Fibrinogen BII patient
- Maternal grandfather: 1 DVT
- Mother: bleeding after childbirth and post-surgery
- Brother: dysfibrinogenemia but asymptomatic
- Daughter: one of two affected

Figure 5-3. Family tree to show family history of bleeding and thrombosis in relatives of Fibrinogen BII patient

Mother showed bleeding after childbirth and post-surgery, maternal grandfather had one DVT, brother has dysfibrinogenemia but is asymptomatic, and one of two of the patient's daughters is affected.

5.2.3 SIFT and Polyphen analysis

SIFT analysis suggested both substitutions were deleterious (both score 0.03) and Polyphen analysis predicted that the Trp334Cys and Asn335Tyr changes were 'possibly damaging' and 'benign' (score 0.85 and 0.41 respectively).

5.2.4 Plasma samples

5.2.4.1 Clot formation and fibrinolysis

Clot structure and lysis of BII plasma could not be analysed using turbidimetric assay as no change in absorbance could be detected when clotting was activated in BII plasma (Figure 5-4). Four repeats were performed in duplicate, confirming this event. In case the low fibrinogen levels were contributing to this, the experiment was

performed with 3x the amount of plasma usually added, but still no change in absorbance was detected. The experiment was also performed using 10x less thrombin than usual in case the clot was forming extremely quickly, before the plate reader began reading, but this proved not to be the case. In each case, BII plasma showed elevated baseline absorbance when compared to control, which could be explained by the plasma having already clotted, however clots formed from this plasma for LSCM and EM analysis.

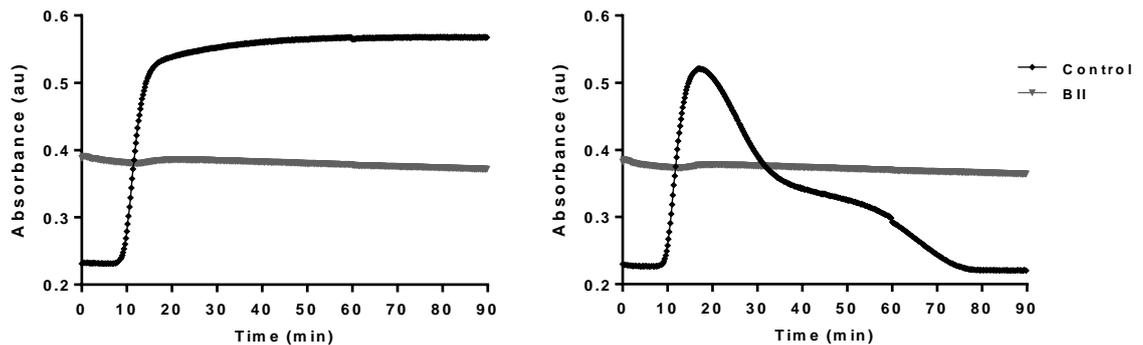


Figure 5-4. Analysis of clot formation and lysis of control and BII plasma by turbidimetric assay

BII plasma showed an elevated baseline compared to control and no change in OD was detected upon activation of clot formation.

5.2.4.2 Clot structure

Confocal microscopy showed clots made from BII plasma to be made of a fluffy texture with no defined fibrin bundle structure (Figure 5-5).

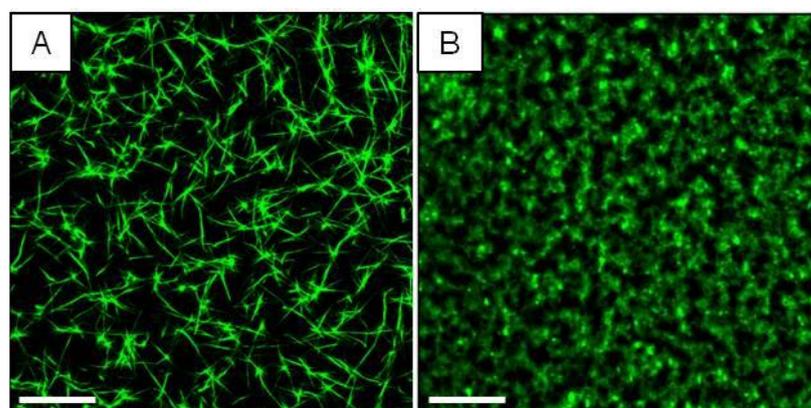


Figure 5-5. LSCM images of clots made from Fibrinogen BII and control plasma
(A) Control and (B) BII plasma clots. Clots made from Fibrinogen BII had an atypical morphology with no defined fibrin bundle structure. Scale bars represent 45 μm .

Electron microscopy revealed clots made from Fibrinogen BII plasma to be made of 67.6% thinner fibres than those of control. Clots had a bizarre morphology of very thin and 'fluffy' fibrin bundles interspersed with large pores (Figure 5-6).

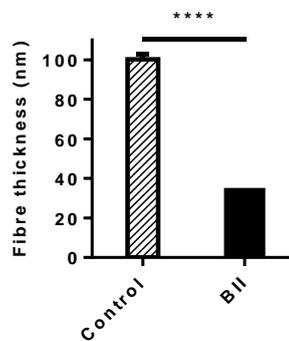
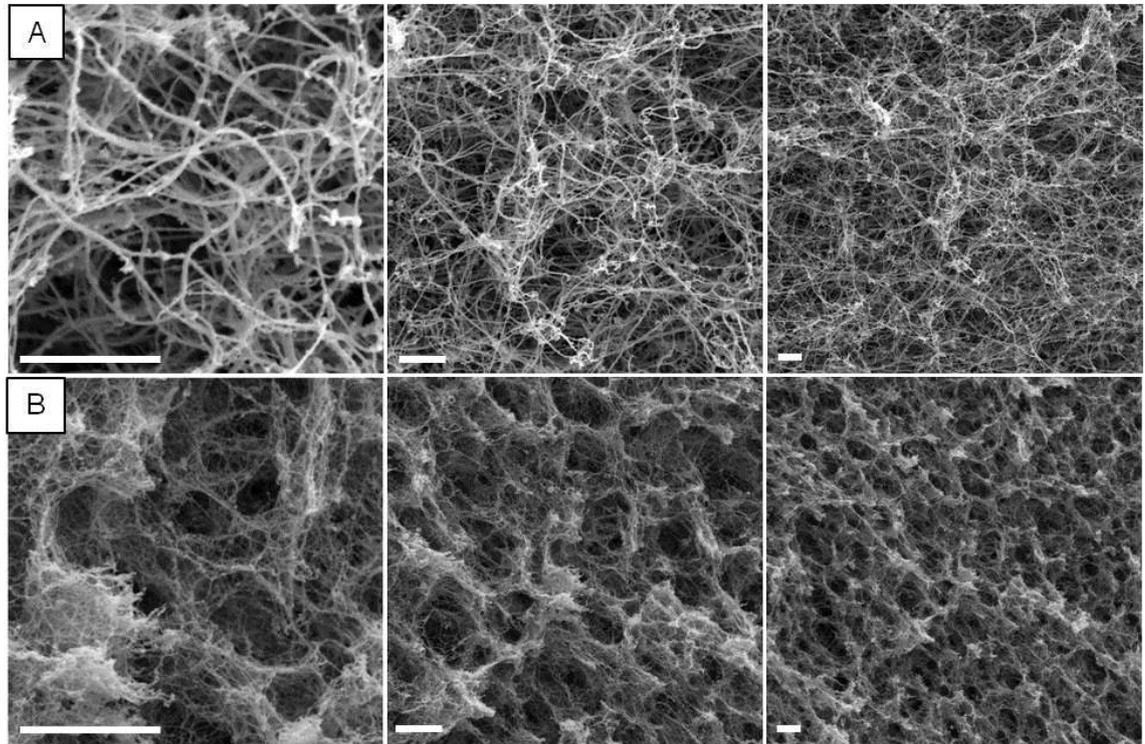


Figure 5-6. EM scans of clots made from of Fibrinogen BII and control plasma and comparison of fibre thickness

(A) Control and (B) BII plasma clots. Scale bars represent 3 μm . BII plasma produced a clot made of an atypical structure with significantly thinner fibres interspersed with large pores.

5.2.5 Purified Protein

5.2.5.1 Integrity of purified patient plasma fibrinogen

Purified fibrinogen showed high purity and integrity with intact chains and absence of significant degradation (Figure 5-7).

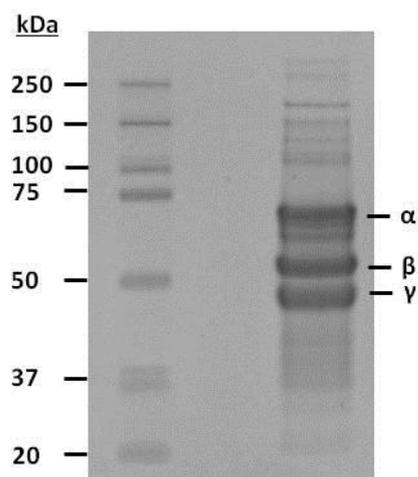


Figure 5-7. Purified Fibrinogen BII fibrinogen resolved on an 4-12% SDS PAGE gel

The gel showed all 3 different fibrinogen chains intact confirming integrity and purity of the fibrinogen purified.

5.2.5.2 Clot formation and fibrinolysis

BII turbidity was 88% lower than that of control fibrinogen. CFT was 368.0% greater in BII fibrinogen than control. Lysis time was 506.3% greater in BII than control. Results summarised in Figure 5-8.

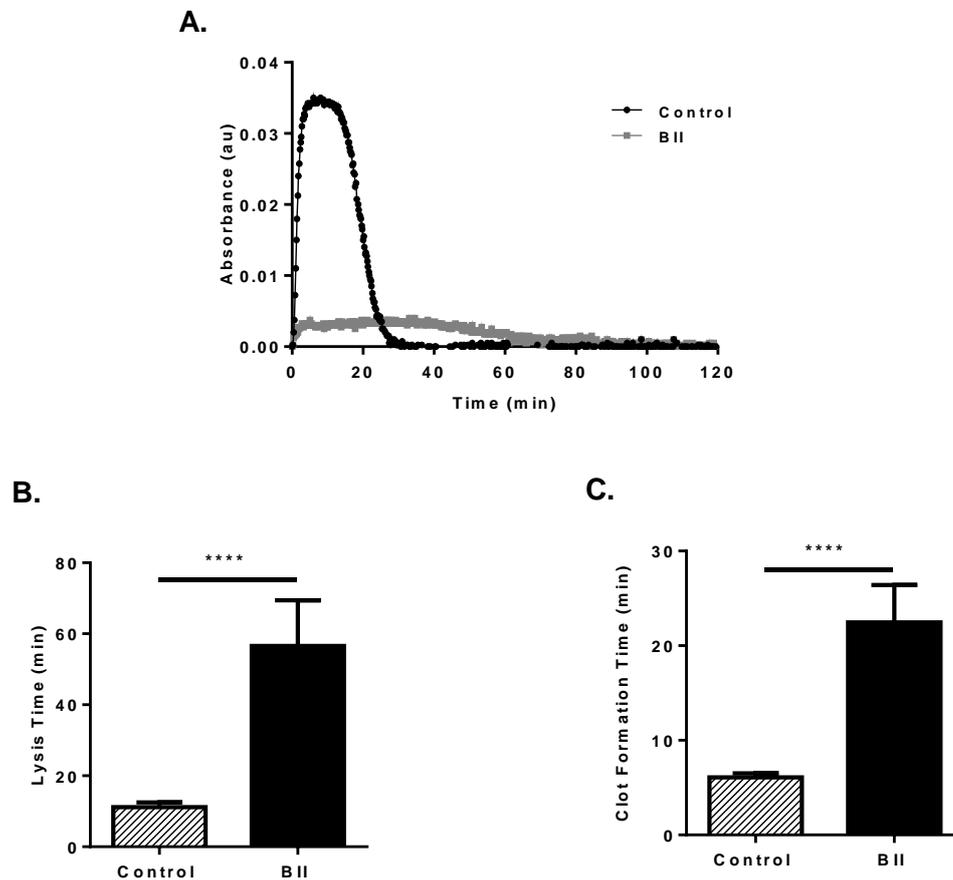


Figure 5-8. Characteristics of clots formed from fibrinogen purified from control and Fibrinogen BII plasma as measured by turbidimetric assay

(A) Maximum absorbance; significant decrease with Fibrinogen BII pointing towards thinner fibres. (B) Lysis time; significant increase with Fibrinogen BII pointing towards clots with increased resistance to lysis. (C) Clot formation time; significant increase with Fibrinogen BII.

5.2.5.3 Clot structure

In standard LSCM preparations, Fibrinogen BII clots appeared to be made of shorter fibres, with more branch points and an ill defined fibre structure (Figure 5-9).

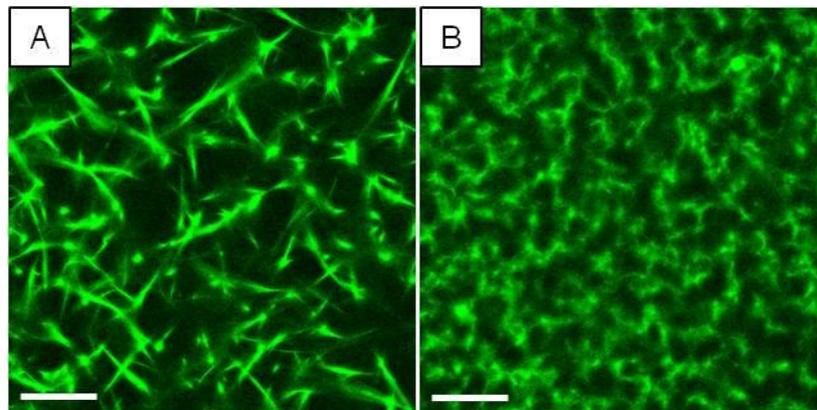


Figure 5-9. LSCM images of clots made from control and Fibrinogen BII plasma-purified fibrinogen

(A) Control and (B) Fibrinogen BII plasma-purified fibrinogen clots. Clots made from Fibrinogen BII had an atypical morphology with no defined fibril structure. Scale bars represent 45 μm .

Fibres of clots made from fibrinogen BII purified fibrinogen were 61.0% thinner than those of control fibrinogen. BII fibrinogen produced a clot made of an atypical structure with thin fibres interspersed with large pores (Figure 5-10).

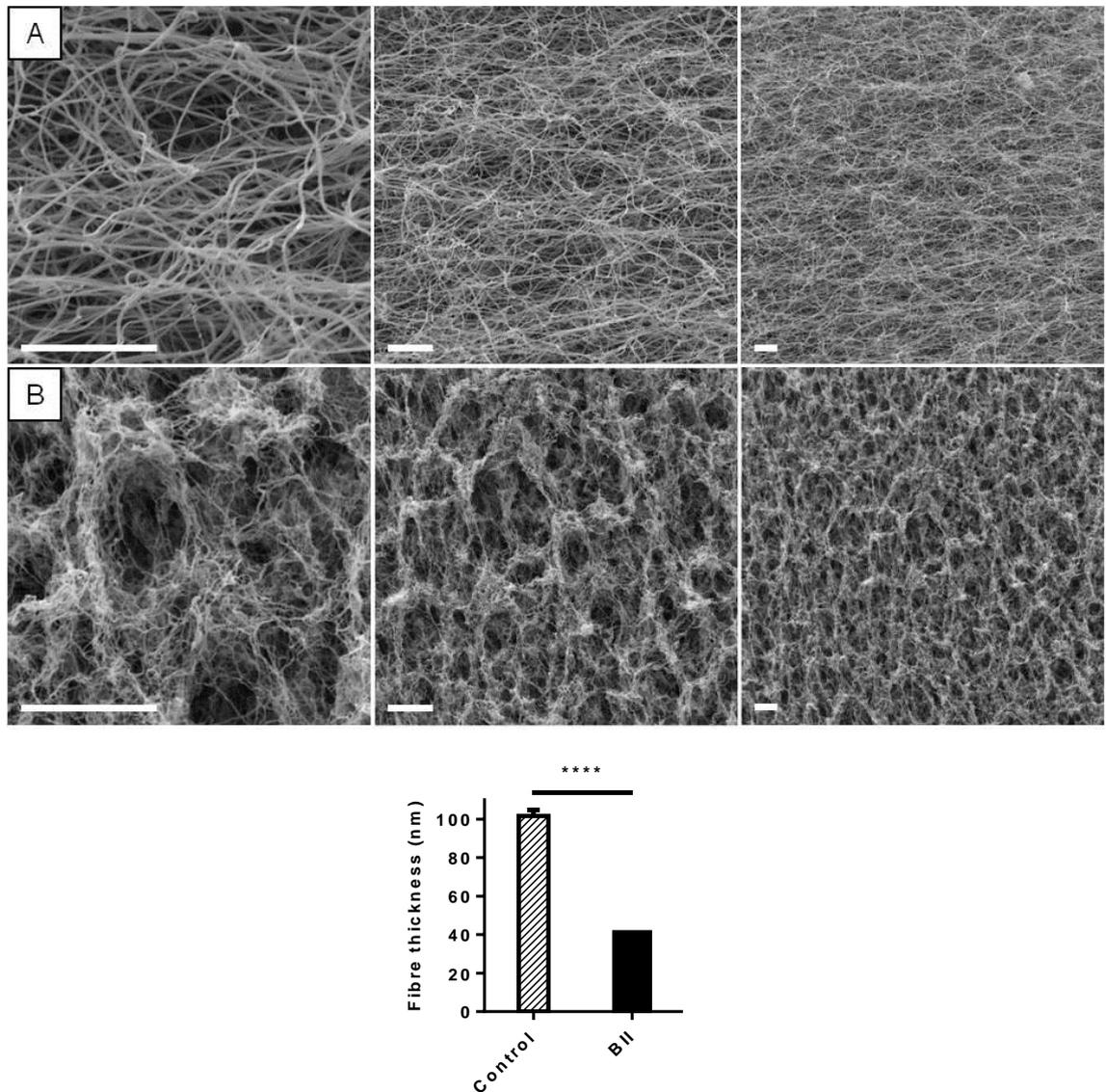


Figure 5-10. EM of clots made from of control and Fibrinogen BII plasma-purified fibrinogen and comparison of fibre thickness

(A) Control and (B) BII plasma-purified fibrinogen clots. Scale bars represent 3 μm . BII fibrinogen produced a clot made of an atypical structure with significantly thinner fibres interspersed with large pores.

5.2.6 Recombinant Protein

Recombinant fibrinogen purified was clean but there was a level of alpha chain degradation (Figure 5-11). This may have been due to the mutations that were introduced which could have caused the molecule to be unstable in the culture conditions and thus prone to degradation. When tested for clottability $61.57 \pm 1.59\%$ of the fibrinogen clotted.

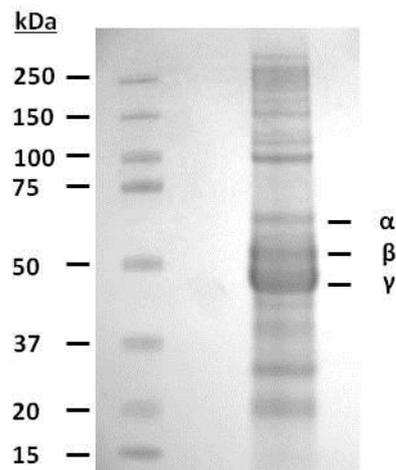


Figure 5-11. Recombinant BII fibrinogen resolved on a 4-12% SDS PAGE gel

The gel shows all 3 different fibrinogen chains though there is a level of α -chain degradation and some high molecular weight bands.

5.2.6.1 Clot formation and fibrinolysis

Final turbidity was 561.3% higher in WT than BII. Clot formation time was 55.9% greater with BII fibrinogen than WT. Lysis time was 67.2% greater in WT when compared to BII. Results are summarised in Figure 5-12.

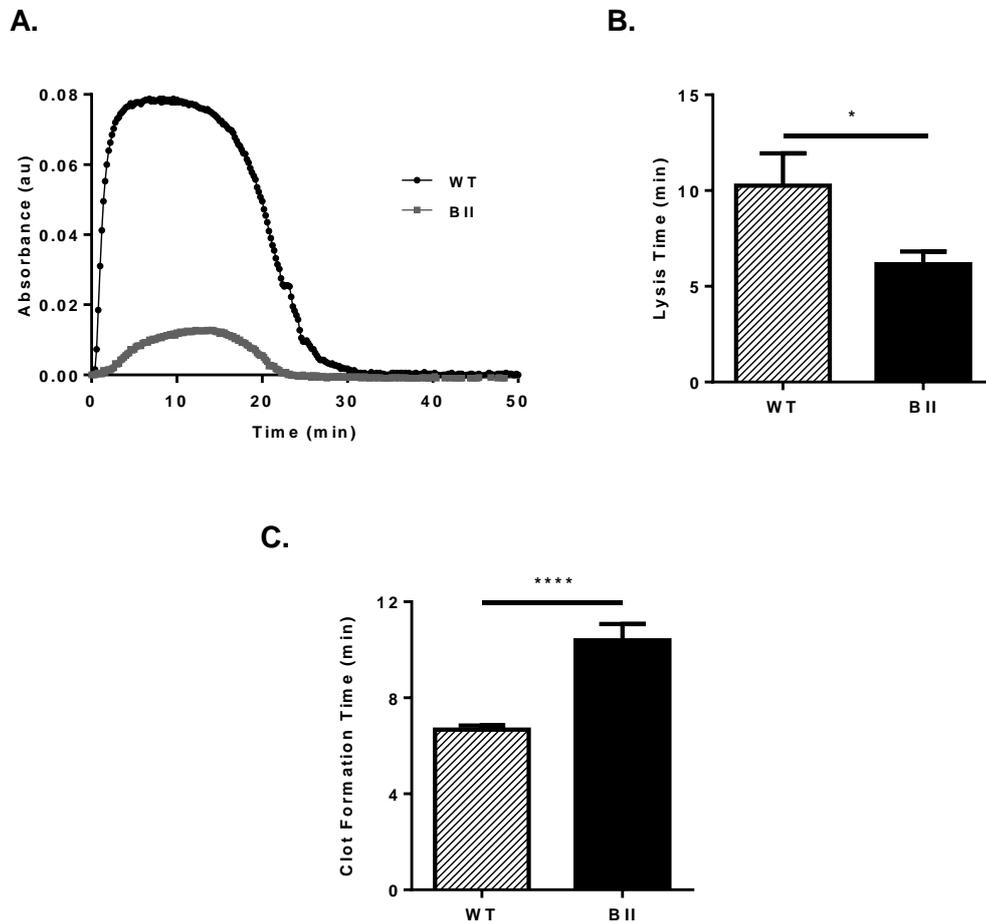


Figure 5-12. Characteristics of clots formed from recombinant WT and BII fibrinogen as measured by turbidimetric assay

(A) Maximum absorbance; significant decrease with Fibrinogen BII pointing towards thinner fibres. (B) Lysis time; significant decrease with Fibrinogen BII. (C) Clot formation time; significant increase with Fibrinogen BII.

5.2.6.2 Clot structure

BII recombinant fibrinogen formed a clot with an atypical architecture with a more dense structure and more curved fibrin bundles and more branch points than WT recombinant fibrinogen. 24 hr after clot formation, BII fibrin clot appeared to retract and dissolve (Figure 5-13).

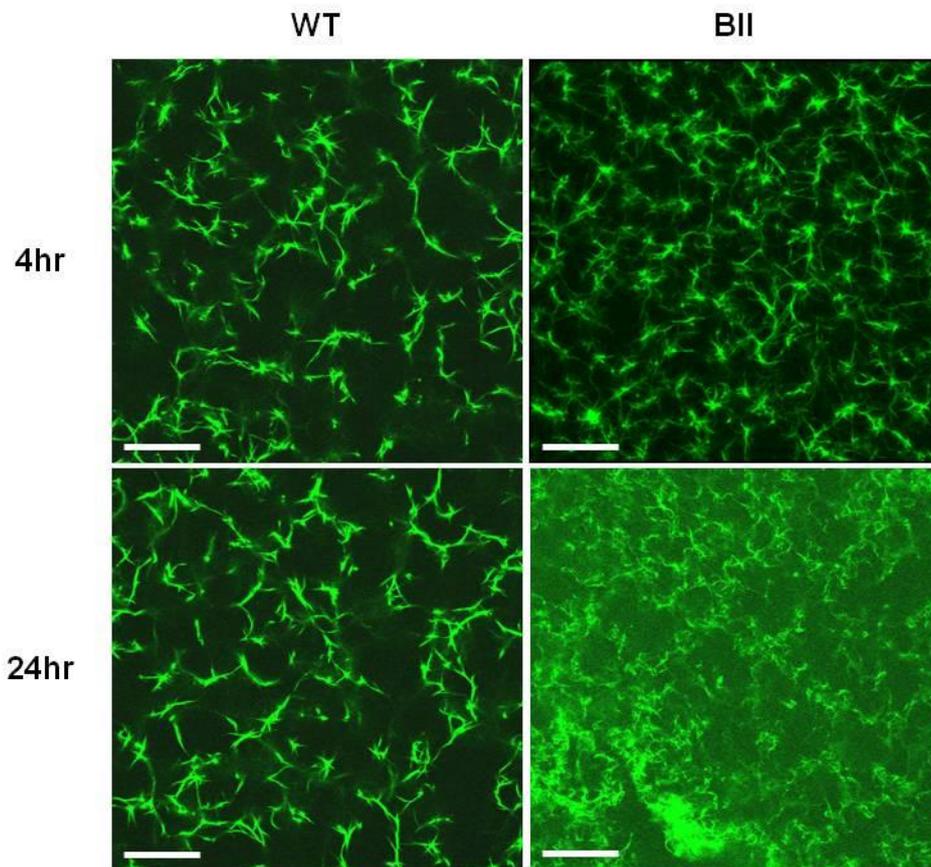


Figure 5-13. LSCM images of clots made from WT and BII recombinant fibrinogen WT and BII recombinant fibrinogen 4 and 24 hr after clot formation. Scale bars represent 45 μ m. BII recombinant fibrinogen formed a clot with an atypical architecture with a more dense structure and more curved fibrin bundles than WT recombinant fibrinogen. 24 hr after clot formation, BII fibrin clot appeared to retract.

Clots made from BII recombinant fibrinogen were more compact and fibre thickness was 61.7% smaller in BII than WT fibrinogen. Results summarised in Figure 5-14.

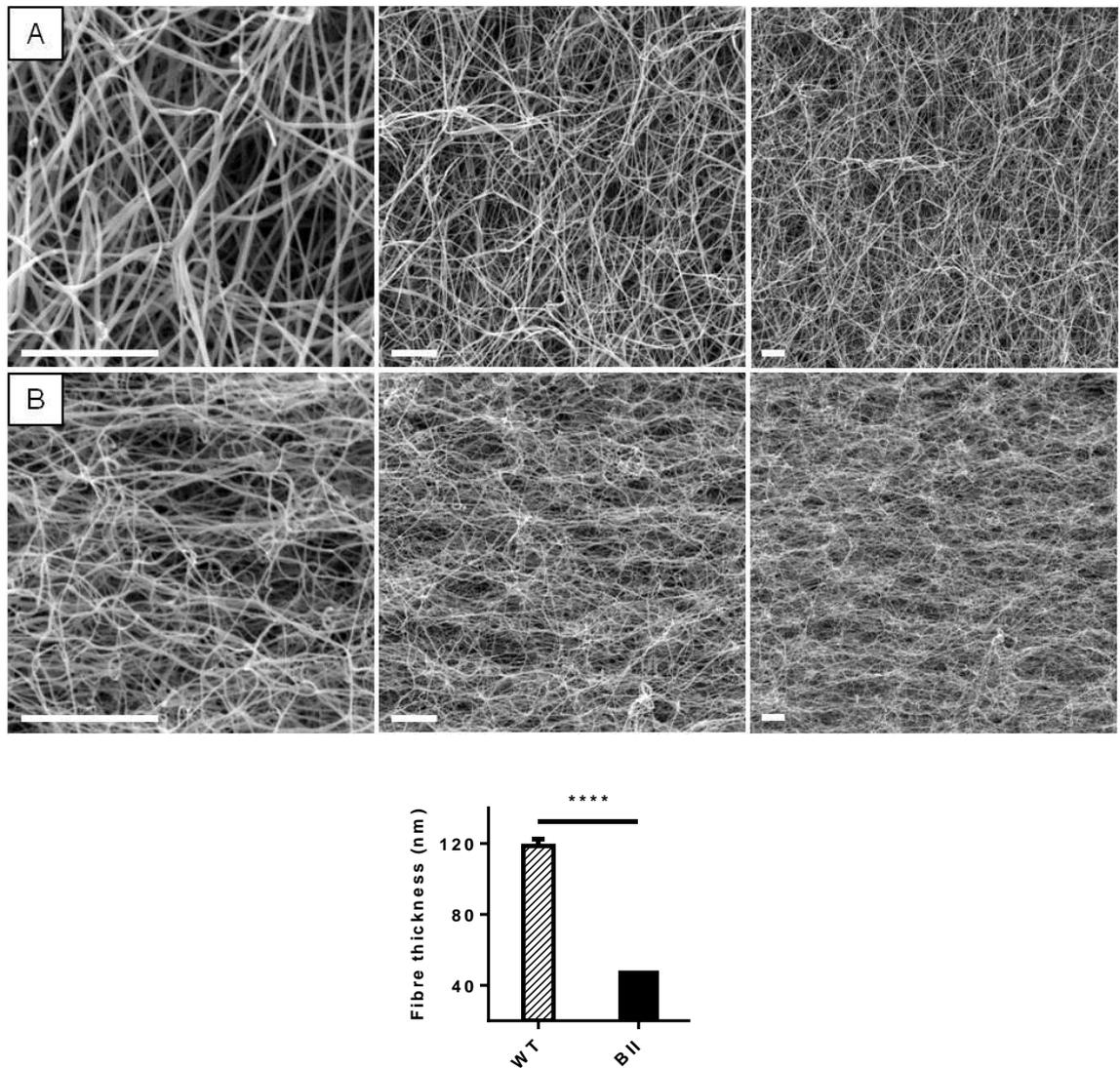


Figure 5-14. EM of clots made from of Fibrinogen BII and WT recombinant fibrinogen and comparison of fibre thickness

(A) WT and (B) BII recombinant fibrinogen clots. Scale bars represent 3 μ m. Fibre thickness as determined by EM scans showed fibre thickness to significantly decrease with Fibrinogen BII fibrinogen.

5.2.7 Clot formation and fibrinolysis in the presence of plasma proteins

The interaction of the fibrinogen variants in the presence of α 2-AP was studied as it has been shown previously (Chapter 4) that α 2-AP is one of the main plasma proteins implicated in causing changes in lysis of clots with differing structures.

In a purified system, absence or presence of α 2-AP had no effect on MA of control fibrinogen. Equally, α 2-AP had no significant effect on MA of BII fibrinogen. Data summarised in Figure 5-15A. In a recombinant system, α 2-AP had no effect on MA of WT fibrinogen. However, α 2-AP significantly increased MA of BII fibrinogen by 16.7%. Data summarised in Figure 5-15B. It should be noted, however, that purified protein had a heterozygous genotype, whereas recombinant protein is homozygous. α 2-AP prolonged CFT by 306% and 200% in BII fibrinogen in purified and recombinant systems respectively, but had no effect in control/WT fibrinogen (Figure 5-16). In a purified system, α 2-AP prolonged lysis by 286.5% in control and BII fibrinogen showed no sign of lysing within 2 hr in the presence of α 2-AP (Figure 5-17A). In a recombinant system α 2-AP prolonged lysis in WT by 277.5% and by 553.3% in BII fibrinogen (Figure 5-17B).

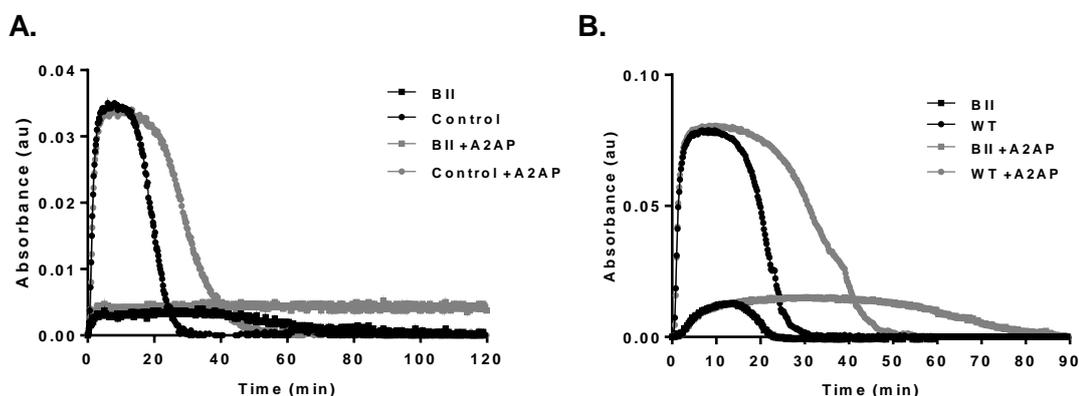


Figure 5-15. Turbidimetric traces of Control/ WT and BII purified and recombinant fibrinogen in the presence of α 2-AP

(A) Purified system and (B) recombinant system. α 2-AP had no effect on MA of control/WT fibrinogen but increased MA of BII fibrinogen.

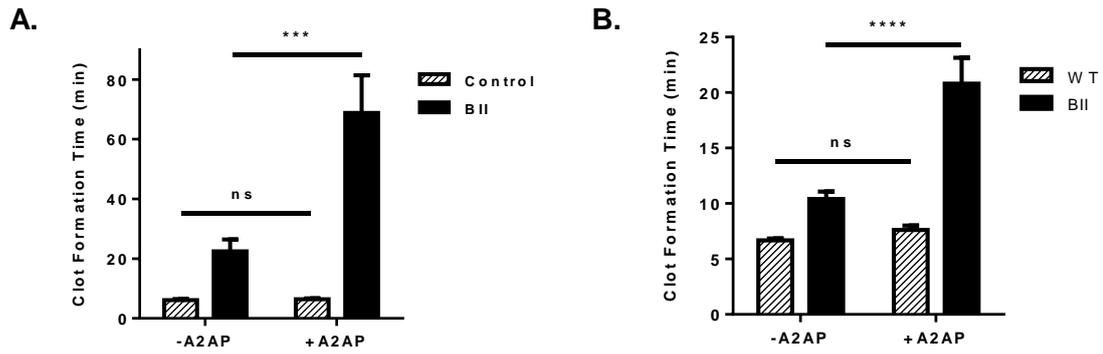


Figure 5-16. Effect of α 2-AP on CFT of control/ WT and BII purified and recombinant fibrinogen

(A) Purified system and (B) recombinant system. α 2-AP significantly prolonged CFT in BII fibrinogen but had no effect in control/WT fibrinogen in purified and recombinant systems.

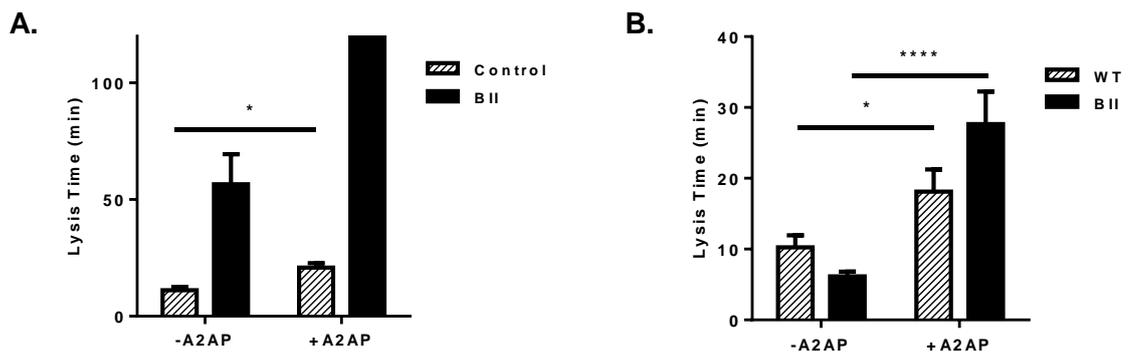


Figure 5-17. Effect of α 2-AP on LT of control/ WT and BII purified and recombinant fibrinogen

(A) Purified system and (B) recombinant system. α 2-AP prolonged lysis in control/WT and BII fibrinogen. α 2-AP prolonged lysis significantly longer in BII than control/WT fibrinogen.

5.2.8 Albumin-Fibrinogen Binding

Human serum albumin has a molecular weight of 67 kDa. The control albumin is BSA which has a molecular weight of 69,324 Da as a full length precursor; this is cleaved of a 2,107 Da signal peptide upon secretion and is cleaved of a further 478 kDa propeptide, producing the mature 66,463 Da protein. The most dense bands picked up by the albumin antibody are between 75 and 50 kDa as predicted by these documented molecular weights (~67 kDa). The other bands are below 25 kDa.

Though albumin is present in the plasma, it does not seem to bind strongly to either BII or control fibrinogen as it separates on the non-reducing gel. There is a very low molecular weight band in the BII purified lane picked up by the albumin antibody; this could be a small cleavage product of albumin which has co-purified with BII fibrinogen, but does not remain bound in solution.

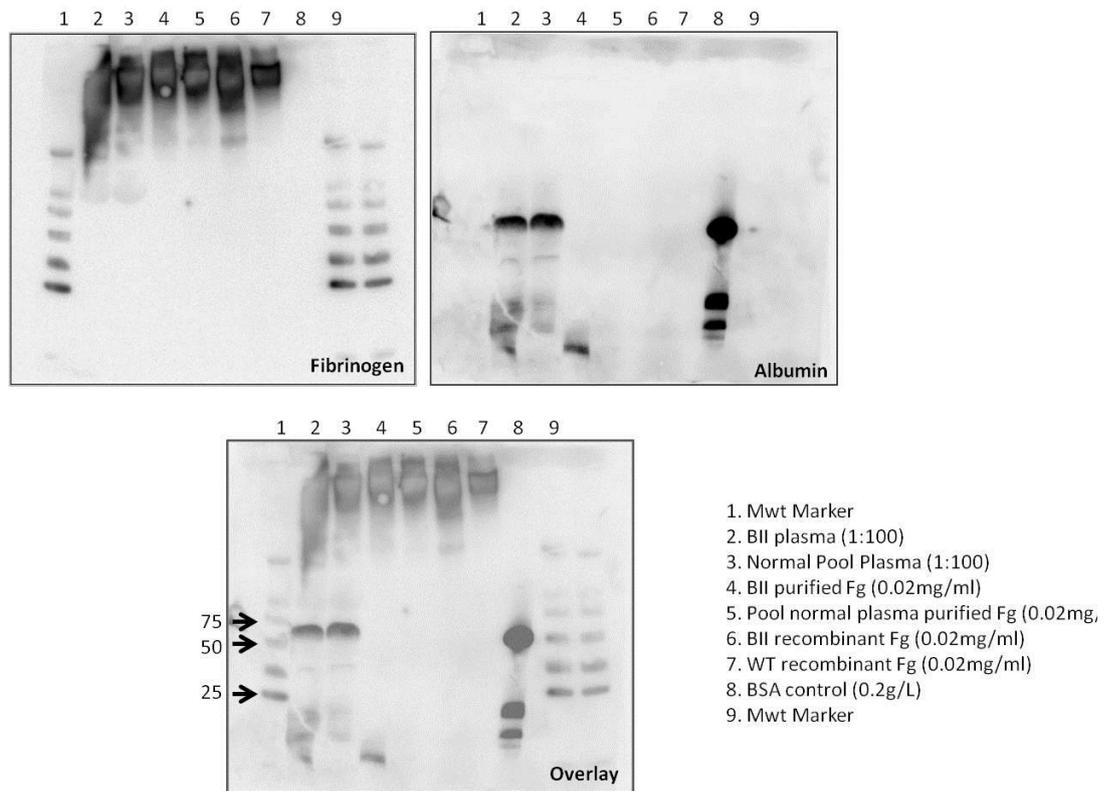


Figure 5-18. Binding of albumin to fibrinogen in plasma, purified and recombinant proteins

Individual blots of fibrinogen (left upper panel) and albumin (right upper panel) and an overlay of the two blots (bottom panel) are shown. Samples containing mutant and wild-type fibrinogen were studied. Albumin does not bind BII or control fibrinogen in plasma, purified or recombinant systems.

5.3 Discussion

We report a novel fibrinogen variant due to co-inheritance of two missense mutations in α C domain (Aa p.Trp334Cys and Aa p.Asn335Tyr). This variant is associated with thin fibrin fibres, impaired clot formation and resistance to fibrinolysis in a plasma environment, and clinically both a thrombotic and bleeding tendency.

When patient plasma was studied, EM revealed the clot to be made of fibrin fibres that were significantly thinner than those of control clots and made of the bizarre fluffy structure rather than defined fibres. Confocal microscopy also showed a lack of defined fibre bundles, again showing an almost fluffy texture to the fibrin network structure. The lack of detection of fibrinogen BII plasma clotting by turbidimetric assay remains unexplained. Though levels of calcium, fibrinogen and thrombin were all investigated, adjusting these factors had no influence on the clotting. The higher baseline absorbance of the BII plasma suggests it may have clotted in storage; however this did not seem to be the case as clots could be formed for both LSCM and EM preparations. It may be that the ultra structure of the clot cannot be detected at the wavelength used to measure the changes in absorbance, but this seems unlikely due to the similar structure of the clot formed from plasma purified protein (as assessed by LSCM and EM) being detected.

When clots made from plasma-purified fibrinogen were studied by EM and LSCM, they showed a similar architecture to that in plasma, with significantly thinner fibres and an atypical structure of fluffy fibre bundles with increased branch points when compared to control plasma-purified fibrinogen clots. Similarly, BII recombinant fibrinogen formed a clot with an atypical architecture with a more dense structure and more curved fibrin bundles than WT recombinant fibrinogen in LSCM preparation. EM showed fibrin fibres to be thinner in the recombinant BII fibrin clot than control, in line with plasma and plasma-purified data. A unique observation relating to recombinant BII fibrinogen is the apparent retraction of the clot in the LSCM preparation 24 hr after clot formation. This may have important clinical implications as retraction of a thrombus may mean that the patient can have a late bleeding following an injury, in line with the thrombotic and bleeding tendency of the BII patient.

In both purified and recombinant preparations, CFT was significantly longer in fibrinogen BII, explaining the tendency for bleeding in the patient. Prolongation of CFT by α 2-AP in BII but not control/ WT further confirms the difference in the interaction of plasma proteins with the fibrinogen variants. The α 2-AP seems to delay lysis enough in the BII in order for it to reach a higher MA, which suggests the clot is not fully formed when it begins to lyse in the absence of α 2-AP whereas control/ WT clots are already fully formed by the time they lyse even without the presence of lysis inhibitors. The mechanisms behind this need further exploration, but differential thrombin binding/

activity, impaired fibrinopeptide release and delayed FXIII crosslinking of fibrils are likely affected by the candidate mutations as discussed.

Lysis of clots made from purified BII fibrinogen was slower than those of control in a purified system, conversely, in the same system, clots formed from recombinant BII fibrinogen lysed significantly faster when compared to WT. Together these purified and recombinant data mirror those in Chapter 4, which show an increase in lysis with the variant plasma-purified B β Arg448Lys fibrinogen in the absence of other plasma proteins. This suggests the structure alone does not confer resistance or susceptibility to lysis, but the disparity in lysis rates may be due to impurities from plasma of the purified protein. Alternatively, since this recombinant protein is homozygous for the A α Trp334Cys/Asn335Tyr, unlike the heterozygous purified protein, it forms a more fragile clot due to the lack of wild-type fibrinogen molecules in the milieu.

In the presence of α 2-AP, lysis time was prolonged significantly more in clots made from fibrinogen BII than those of control/WT fibrinogen in both plasma-purified and recombinant systems. This suggests that the interaction between the fibrin ultra-structure and fibrinolytic plasma proteins between the variants confers resistance to lysis of the BII fibrinogen in the plasma environment, and that α 2-AP is one of the main proteins in the blood responsible for this phenomenon.

Western blot analysis revealed that although a small amount of a low molecular weight product picked up by the albumin antibody co-purified out of plasma with the BII fibrinogen, it was not bound to fibrinogen. This mirrored the interaction of albumin and BII fibrinogen in plasma samples, although there was albumin present in plasma, it was not bound to either control or BII fibrinogen. Expectedly, there was no albumin present in the BII or WT recombinant fibrinogen as it was not involved at any point in the growth or purification or preparation of the protein. This rules-out the possibility that increased albumin binding to the extra cysteine residue in BII fibrinogen accounts for the observed changes in clot structure and lysis.

The amino acid residues affected by the candidate mutations of fibrinogen BII are both highly conserved residues in the α C domain of fibrinogen, which have a role in crosslinking during fibrin polymerisation²⁶. Changes in binding of fibrinolytic modulators, altered clot structure and increased resistance to lysis have been linked to other mutations in this region. Molecular modelling of the effect of these substitutions could not go any further than guessing at secondary structure of this region of the protein due to its crystal structure not yet being defined due to the intrinsic flexibility of the α C domain of the fibrinogen molecule. Despite difficulties with lack of certainty of the structural context of this area, in collaboration with Mike Harrison (University of Leeds) we have made some effort to predict the effects of the mutations by looking at the characteristics of the residues. Internalisation of the hydrophobic tryptophan could be a

key factor in stabilizing the fold of a globular domain, or it could contribute to a hydrophobic patch on the surface involved in protein-protein interactions, however it is hard to determine which without some basic idea of the fold. Cysteine has the potential for forming aberrant disulfides that could impact on function. At the most basic level, the indole side chain of tryptophan is large, rigid, planar and hydrophobic, whereas the cysteine side chain is smaller and essentially polar, which could cause a relatively simple packing (steric) effect. Asparagine to tyrosine substitution could have a number of impacts; asparagine is a good H-bond donor on a long side chain that is conformationally quite flexible, this allows it to H-bond back to the peptide backbone, effectively capping-off the main chain -NH to =OH-bonding that maintains secondary structures like alpha-helices, hence it often terminates these helices. In principle, this could impact on β -structures too, it tends to be found at turns so removal could cause disruption to the overall fold, but again overall context is important here. Tyrosine is also a good H-bonder, but the side chain can only rotate about its bond to the alpha carbon, hence only forms "outward facing" bonds. In terms of polarity, Tyr is a little hydrophobic, but this is moderated by its -OH group, the side chain is relatively large though, and its lack of rotational movement could have an impact on packing. Additionally, sulphation of tyrosine is quite common, which could lead to aberrant modification of the side chain that could affect function. These stark differences in the amino acid residues at these positions may explain why they have such a significant effect on the clotting of this fibrinogen. This is reflected in SIFT analysis suggesting both substitutions to be deleterious and PolyPhen identifying the Trp334Cys change as possibly damaging. Changes in binding of fibrinolytic modulators, altered clot structure and increased resistance to lysis have been linked to other mutations in this region.

It has been shown in some dysfibrinogenemia cases that changes in clot structure due to mutant fibrinogens compared to healthy controls are solely due to changes in fibrinogen levels as in Novy Jicin¹⁰³. In this fibrinogen variant, diluting healthy controls plasma with TRIS buffer to the patients Clauss fibrinogen levels gave similar clot structure to those of control subjects, revealing differences in morphology of the fibrin network to be concentration dependent and independent of the substitution. In Novy Jicin dysfibrinogenemia, the mutant molecules are not found in the clot, which is made up solely of the subpopulation of wildtype homodimers even though both homo and heterodimers were present in the circulation. Despite the hypofibrinogenemia in Fibrinogen BII, alterations in clot structure were not related to quantitative but to qualitative changes in fibrinogen, as our purified and recombinant protein data clearly demonstrate. It is worth bearing in mind that my recombinant protein is homogenous for the polymorphism as the cell line set up is inherently homozygous for a transfected mutation; which means all recombinant fibrinogen molecules were homodimers. This

confirms that the mutant fibrinogen is capable of participating in clot formation, ruling out the patient's hypofibrinogenemia as the sole cause for the changes in clotting.

Fibrinopeptide A release has repeatedly been shown to be impaired by α -chain mutations by as much as 75% and fibrinopeptide B by up to 25% (notably the A α 16Arg-Cys (Novy Jicin) mutation). This is due to the inability of thrombin to cleave the altered arginine 16-glycine 17 peptide bond, essential for fibrinopeptide release and fibrin clot formation. This impaired cleavage of fibrinopeptide A results in delayed fibrin clot formation and unusual fibrin clot morphology. The candidate mutations in our patient lead to impaired fibrinogen polymerisation with a prolonged lag phase, decreased final turbidity and fibre thickness: changes in fibrinopeptide release caused by the mutations could go some way to explaining this, though further investigation is needed to confirm this. Since the candidate mutations lie in the α C domain, they most likely influence its structure and movement, in this way they could influence fpA release as the α C domain may interact differently with the central E domain of the fibrinogen molecule to which the fibrinopeptides are anchored. It is well established that the α C domain of fibrinogen plays a critical role in the lateral association of fibrin fibrils, constituting at least part of the fibrin molecule b polymerisation site, as it has been shown that fibrinogen molecules missing this region form polymers that produce clots made of thinner fibres and reduced turbidity³⁵. This could also go some way to explaining the reduced turbidity and thinner fibres in our patient.

Koopman and colleagues³⁵ showed prolongation of lysis of fibrinogen Dusart to be due to reduced binding of Lys-plasminogen and defective tPA induced plasminogen activation. This dysfibrinogenemia, similarly, displays thinner fibres due to impaired lateral association of fibrin fibrils which they conclude are responsible for the changes in plasminogen binding and activation as it has been shown that inhibition of fibrin polymerisation reduces the acceleratory effect of fibrin on tPA induced plasminogen activation. This mechanism could have implications for fibrinogen BII due to the similar morphology of thin fibrin fibres and delayed fibrin polymerisation.

In conclusion, we report a novel fibrinogen variant due to co-inheritance of two missense mutations in α C domain (A α Trp334Cys and A α Asn335Tyr). This variant is associated with impaired clot formation, thin fibrin fibres and resistance to fibrinolysis in the plasma environment. Our data using plasma-purified and recombinant fibrinogen confirm the mutations are directly responsible for the changes in clot structure, formation and lysis. Both Albumin binding to the variant fibrinogen and overall quantitative changes of the protein in the blood have been ruled out as mechanisms by which the BII fibrinogen has altered clotting parameters.

These findings provide mechanistic explanations for the clinical presentation of our patient: The excessively low clot turbidity and thin fibres are consistent with less

robust clots predisposing to bleeding, confounded by the greater time take for clot formation. On the other hand, impaired fibrinolysis of clots made from BII fibrinogen may explain the increased thrombotic tendency in our patient.

Chapter 6. Summary and Conclusions

Clot formation is an essential physiological process to prevent blood loss upon vessel injury. However changes in various parameters involved in the clotting cascade can lead to pathological complications with serious clinical implications. Alterations in clot formation, morphology and lysis can increase risk of CVD and can result in irreversible end organ damage. Consequently it is important to understand the mechanisms behind the clotting process to elucidate the impact on atherothrombosis and CVD in the hope of developing preventative and therapeutic targets.

Fibrinogen is essential in the blood clotting process, which upon conversion to fibrin, forms the skeleton of the blood clot. A large number of factors affect clot structure and fibrinolysis, which modulate predisposition to vascular occlusive events, and studies often ignore the possible effects of genetic alterations in fibrinogen when investigating fibrin-related thrombosis risk in multifactorial vascular occlusive conditions.

On top of a genetic effect on clot structure, environmental factors can affect clot formation, morphology and lysis. Diabetes is a complex multifactorial condition in which both genetic and environmental factors play a role. It is associated with qualitative and quantitative changes in procoagulant and anti-fibrinolytic proteins leading to fibrin clots with tight network structure and resistance to lysis, consequently predisposing to a thrombotic environment and premature atherosclerosis.

While a number of studies have investigated various fibrinogen mutations, the role of the common B β Arg448Lys variants of fibrinogen in clinical disease was only partially characterised. In particular, the effects of this polymorphism on fibrin clot structure/function and predisposition to thrombosis in high risk subjects remained unclear. In other words, the effect of this common polymorphism on clot structure and vascular events in complex multifactorial pathological conditions is unknown. Moreover, the mechanisms by which these genetic variants of fibrinogen cause alterations in clot structure were not defined. In contrast to the common B β Arg448Lys substitution, a rare genetic mutation (A α Trp334Cys/Asn335Tyr) in the α -chain of fibrinogen has been found in an individual with unusual phenotype that includes both bleeding and thrombotic tendencies. It was unclear, however, whether this rare genetic mutation was directly responsible for the clinical picture in this individual.

Therefore the three main aims of this work were to; i) identify any additional effect of B β Arg448Lys on fibrin network in a complex multifactorial disease with increased vascular risk such as T2DM, ii) study the mechanisms behind the effects of the B β Arg448Lys mutation on fibrin clot structural and functional properties and iii) investigate the role of fibrinogen A α Trp334Cys/Asn335Tyr on clot structure and lysis and its potential responsibility for abnormal patient symptoms.

Diabetes and B β Arg448Lys polymorphism

In the clinical part of this work, we have shown that B β 448Lys has an effect on fibrin clot structure and fibrinolysis in people with type 2 diabetes. In addition we suggest these changes may add to an already increased level of vascular risk in diabetes.

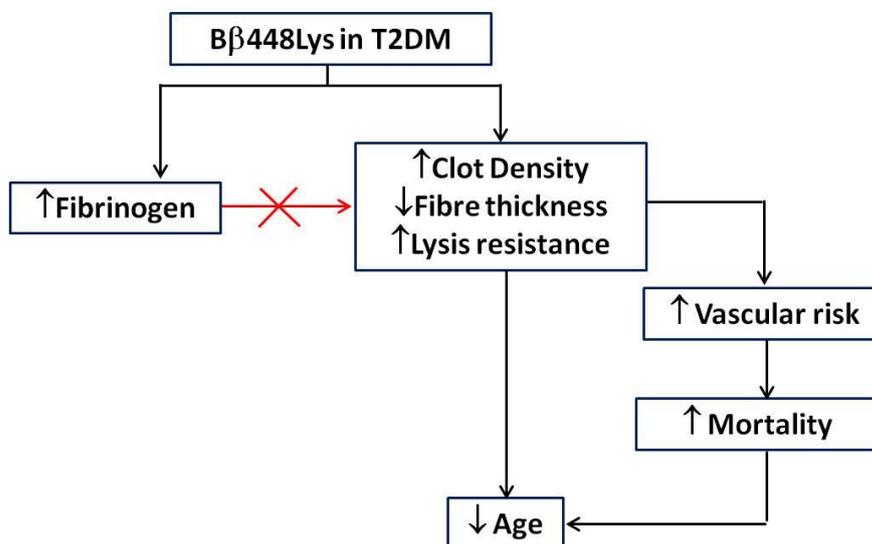


Figure 6-1. The effect of B β Arg448Lys fibrinogen on the structure and lysis of fibrin clots and risk of vascular events in patients with T2DM

When compared to B β 448Arg, B β 448Lys leads to formation of clots with a more dense structure with thinner fibres and an increased resistance to lysis independent of the increased fibrinogen levels in this variant. This may lead to increased vascular risk leading to early mortality in this patient group. Increased fibrinogen levels are not directly related to the polymorphism but secondary to linkage disequilibrium with another polymorphism in fibrinogen that controls protein levels.

The observation that clots prepared from plasma of T2DM individuals with B β 448Lys were more compact and more resistant to lysis indicates a more thrombotic environment in these individuals when compared to carriers of B β 448Arg. A direct role of the polymorphism in this resistance to fibrinolysis was suggested by the fact that the significant difference in plasma lysis time remained after adjustment for the higher fibrinogen levels in these patients. Interestingly, a greater impact of environmental or alternative genetic factors on fibrinogen levels in men with diabetes was indicated by the observation that the difference in fibrinogen levels between the B β Arg448Lys variants was only significant in women.

To explore the role of B β Arg448Lys on clot structure, and the influence of the disparity in fibrinogen levels between the variants, I controlled for fibrinogen levels using plasma-purified protein and show similar differences in clot density between the B β Arg448Lys variants. The increasing clot density of Arg/Lys and Lys/Lys fibrin clots,

demonstrates that B β 448Lys is associated with more compact clots and thinner fibrin fibres, confirming the direct role of the polymorphism on fibrin network structure. Similarly, the stepwise increase in lysis time in clots made from Arg/Arg, Arg/Lys and Lys/Lys fibrinogen directly implicates the polymorphism in altered clot lysis in diabetes. One possible explanation for these findings may be related to reduced lateral aggregation due to the failure of α C dissociation caused by B β 448Lys, resulting in a greater density of thinner fibres, which interact with plasma proteins, prolonging lysis time. Alternatively we suggest a differential post-translational modification between the B β Arg448Lys variants affecting clot structure and fibrinolysis. Most likely glycation or oxidation of the B β 448Lys fibrinogen occurs resulting in altered clot morphology and lysis, particularly as lysine residues are amenable to such modifications. Though the mechanisms by which B β Arg448Lys affect clot structure and lysis in T2DM are as yet unclear, we have identified a definitive mechanistic role of the polymorphism and shown that the changes in fibrinogen levels elicited by the B β Arg448Lys substitution do not explain the difference

Exploring the possible effects of B β Arg448Lys variants on previous cardiovascular events, we discovered a predictive effect of B β Arg448Lys on vascular risk, revealing B β 448Lys variant to be associated with a history of cerebrovascular disease. This finding gives further weight to the suggestion that this polymorphism adds to the thrombotic environment already present in diabetes. Of particular interest in this observation is the fact that the association was only significant in women, mirroring a similar association between this polymorphism and cerebrovascular disease in non-diabetic women¹⁰¹. Furthermore, the identification of an age difference between the B β Arg448Lys variants, with mean age of B β 448Lys carriers lower compared to B β 448Arg (and of note, only in women), leads to speculation that mortality is increased in individuals with this genotype through predisposition to a thrombotic environment, an effect that may be gender-specific. In further support of a gender-specific association between this polymorphism and vascular pathology is the lower ABI in women carrying B β 448Lys variant of fibrinogen.

From this part of the work we make a number of novel observations: i) Carriers of B β 448Lys variant of fibrinogen display compact plasma fibrin clots and impaired fibrinolysis compared with B β 448Arg even in complex multifactorial conditions, ii) the polymorphism plays a direct role in the changes observed with plasma clots and iii) carriers of B β 448Lys variant are younger, suggesting early mortality in this group, which may be gender specific and attributable to increased vascular disease.

B β Arg448Lys and altered clot structure: potential mechanisms

To further characterise the interaction between B β Arg448Lys fibrinogen, clot structure and fibrinolysis, I produced recombinant protein to isolate 'pure' fibrinogen, removing the possibility of other alterations of the molecule that could affect its behaviour (e.g. additional genetic modification, protein level, co-purification contaminants).

These data using recombinant fibrinogen showed similar differences in clot structure between B β 448Arg and B β 448Lys variants, confirming recombinant B β 448Lys fibrin is made of thinner fibres and a more compact structure than B β 448Arg. Analysis of the possible mechanisms behind this change in structure by molecular modelling found the B β Arg448Lys substitution could lead to weakening or loss of hydrogen bonds close to the b-site, and in the coiled-coil structure, as well as causing increased mobility of residues in the B-b interaction site. These changes likely affect fibrin polymerisation and final clot ultra-structure as they are all regions proposed to be involved in lateral aggregation, via B-b and α C domain interactions¹⁰².

The relationship between clot structure and fibrinolysis

Previous work suggested a direct interaction between clot structure and lysis²³. However, analysing lysis of clots formed from the B β Arg448Lys variants, a difference in clot lysis between the two variants was only evident in the presence of anti-fibrinolytic proteins, with equivalent lysis times seen between the variants in their absence. We postulate that there is a delicate balance between the number of fibres in the clot, fibre thickness, porosity of the clot and plasmin generation which controls the lysis rates of these clots.

In this proposed model, in the absence of other plasma proteins, the increased number of thin fibres of the B β 448Lys clot lyse at a faster rate at the level of individual fibres than the thick fibres of B β 448Arg. This evens out the disparity in fibre number¹⁰², having a neutral effect on fibrinolytic activity. In addition, an anti-fibrinolytic influence on the B β 448Lys clot comes from the decreased distribution, movement and binding of fibrinolytic components through the fibrin network due to the decreased porosity of the clot. Finally, an initial increase in plasmin generation in B β 448Lys enhances fibrinolytic activity, possibly attributable to the increased density of fibres in the B β 448Lys clot providing more binding sites for plasminogen at the lysis front, where it is most rapidly converted to plasmin by tPA. In this system, the pro-fibrinolytic influence of increased plasmin generation is balanced by the anti-fibrinolytic influence of the decreased clot permeability (Figure 6-2). On the other hand, clots made from purified fibrinogen of diabetes patients showed longer lysis time in Lys/Lys compared with Arg/Lys variant. This may have been due to different post-translational modification in diabetes or

contamination with plasma proteins that were not visualised on SDS-PAGE gel (further discussed below).

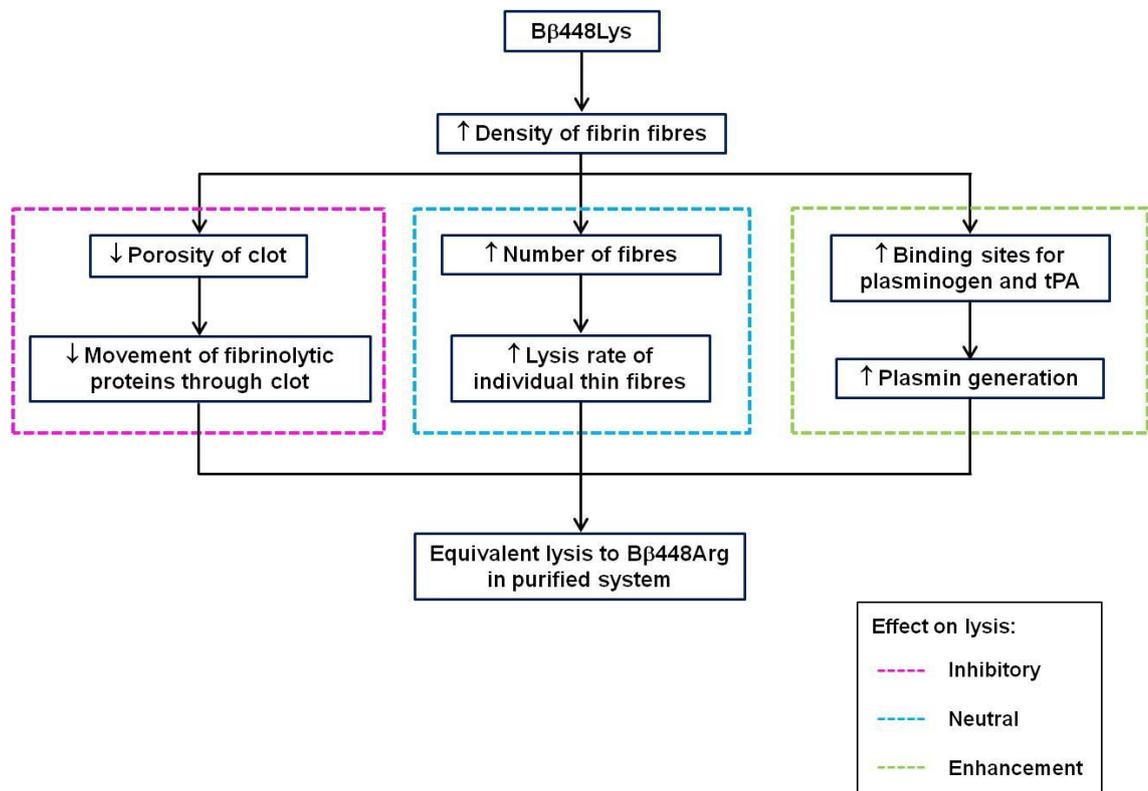


Figure 6-2. Mechanisms behind similarity in lysis times between BβArg448Lys fibrinogen variants in a purified system

In the absence of fibrinolytic inhibitory proteins, the decreased movement of fibrinolytic proteins through the clot is balanced by an increased plasmin generation

Interactions between clot structure and α2-AP: role in fibrinolytic efficiency

When studied in a system more akin to that *in-vivo*, our data show significantly greater prolongation of lysis in the presence of α2-AP of clots made from recombinant Bβ448Lys than Bβ448Arg fibrinogen, implicating the interaction of clot structure and plasma proteins in the difference in lysis times between the variant fibrins. This may also explain the data with purified fibrinogen from diabetes subjects as small amounts of plasma proteins (e.g. α2-AP) could have co-purified with the fibrinogen.

Our data identifies α2-AP as one of the main factors responsible for this prolongation in lysis, as well as the disparity in lysis rates between the BβArg448Lys variants in the plasma environment previously observed by our group¹⁰². This can be explained by the same model as above, in which the increased number of Bβ448Lys fibrin fibres being lysed at a faster rate at the level of individual fibres has a neutral effect on fibrinolytic activity. Similarly, the anti-fibrinolytic influence on the Bβ448Lys clot of the decreased porosity of the thin fibred clot is still at work. The difference

identified in this system is in the plasmin generation between the B β Arg448Lys variants, which becomes equivalent in the presence of α 2-AP. These data indicate α 2-AP has a greater effect on the initially enhanced plasmin generation in the B β 448Lys variant, the mechanisms of which may mirror that of plasminogen and tPA discussed above; the increased surface area of the B β 448Lys fibrin clot could provide more binding sites for α 2-AP, allowing it to exert a greater effect on both resistance to lysis and conversion of plasminogen to plasmin by tPA. Consequently, in this system with α 2-AP, the pro-fibrinolytic influence of increased plasmin generation in B β 448Lys is removed and thus the anti-fibrinolytic influence of the decreased clot permeability prevails causing the B β 448Lys variant to lyse slower (Figure 6-3). The effect of B β Arg448Lys variant on plasmin generation and lysis rate are summarised in

Table 6-1.

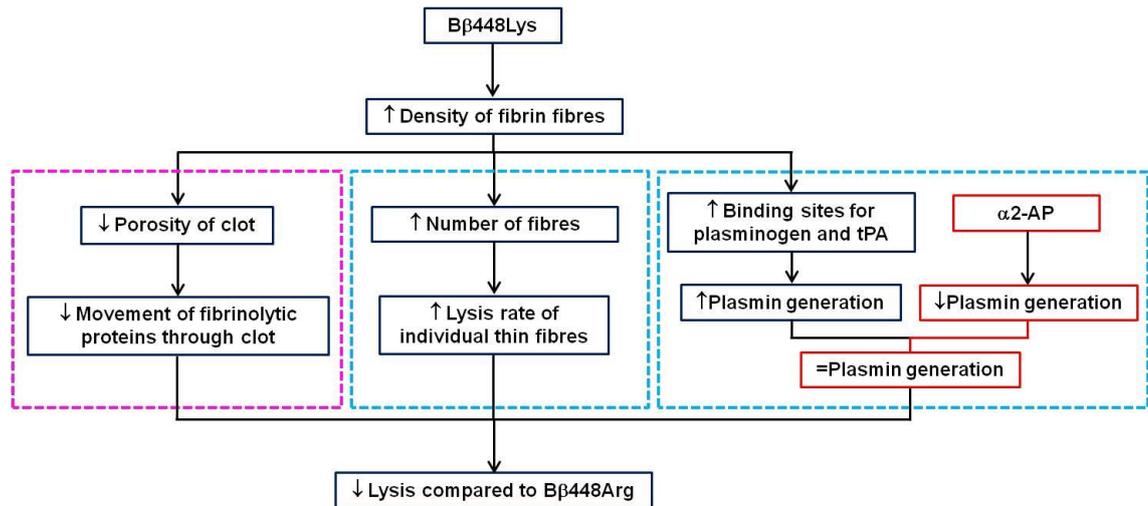


Figure 6-3. Mechanisms behind differences in lysis times between BβArg448Lys fibrinogen variants in a purified system with α2-AP

In the presence of α2-AP, plasmin generation is equivalent between BβArg448Lys fibrinogen variants so the inhibitory effect of decreased porosity of the Bβ448Lys clot on lysis is not balanced out.

Table 6-1. The effect of B β Arg448Lys allele on plasmin generation and lysis rate in the presence and absence of α 2-AP

B β 448allele	- α 2-AP		+ α 2-AP	
	Arg	Lys	Arg	Lys
Plasmin generation	↓	↑	=	=
Lysis rate	=	=	↑	↓

Analysing the interaction between α 2-AP and the B β Arg448Lys variants revealed some unexpected data; despite the differences in the effect of α 2-AP between the variants, the rate and amount of incorporation of the protein by FXIII into the fibrin network did not differ between B β 448Arg448Lys variants. Given the equivalent incorporation of α 2-AP between the variants in crosslinking assay analysis, we suggest the protein is incorporated to the same degree between the B β Arg448Lys variants, ruling out quantitative changes in α 2-AP as a mechanism by which lysis is prolonged in B β 448Lys variant. We suggest differences in lysis are due to a different structural arrangement or interaction of the α 2-AP between the two B β 448 variants whereby α 2-AP may be incorporated more tightly into the fibrin fibres of the B β 448Lys variant, or embedded deeper in the fibre. This concept is supported by the confocal data that showed fluorescent α 2-AP to only be detected in clots formed from B β 448Arg, and not B β 448Lys fibrinogen.

These recombinant data lead to the conclusion that the fibrin network structure plays an important role in conferring a much greater resistance to lysis/ plasmin activity of clots formed from thin fibres with a compact structure, most likely due to the difference in porosity and permeability of the clots to fibrinolytic components. Together the data suggest that α 2-AP is crosslinked to B β Arg448Lys fibrin variants to a similar extent. However, the structural conformation of these binding and crosslinking interactions is markedly different, such that an initial increase in plasmin activity in the absence of α 2-AP, eliciting similar lysis rates between the variants due to the inherent increased resistance to lysis of the B β 448Lys clot, is normalised in the presence of α 2-AP. This results in an increased resistance to lysis of the B β 448Lys fibrin in the presence of α 2-AP. It follows that α 2-AP has a more marked effect on plasmin generation in the B β 448Lys clot, and this may be because of the mentioned difference in conformation of the interaction between α 2-AP and the two B β Arg448Lys fibrin variants.

A α Trp334Cys/Asn335Tyr and clot structure and lysis

Plasma clots made from an individual with double mutation in the α -chain of fibrinogen (A α Trp334Cys/Asn335Tyr) showed impaired clot formation, with a fibrin network of densely packed thin fibres, increased branch points, forming a bizarre fluffy structure rather than defined fibre bundles. This structure may account, at least in part, for the clinical presentation of both a thrombotic and bleeding tendency. However, this did not prove that the mutation was the direct cause of change in structure and therefore I undertook further studies using purified and recombinant fibrinogen. The direct role of the amino acid substitutions in altered clot morphology were confirmed when clots made from plasma-purified and recombinant BII fibrinogen were studied showing a similar architecture to that in plasma.

Analysis of fibrinolysis of Fibrinogen BII revealed prolonged lysis time in clots made from fibrinogen BII when compared to those of control/WT fibrinogen in the presence of α 2-AP, explaining the thrombotic phenotype of the patient. This finding indicates the interaction between the fibrin ultra-structure and fibrinolytic plasma proteins between the variants confers resistance to lysis of the BII fibrinogen in the plasma environment, and that α 2-AP is one of the main proteins in the blood responsible for this phenomenon.

Furthermore, detection of retraction of the recombinant BII clot may have important clinical implications as retraction of a thrombus may cause the patient to have a late bleed following an injury, in line with the thrombotic and bleeding tendency of the BII patient. Moreover, the increased clot formation time of Fibrinogen BII may also have important implications for the tendency for bleeding.

Despite the hypofibrinogenemia in Fibrinogen BII, alterations in clot structure were not related to quantitative but to qualitative changes in fibrinogen, as our purified and recombinant protein data clearly demonstrate. Furthermore, the homogenous nature of my recombinant protein confirms that the mutant fibrinogen is capable of participating in clot formation, ruling out the patient's hypofibrinogenemia as the sole cause for the changes in clotting.

The mechanisms by which the BII substitutions affect clot structure and lysis remain unclear. However, the two substitutions are both highly conserved residues in the α C domain of fibrinogen, an area in which other mutations have displayed changes in binding of fibrinolytic modulators, altered clot structure and increased resistance to lysis. These fibrinogen α C domain alterations have been linked to impaired fibrinopeptide release and disruption of the b-polymerisation site which lead to delayed fibrin clot formation and unusual fibrin clot morphology as well as impaired lateral aggregation and thus clots with thinner fibres. These mechanisms could go some way

to explain the prolonged lag phase and decreased final turbidity and fibre thickness in Fibrinogen BII.

The mechanisms behind prolongation in lysis may mirror those of fibrinogen Dusart³⁵ which was shown to support reduced plasminogen binding and activation due to the thinner fibres as inhibition of fibrin polymerisation reduces the acceleratory effect of fibrin on tPA induced plasminogen activation. Potential mechanisms for the bleeding and thrombotic phenotype of the Fibrinogen BII patient are described in Figure 6-4.

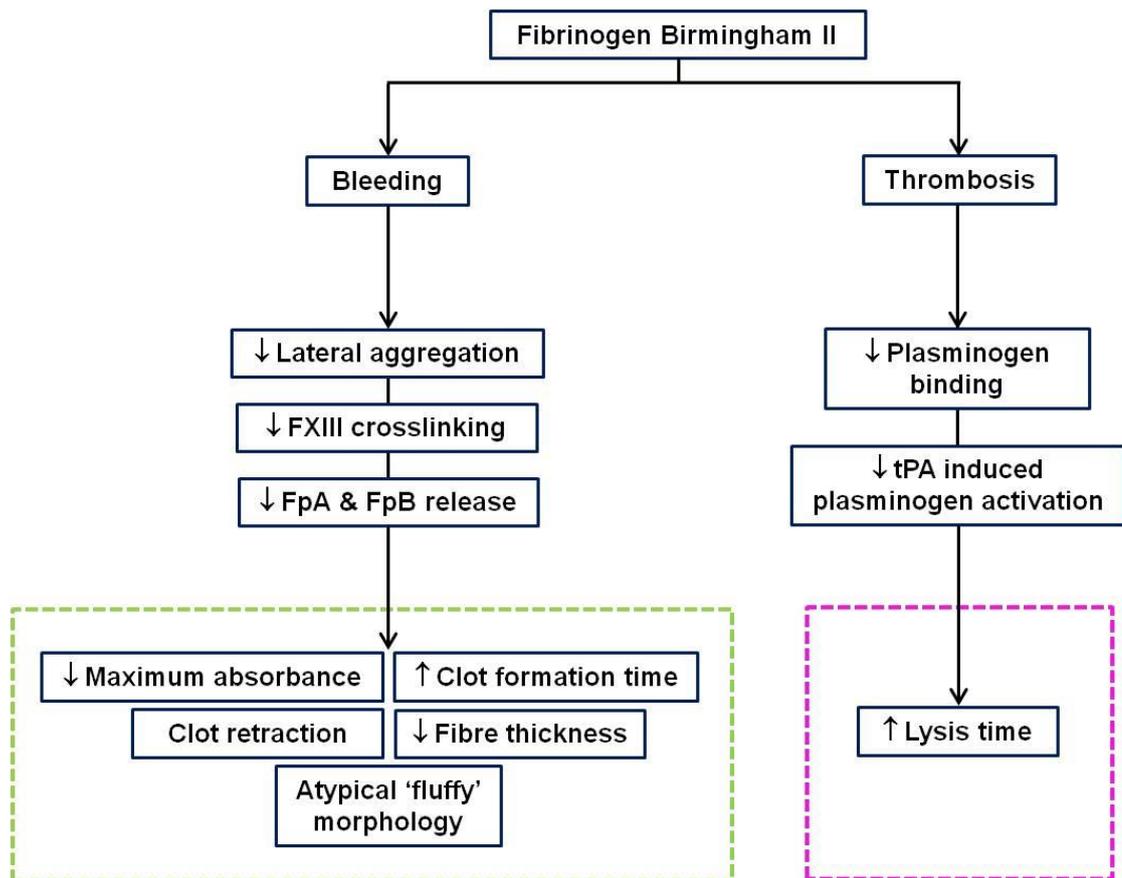


Figure 6-4. Explanations for clinical presentation of bleeding and thrombosis of Fibrinogen Birmingham II patient

Decreased lateral aggregation, FXIII crosslinking and FpA & B release may explain decreased MA, CFT and fibre thickness as well as clot retraction, leading to the bleeding presentation. Decreased plasminogen binding and tPA induced plasminogen activation may explain increased lysis time, leading to the thrombotic phenotype.

To conclude this part of my work, we confirm the changes caused by the fibrinogen BII variant are likely to be responsible for the clinical presentation of bleeding and thrombosis in our patient. Our data prove the mutations play a direct role in changes in clot structure, formation and lysis, providing mechanistic explanations for the clinical presentation of our patient: The excessively low clot turbidity and thin fibres

are consistent with less robust clots predisposing to bleeding, confounded by the greater time taken for clot formation and clot retraction, whereas impaired fibrinolysis may explain the increased thrombotic tendency.

To summarise, studying the B β Arg448Lys polymorphism in T2DM, I found B β 448Lys has an additional effect on fibrin clot structure and fibrinolysis in these patients which may add to an already increased level of vascular risk in diabetes. Examining the same polymorphism in a recombinant system, I found differences in lysis between B β Arg448Lys variants in the plasma environment are attributable to the interplay between permeability of the clot, the fibrin- α 2-AP interaction and plasmin generation. Furthermore, looking at the so far unique fibrinogen BII dysfibrinogenemia, I established A α Trp334Cys/Asn335Tyr substitutions are directly responsible for changes in clot structure, formation and lysis, providing mechanistic explanations for the bleeding and thrombotic clinical presentation of our patient.

Future work

In relation to the work in patients with T2DM, investigation into the effect of B β Arg448Lys on vascular risk revealed B β 448Lys variant was associated with a history of cerebrovascular disease in women. A similar association between this polymorphism and cerebrovascular disease in non-diabetic women was documented before and therefore this finding merits further investigation. This can only be done using longitudinal prospective studies which should concentrate on the dichotomy of vascular risk in males and females and role of SNPs in complex clinical conditions, this could identify high risk patient groups and give the opportunity to provide more targeted therapeutic interventions.

While important associations between clot structure and lysis have been shown in this work, further studies are needed to address the mechanisms behind differences in clot formation and consequently those by which clot structure governs lysis rates. The work on recombinant B β Arg448Lys and A α Trp334Cys/Asn335Tyr protein has opened up a number of opportunities to further study the molecular mechanisms surrounding fibrin clot formation and lysis, including interaction with plasma proteins.

Firstly, it is important to elucidate the mechanisms by which the fibrin fibres differ structurally between B β Arg448Lys and A α Trp334Cys/Asn335Tyr variants which will clarify the effect these substitutions have on the structure and function of the fibrinogen molecule and consequently aid the direction of work on the disparity in lysis rates between the variants. AFM analysis of the protofibril formation would be valuable in this study, however due to the flaws of this technique, alternative approaches such as real time confocal and sequential EM may be of value in future work.

While analysis of the A α Trp334Cys/Asn335Tyr substitutions in the fibrinogen BII patient has confirmed their responsibility for the clinical presentation of our patient, future work should focus on the mechanisms behind altered clot formation time, structure and lysis. Crystallisation of the flexible portion of the α C domain to determine its structure may aid these studies and production of recombinant fibrinogen with only one of the substitutions could identify if either one is causing the effect on fibrin behaviour, or if both are required for this phenotype.

Perhaps most importantly, a large study is warranted to clarify the mechanisms behind resistance to lysis of clots made of thinner fibres that are more compact. The observation that plasminogen activity is increased in the presence of clots made of thin fibres requires further investigation, especially given the discrepancy with previous studies that contradict our data. This and the increased lysis time in the presence of FXIII when α 2-AP is present merit future investigation of the complex interactions between B β Arg448Lys, clot structure, plasmin generation, FXIII cross-linking, α 2-AP and lysis rates. Further characterisation of the binding and crosslinking of α 2-AP to fibres of differing diameters and/ or B β Arg448Lys variants in a full clot based system, alongside investigation of the structural conformation of the α 2-AP-fibrin interaction, would help to ascertain the effect α 2-AP has in these preparations.

Lastly, in both B β Arg448Lys and A α Trp334Cys/Asn335Tyr variants, lysis of clots made from variant plasma-purified fibrinogen was slower than those of control in a purified system in the absence of other anti-fibrinolytic plasma proteins. Conversely, in the same system, clots formed from recombinant fibrinogen lysed at equivalent rates (B β 448Lys) or faster (A α 334Cys/335Tyr) when compared to WT. This disparity in lysis rates warrants further investigation to investigate the possibility of impurities from plasma of the purified protein and/ or posttranslational modifications.

Though this study has provided valuable insight into clot formation and lysis, improvement and development of new techniques could greatly enhance the clinical applicability of the knowledge gained. Whilst turbidimetric techniques used in this work have the advantage of studying clot formation and lysis as a dynamic process, this system does not take into account other factors that affect coagulation such as platelets and blood flow. Techniques to visualise the clot (EM and LSCM), whilst providing both high resolution and hydrated conditions respectively, also fail to represent these *in-vivo* conditions. Development of a flow model to study all these components together would thus be greatly beneficial to this field of research.

The lack of a reliable technique to assess binding/crosslinking of α 2-AP to fibrin was a severe drawback to this work. Whilst the plate based FXIII crosslinking assays hold merit in their reproducibility, they have the limitation that although fibrin is formed (arguably more relevant than un-polymerised fibrinogen) it is formed on a surface,

which does not represent the *in-vivo* environment fully. Whilst this problem is overcome in FXIII crosslinking gels, these have the drawback of poor reproducibility and an element of subjectivity. Though attempts were made to produce a method by which α 2-AP incorporation could be assessed in a full clot, these were unsuccessful. LSCM analysis, whilst promising, proved complicated due to the lack of α 2-AP signal detected in the B β 448Lys preparations. Furthermore an ELISA to measure α 2-AP incorporation by assessing α 2-AP concentration in the clotting reaction and subsequent clot supernatant was complicated by the fragility of the recombinant and plasma-purified fibrin clot. It would thus be beneficial to develop a technique by which this could be assessed, perhaps by ELISA of a formed clot, or radioactive assay with labelled α 2-AP.

References

- (1) Ajjan R, Grant PJ. Coagulation and atherothrombotic disease. *Atherosclerosis* 2006 June;186(2):240-59.
- (2) Knighton DR, Ciresi KF, Fiegel VD, Austin LL, Butler EL. Classification and treatment of chronic nonhealing wounds. Successful treatment with autologous platelet-derived wound healing factors (PDWHF). *Ann Surg* 1986 September;204(3):322-30.
- (3) Wolberg AS. Thrombin generation and fibrin clot structure. *Blood Rev* 2007 May;21(3):131-42.
- (4) Ajjan RA, Standeven KF, Khanbhai M, Phoenix F, Gersh KC, Weisel JW, Kearney MT, Ariens RA, Grant PJ. Effects of aspirin on clot structure and fibrinolysis using a novel in vitro cellular system. *Arterioscler Thromb Vasc Biol* 2009 May;29(5):712-7.
- (5) Schaller J, Gerber SS. The plasmin-antiplasmin system: structural and functional aspects. *Cell Mol Life Sci* 2011 March;68(5):785-801.
- (6) Huntington JA, Read RJ, Carrell RW. Structure of a serpin-protease complex shows inhibition by deformation. *Nature* 2000 October 19;407(6806):923-6.
- (7) Wang H, Pap S, Wiman B. Inactivation of antiplasmin at low pH: evidence for the formation of latent molecules. *Thromb Res* 2004;114(4):301-6.
- (8) Fuss C, Palmaz JC, Sprague EA. Fibrinogen: structure, function, and surface interactions. *J Vasc Interv Radiol* 2001 June;12(6):677-82.
- (9) Mosesson MW, Finlayson JS, Galanakis DK. The essential covalent structure of human fibrinogen evinced by analysis of derivatives formed during plasminic hydrolysis. *J Biol Chem* 1973 November 25;248(22):7913-29.
- (10) Zhang JZ, Redman C. Fibrinogen assembly and secretion. Role of intrachain disulfide loops. *J Biol Chem* 1996 November 22;271(47):30083-8.
- (11) Henschen A, Lottspeich F, Kehl M, Southan C. Covalent structure of fibrinogen. *Ann N Y Acad Sci* 1983 June 27;408:28-43.
- (12) Acuna AU, Gonzalez-Rodriguez J, Lillo MP, Naqvi KR. Protein structure probed by polarization spectroscopy. II. A time-resolved fluorescence study of human fibrinogen. *Biophys Chem* 1987 April;26(1):63-70.
- (13) Wolfenstein-Todel C, Mosesson MW. Human plasma fibrinogen heterogeneity: evidence for an extended carboxyl-terminal sequence in a normal gamma chain variant (gamma'). *Proc Natl Acad Sci U S A* 1980 September;77(9):5069-73.
- (14) Huang S, Mulvihill ER, Farrell DH, Chung DW, Davie EW. Biosynthesis of human fibrinogen. Subunit interactions and potential intermediates in the assembly. *J*

Biol Chem 1993 April 25;268(12):8919-26.

(15) Scheraga HA. The thrombin-fibrinogen interaction. *Biophys Chem* 2004 December 20;112(2-3):117-30.

(16) Doolittle RF. Structural basis of the fibrinogen-fibrin transformation: contributions from X-ray crystallography. *Blood Rev* 2003 March;17(1):33-41.

(17) Carr ME, Jr., Qureshi GD. The impact of delayed fibrinopeptide-A release on fibrin structure. Studies of an abnormal fibrinogen. *J Biol Chem* 1987 November 15;262(32):15568-74.

(18) Medved' LV, Gorkun OV, Manyakov VF, Belitser VA. The role of fibrinogen alpha C-domains in the fibrin assembly process. *FEBS Lett* 1985 February 11;181(1):109-12.

(19) Collet JP, Moen JL, Veklich YI, Gorkun OV, Lord ST, Montalescot G, Weisel JW. The alphaC domains of fibrinogen affect the structure of the fibrin clot, its physical properties, and its susceptibility to fibrinolysis. *Blood* 2005 December 1;106(12):3824-30.

(20) Rixon MW, Chan WY, Davie EW, Chung DW. Characterization of a complementary deoxyribonucleic acid coding for the alpha chain of human fibrinogen. *Biochemistry* 1983 June 21;22(13):3237-44.

(21) Chung DW, Que BG, Rixon MW, Mace M, Jr., Davie EW. Characterization of complementary deoxyribonucleic acid and genomic deoxyribonucleic acid for the beta chain of human fibrinogen. *Biochemistry* 1983 June 21;22(13):3244-50.

(22) Chung DW, Chan WY, Davie EW. Characterization of a complementary deoxyribonucleic acid coding for the gamma chain of human fibrinogen. *Biochemistry* 1983 June 21;22(13):3250-6.

(23) Collet JP, Park D, Lesty C, Soria J, Soria C, Montalescot G, Weisel JW. Influence of fibrin network conformation and fibrin fiber diameter on fibrinolysis speed: dynamic and structural approaches by confocal microscopy. *Arterioscler Thromb Vasc Biol* 2000 May;20(5):1354-61.

(24) Longstaff C, Thelwell C, Williams SC, Silva MM, Szabo L, Kolev K. The interplay between tissue plasminogen activator domains and fibrin structures in the regulation of fibrinolysis: kinetic and microscopic studies. *Blood* 2011 January 13;117(2):661-8.

(25) Kroll MH, Hellums JD, McIntire LV, Schafer AI, Moake JL. Platelets and shear stress. *Blood* 1996 September 1;88(5):1525-41.

(26) Siebenlist KR, Mosesson MW. Factors affecting gamma-chain multimer formation in cross-linked fibrin. *Biochemistry* 1992 January 28;31(3):936-41.

(27) Muszbek L, Yee VC, Hevessy Z. Blood coagulation factor XIII: structure and function. *Thromb Res* 1999 June 1;94(5):271-305.

- (28) Tsurupa G, Yakovlev S, McKee P, Medved L. Noncovalent interaction of alpha(2)-antiplasmin with fibrin(ogen): localization of alpha(2)-antiplasmin-binding sites. *Biochemistry* 2010 September 7;49(35):7643-51.
- (29) Muszbek L, Bereczky Z, Bagoly Z, Komaromi I, Katona E. Factor XIII: a coagulation factor with multiple plasmatic and cellular functions. *Physiol Rev* 2011 July;91(3):931-72.
- (30) Collet JP, Shuman H, Ledger RE, Lee S, Weisel JW. The elasticity of an individual fibrin fiber in a clot. *Proc Natl Acad Sci U S A* 2005 June 28;102(26):9133-7.
- (31) Weisel JW. The mechanical properties of fibrin for basic scientists and clinicians. *Biophys Chem* 2004 December 20;112(2-3):267-76.
- (32) Brown AE, Litvinov RI, Discher DE, Purohit PK, Weisel JW. Multiscale mechanics of fibrin polymer: gel stretching with protein unfolding and loss of water. *Science* 2009 August 7;325(5941):741-4.
- (33) Averett LE, Schoenfish MH, Akhremitchev BB, Gorkun OV. Kinetics of the multistep rupture of fibrin 'A-a' polymerization interactions measured using atomic force microscopy. *Biophys J* 2009 November 18;97(10):2820-8.
- (34) Falvo MR, Millard D, O'Brien ET, III, Superfine R, Lord ST. Length of tandem repeats in fibrin's alphaC region correlates with fiber extensibility. *J Thromb Haemost* 2008 November;6(11):1991-3.
- (35) Koopman J, Haverkate F, Grimbergen J, Lord ST, Mosesson MW, DiOrio JP, Siebenlist KS, Legrand C, Soria J, Soria C, . Molecular basis for fibrinogen Dusart (A alpha 554 Arg-->Cys) and its association with abnormal fibrin polymerization and thrombophilia. *J Clin Invest* 1993 April;91(4):1637-43.
- (36) Ariens RA, Philippou H, Nagaswami C, Weisel JW, Lane DA, Grant PJ. The factor XIII V34L polymorphism accelerates thrombin activation of factor XIII and affects cross-linked fibrin structure. *Blood* 2000 August 1;96(3):988-95.
- (37) Takagi T, Doolittle RF. Amino acid sequence studies on factor XIII and the peptide released during its activation by thrombin. *Biochemistry* 1974 February 12;13(4):750-6.
- (38) Robinson BR, Houg AK, Reed GL. Catalytic life of activated factor XIII in thrombi. Implications for fibrinolytic resistance and thrombus aging. *Circulation* 2000 September 5;102(10):1151-7.
- (39) Valnickova Z, Enghild JJ. Human procarboxypeptidase U, or thrombin-activable fibrinolysis inhibitor, is a substrate for transglutaminases. Evidence for transglutaminase-catalyzed cross-linking to fibrin. *J Biol Chem* 1998 October 16;273(42):27220-4.
- (40) Ritchie H, Robbie LA, Kinghorn S, Exley R, Booth NA. Monocyte plasminogen activator inhibitor 2 (PAI-2) inhibits u-PA-mediated fibrin clot lysis and is cross-linked to

- fibrin. *Thromb Haemost* 1999 January;81(1):96-103.
- (41) Bale MD, Westrick LG, Mosher DF. Incorporation of thrombospondin into fibrin clots. *J Biol Chem* 1985 June 25;260(12):7502-8.
- (42) Sane DC, Moser TL, Phippen AM, Parker CJ, Achyuthan KE, Greenberg CS. Vitronectin is a substrate for transglutaminases. *Biochem Biophys Res Commun* 1988 November 30;157(1):115-20.
- (43) Bendixen E, Borth W, Harpel PC. Transglutaminases catalyze cross-linking of plasminogen to fibronectin and human endothelial cells. *J Biol Chem* 1993 October 15;268(29):21962-7.
- (44) Mosher DF. Cross-linking of cold-insoluble globulin by fibrin-stabilizing factor. *J Biol Chem* 1975 August 25;250(16):6614-21.
- (45) Mosher DF, Schad PE. Cross-linking of fibronectin to collagen by blood coagulation Factor XIIIa. *J Clin Invest* 1979 September;64(3):781-7.
- (46) Barry EL, Mosher DF. Factor XIII cross-linking of fibronectin at cellular matrix assembly sites. *J Biol Chem* 1988 July 25;263(21):10464-9.
- (47) Prince CW, Dickie D, Krumdieck CL. Osteopontin, a substrate for transglutaminase and factor XIII activity. *Biochem Biophys Res Commun* 1991 June 28;177(3):1205-10.
- (48) Bockenstedt P, McDonagh J, Handin RI. Binding and covalent cross-linking of purified von Willebrand factor to native monomeric collagen. *J Clin Invest* 1986 August;78(2):551-6.
- (49) Hada M, Kaminski M, Bockenstedt P, McDonagh J. Covalent crosslinking of von Willebrand factor to fibrin. *Blood* 1986 July;68(1):95-101.
- (50) Cohen I, Young-Bandala L, Blankenberg TA, Siefring GE, Jr., Bruner-Lorand J. Fibrinolytic-catalyzed cross-linking of myosin from platelet and skeletal muscle. *Arch Biochem Biophys* 1979 January;192(1):100-11.
- (51) Inbal A, Lubetsky A, Krapp T, Castel D, Shaish A, Dickneite G, Modis L, Muszbek L, Inbal A. Impaired wound healing in factor XIII deficient mice. *Thromb Haemost* 2005 August;94(2):432-7.
- (52) Brown LF, Lanir N, McDonagh J, Tognazzi K, Dvorak AM, Dvorak HF. Fibroblast migration in fibrin gel matrices. *Am J Pathol* 1993 January;142(1):273-83.
- (53) Grinnell F, Feld M, Minter D. Fibroblast adhesion to fibrinogen and fibrin substrata: requirement for cold-insoluble globulin (plasma fibronectin). *Cell* 1980 February;19(2):517-25.
- (54) Dardik R, Krapp T, Rosenthal E, Loscalzo J, Inbal A. Effect of FXIII on monocyte and fibroblast function. *Cell Physiol Biochem* 2007;19(1-4):113-20.
- (55) Sarvary A, Szucs S, Balogh I, Becsky A, Bardos H, Kawai M, Seligsohn U, Egbring R, Lopaciuk S, Muszbek L, Adany R. Possible role of factor XIII subunit A in

Fcgamma and complement receptor-mediated phagocytosis. *Cell Immunol* 2004 April;228(2):81-90.

(56) Dardik R, Solomon A, Loscalzo J, Eskaraev R, Bialik A, Goldberg I, Schiby G, Inbal A. Novel proangiogenic effect of factor XIII associated with suppression of thrombospondin 1 expression. *Arterioscler Thromb Vasc Biol* 2003 August 1;23(8):1472-7.

(57) Asahina T, Kobayashi T, Takeuchi K, Kanayama N. Congenital blood coagulation factor XIII deficiency and successful deliveries: a review of the literature. *Obstet Gynecol Surv* 2007 April;62(4):255-60.

(58) Kobayashi T, Terao T, Kojima T, Takamatsu J, Kamiya T, Saito H. Congenital factor XIII deficiency with treatment of factor XIII concentrate and normal vaginal delivery. *Gynecol Obstet Invest* 1990;29(3):235-8.

(59) Asahina T, Kobayashi T, Okada Y, Goto J, Terao T. Maternal blood coagulation factor XIII is associated with the development of cytotrophoblastic shell. *Placenta* 2000 May;21(4):388-93.

(60) Nahrendorf M, Hu K, Frantz S, Jaffer FA, Tung CH, Hiller KH, Voll S, Nordbeck P, Sosnovik D, Gattenlohner S, Novikov M, Dickneite G, Reed GL, Jakob P, Rosenzweig A, Bauer WR, Weissleder R, Ertl G. Factor XIII deficiency causes cardiac rupture, impairs wound healing, and aggravates cardiac remodeling in mice with myocardial infarction. *Circulation* 2006 March 7;113(9):1196-202.

(61) AbdAlla S, Lothar H, Langer A, el FY, Quitterer U. Factor XIIIa transglutaminase crosslinks AT1 receptor dimers of monocytes at the onset of atherosclerosis. *Cell* 2004 October 29;119(3):343-54.

(62) Lucas MA, Fretto LJ, McKee PA. The binding of human plasminogen to fibrin and fibrinogen. *J Biol Chem* 1983 April 10;258(7):4249-56.

(63) Christensen U, Bangert K, Thorsen S. Reaction of human alpha2-antiplasmin and plasmin stopped-flow fluorescence kinetics. *FEBS Lett* 1996 May 27;387(1):58-62.

(64) Rijken DC, Sakharov DV. Basic principles in thrombolysis: regulatory role of plasminogen. *Thromb Res* 2001 September 30;103 Suppl 1:S41-S49.

(65) Wolberg AS, Campbell RA. Thrombin generation, fibrin clot formation and hemostasis. *Transfus Apher Sci* 2008 February;38(1):15-23.

(66) Lee KN, Jackson KW, Christiansen VJ, Chung KH, McKee PA. A novel plasma proteinase potentiates alpha2-antiplasmin inhibition of fibrin digestion. *Blood* 2004 May 15;103(10):3783-8.

(67) Lijnen HR, Van Hoef B, Collen D. Inactivation of the serpin alpha(2)-antiplasmin by stromelysin-1. *Biochim Biophys Acta* 2001 June 11;1547(2):206-13.

(68) Lee KN, Jackson KW, Christiansen VJ, Lee CS, Chun JG, McKee PA. Why alpha-antiplasmin must be converted to a derivative form for optimal function. *J Thromb*

Haemost 2007 October;5(10):2095-104.

(69) Lee KN, Lee CS, Tae WC, Jackson KW, Christiansen VJ, McKee PA. Cross-linking of wild-type and mutant alpha 2-antiplasmins to fibrin by activated factor XIII and by a tissue transglutaminase. *J Biol Chem* 2000 December 1;275(48):37382-9.

(70) Law RH, Sofian T, Kan WT, Horvath AJ, Hitchen CR, Langendorf CG, Buckle AM, Whisstock JC, Coughlin PB. X-ray crystal structure of the fibrinolysis inhibitor alpha2-antiplasmin. *Blood* 2008 February 15;111(4):2049-52.

(71) Lu BG, Sofian T, Law RH, Coughlin PB, Horvath AJ. Contribution of conserved lysine residues in the alpha2-antiplasmin C terminus to plasmin binding and inhibition. *J Biol Chem* 2011 July 15;286(28):24544-52.

(72) Heinrich J, Funke H, Rust S, Schulte H, Schonfeld R, Kohler E, Assmann G. Impact of polymorphisms in the alpha- and beta-fibrinogen gene on plasma fibrinogen concentrations of coronary heart disease patients. *Thromb Res* 1995 February 1;77(3):209-15.

(73) Jacquemin B, Antoniades C, Nyberg F, Plana E, Muller M, Greven S, Salomaa V, Sunyer J, Bellander T, Chalamandaris AG, Pistelli R, Koenig W, Peters A. Common genetic polymorphisms and haplotypes of fibrinogen alpha, beta, and gamma chains affect fibrinogen levels and the response to proinflammatory stimulation in myocardial infarction survivors: the AIRGENE study. *J Am Coll Cardiol* 2008 September 9;52(11):941-52.

(74) Standeven KF, Grant PJ, Carter AM, Scheiner T, Weisel JW, Ariens RA. Functional analysis of the fibrinogen Aalpha Thr312Ala polymorphism: effects on fibrin structure and function. *Circulation* 2003 May 13;107(18):2326-30.

(75) Rasmussen-Torvik LJ, Cushman M, Tsai MY, Zhang Y, Heckbert SR, Rosamond WD, Folsom AR. The association of alpha-fibrinogen Thr312Ala polymorphism and venous thromboembolism in the LITE study. *Thromb Res* 2007;121(1):1-7.

(76) Kapczynska K, Stefanowicz P, Jaremko L, Jaremko M, Kluczyk A, Szewczuk Z. The efficient synthesis of isotopically labeled peptide-derived Amadori products and their characterization. *Amino Acids* 2011 March;40(3):923-32.

(77) Kijewska M, Stefanowicz P, Kluczyk A, Szewczuk Z. The isotopic exchange of oxygen as a tool for detection of the glycation sites in proteins. *Anal Biochem* 2011 December 15;419(2):81-7.

(78) Thornalley PJ, Battah S, Ahmed N, Karachalias N, Agalou S, Babaei-Jadidi R, Dawnay A. Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. *Biochem J* 2003 November 1;375(Pt 3):581-92.

(79) Andrades ME, Lorenzi R, Berger M, Guimaraes JA, Moreira JC, Dal-Pizzol F.

Glycolaldehyde induces fibrinogen post-translational modification, delay in clotting and resistance to enzymatic digestion. *Chem Biol Interact* 2009 August 14;180(3):478-84.

(80) Ajjan RA, Gamlen T, Standeven KF, Mughal S, Hess K, Smith KA, Dunn EJ, Anwar MM, Rabbani N, Thornalley PJ, Philippou H, Grant PJ. Diabetes is associated with posttranslational modifications in plasminogen resulting in reduced plasmin generation and enzyme-specific activity. *Blood* 2013 July 4;122(1):134-42.

(81) Henschen-Edman AH. Fibrinogen non-inherited heterogeneity and its relationship to function in health and disease. *Ann N Y Acad Sci* 2001;936:580-93.

(82) Upchurch Jr GR, Ramdev N, Walsh MT, Loscalzo J. Prothrombotic Consequences of the Oxidation of Fibrinogen and their Inhibition by Aspirin. *J Thromb Thrombolysis* 1998;5(1):9-14.

(83) Azizova OA, Aseichev AV, Piryazev AP, Roitman EV, Shcheglovitova ON. Effects of oxidized fibrinogen on the functions of blood cells, blood clotting, and rheology. *Bull Exp Biol Med* 2007 September;144(3):397-407.

(84) Ajjan RA, Ariens RA. Cardiovascular disease and heritability of the prothrombotic state. *Blood Rev* 2009 March;23(2):67-78.

(85) Svensson J, Bergman AC, Adamson U, Blomback M, Wallen H, Jorneskog G. Acetylation and glycation of fibrinogen in vitro occur at specific lysine residues in a concentration dependent manner: a mass spectrometric and isotope labeling study. *Biochem Biophys Res Commun* 2012 May 4;421(2):335-42.

(86) Haglund AC, Ronquist G, Frithz G, Ek P. Alteration of the fibrinogen molecule and its phosphorylation state in myocardial infarction patients undergoing thrombolytic treatment. *Thromb Res* 2000 April 15;98(2):147-56.

(87) Sauls DL, Warren M, Hoffman M. Homocysteinylated fibrinogen forms disulfide-linked complexes with albumin. *Thromb Res* 2011 June;127(6):576-81.

(88) Parastatidis I, Thomson L, Burke A, Chernysh I, Nagaswami C, Visser J, Stamer S, Liebler DC, Koliakos G, Heijnen HF, Fitzgerald GA, Weisel JW, Ischiropoulos H. Fibrinogen beta-chain tyrosine nitration is a prothrombotic risk factor. *J Biol Chem* 2008 December 5;283(49):33846-53.

(89) Veldman FJ, Nair CH, Vorster HH, Vermaak WJ, Jerling JC, Oosthuizen W, Venter CS. Dietary pectin influences fibrin network structure in hypercholesterolaemic subjects. *Thromb Res* 1997 May 1;86(3):183-96.

(90) Hess K, Alzahrani SH, Mathai M, Schroeder V, Carter AM, Howell G, Koko T, Strachan MW, Price JF, Smith KA, Grant PJ, Ajjan RA. A novel mechanism for hypofibrinolysis in diabetes: the role of complement C3. *Diabetologia* 2012 April;55(4):1103-13.

(91) Jorneskog G, Egberg N, Fagrell B, Fatah K, Hessel B, Johnsson H, Brismar K, Blomback M. Altered properties of the fibrin gel structure in patients with IDDM.

Diabetologia 1996 December;39(12):1519-23.

(92) Collet JP, Allali Y, Lesty C, Tanguy ML, Silvain J, Ankri A, Blanchet B, Dumaine R, Gianetti J, Payot L, Weisel JW, Montalescot G. Altered fibrin architecture is associated with hypofibrinolysis and premature coronary atherothrombosis. *Arterioscler Thromb Vasc Biol* 2006 November;26(11):2567-73.

(93) Stone MC, Thorp JM. Plasma fibrinogen--a major coronary risk factor. *J R Coll Gen Pract* 1985 December;35(281):565-9.

(94) Smith FB, Lee AJ, Fowkes FG, Price JF, Rumley A, Lowe GD. Hemostatic factors as predictors of ischemic heart disease and stroke in the Edinburgh Artery Study. *Arterioscler Thromb Vasc Biol* 1997 November;17(11):3321-5.

(95) Carter AM, Catto AJ, Grant PJ. Association of the alpha-fibrinogen Thr312Ala polymorphism with poststroke mortality in subjects with atrial fibrillation. *Circulation* 1999 May 11;99(18):2423-6.

(96) Carter AM, Catto AJ, Kohler HP, Ariens RA, Stickland MH, Grant PJ. alpha-fibrinogen Thr312Ala polymorphism and venous thromboembolism. *Blood* 2000 August 1;96(3):1177-9.

(97) Yarnell JW, Baker IA, Sweetnam PM, Bainton D, O'Brien JR, Whitehead PJ, Elwood PC. Fibrinogen, viscosity, and white blood cell count are major risk factors for ischemic heart disease. The Caerphilly and Speedwell collaborative heart disease studies. *Circulation* 1991 March;83(3):836-44.

(98) Vadseth C, Souza JM, Thomson L, Seagraves A, Nagaswami C, Scheiner T, Torbet J, Vilaire G, Bennett JS, Murciano JC, Muzykantov V, Penn MS, Hazen SL, Weisel JW, Ischiropoulos H. Pro-thrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species. *J Biol Chem* 2004 March 5;279(10):8820-6.

(99) Behague I, Poirier O, Nicaud V, Evans A, Arveiler D, Luc G, Cambou JP, Scarabin PY, Bara L, Green F, Cambien F. Beta fibrinogen gene polymorphisms are associated with plasma fibrinogen and coronary artery disease in patients with myocardial infarction. The ECTIM Study. Etude Cas-Temoins sur l'Infarctus du Myocarde. *Circulation* 1996 February 1;93(3):440-9.

(100) French JK, Van de Water NS, Sutton TM, Lund M, Gao W, McDowell J, Liu-Stratton Y, Pohorence J, Szymanski D, Goldschmidt-Clermont P, White HD, Browett PJ, Cooke G. Potential thrombophilic mutations/polymorphisms in patients with no flow-limiting stenosis after myocardial infarction. *Am Heart J* 2003 January;145(1):118-24.

(101) Carter AM, Catto AJ, Bamford JM, Grant PJ. Gender-specific associations of the fibrinogen B beta 448 polymorphism, fibrinogen levels, and acute cerebrovascular disease. *Arterioscler Thromb Vasc Biol* 1997 March;17(3):589-94.

(102) Ajjan R, Lim BC, Standeven KF, Harrand R, Dolling S, Phoenix F, Greaves R,

- Abou-Saleh RH, Connell S, Smith DA, Weisel JW, Grant PJ, Ariens RA. Common variation in the C-terminal region of the fibrinogen beta-chain: effects on fibrin structure, fibrinolysis and clot rigidity. *Blood* 2008 January 15;111(2):643-50.
- (103) Kotlin R, Chytilova M, Suttner J, Riedel T, Salaj P, Blatny J, Santrucek J, Klener P, Dyr JE. Fibrinogen Novy Jicin and Praha II: cases of hereditary Aalpha 16 Arg-->Cys and Aalpha 16 Arg-->His dysfibrinogenemia. *Thromb Res* 2007;121(1):75-84.
- (104) Mimuro J, Hamano A, Tanaka T, Madoiwa KS, Sugo T, Matsuda M, Sakata Y. Hypofibrinogenemia caused by a nonsense mutation in the fibrinogen Bbeta chain gene. *J Thromb Haemost* 2003 November;1(11):2356-9.
- (105) Lee MH, Kaczmarek E, Chin DT, Oda A, McIntosh S, Bauer KA, Clyne LP, McDonagh J. Fibrinogen Ledyard (A alpha Arg16---Cys): biochemical and physiologic characterization. *Blood* 1991 October 1;78(7):1744-52.
- (106) Bogli C, Hofer A, Baudo F, Redaelli R, Furlan M. Fibrinogen Milano IV, another case of congenital dysfibrinogenemia with an abnormal fibrinopeptide A release (A alpha 16 Arg----His). *Haemostasis* 1992;22(1):7-11.
- (107) Furlan M, Leupin L, Biasiutti FD, Lammle B. [Fibrinogen Bern III: a further case of hereditary fibrinogen variants with substitution A alpha 16 Arg----Cys]. *Schweiz Med Wochenschr* 1991 July 20;121(29):1068-71.
- (108) Hill M, Thompson N, Gordon B, Ajjan R, Ariens RAS, Williams M, Dolan G. Fibrinogen Birmingham II - A novel variant associated with hypodysfibrinogenemia, due to compound heterozygosity for TRP334CYS and ASN335TYR in the fibrinogen A alpha chain. 2009.
- (109) Kannel WB, D'Agostino RB, Wilson PW, Belanger AJ, Gagnon DR. Diabetes, fibrinogen, and risk of cardiovascular disease: the Framingham experience. *Am Heart J* 1990 September;120(3):672-6.
- (110) Pieters M, van Zyl DG, Rheeder P, Jerling JC, Loots dT, van der Westhuizen FH, Gottsche LT, Weisel JW. Glycation of fibrinogen in uncontrolled diabetic patients and the effects of glycaemic control on fibrinogen glycation. *Thromb Res* 2007;120(3):439-46.
- (111) Dunn EJ, Ariens RA, Grant PJ. The influence of type 2 diabetes on fibrin structure and function. *Diabetologia* 2005 June;48(6):1198-206.
- (112) Raynaud E, Perez-Martin A, Brun J, Aissa-Benhaddad A, Fedou C, Mercier J. Relationships between fibrinogen and insulin resistance. *Atherosclerosis* 2000 June;150(2):365-70.
- (113) Diamant M, Nieuwland R, Pablo RF, Sturk A, Smit JW, Radder JK. Elevated numbers of tissue-factor exposing microparticles correlate with components of the metabolic syndrome in uncomplicated type 2 diabetes mellitus. *Circulation* 2002 November 5;106(19):2442-7.

- (114) Vaidyula VR, Rao AK, Mozzoli M, Homko C, Cheung P, Boden G. Effects of hyperglycemia and hyperinsulinemia on circulating tissue factor procoagulant activity and platelet CD40 ligand. *Diabetes* 2006 January;55(1):202-8.
- (115) Mansfield MW, Heywood DM, Grant PJ. Circulating levels of factor VII, fibrinogen, and von Willebrand factor and features of insulin resistance in first-degree relatives of patients with NIDDM. *Circulation* 1996 November 1;94(9):2171-6.
- (116) Ceriello A, Esposito K, Ihnat M, Zhang J, Giugliano D. Simultaneous control of hyperglycemia and oxidative stress normalizes enhanced thrombin generation in type 1 diabetes. *J Thromb Haemost* 2009 July;7(7):1228-30.
- (117) Dunn EJ, Philippou H, Ariens RA, Grant PJ. Molecular mechanisms involved in the resistance of fibrin to clot lysis by plasmin in subjects with type 2 diabetes mellitus. *Diabetologia* 2006 May;49(5):1071-80.
- (118) Watala C, Pluta J, Golanski J, Rozalski M, Czyz M, Trojanowski Z, Drzewoski J. Increased protein glycation in diabetes mellitus is associated with decreased aspirin-mediated protein acetylation and reduced sensitivity of blood platelets to aspirin. *J Mol Med (Berl)* 2005 February;83(2):148-58.
- (119) Carter AM, Cymbalista CM, Spector TD, Grant PJ. Heritability of clot formation, morphology, and lysis: the EuroCLOT study. *Arterioscler Thromb Vasc Biol* 2007 December;27(12):2783-9.
- (120) Alzahrani SH, Ajjan RA. Coagulation and fibrinolysis in diabetes. *Diab Vasc Dis Res* 2010 October;7(4):260-73.
- (121) Undas A, Kolarz M, Kopec G, Tracz W. Altered fibrin clot properties in patients on long-term haemodialysis: relation to cardiovascular mortality. *Nephrol Dial Transplant* 2008 June;23(6):2010-5.
- (122) Alzahrani SH, Hess K, Price JF, Strachan M, Baxter PD, Cubbon R, Phoenix F, Gamlen T, Ariens RA, Grant PJ, Ajjan RA. Gender-specific alterations in fibrin structure function in type 2 diabetes: associations with cardiometabolic and vascular markers. *J Clin Endocrinol Metab* 2012 December;97(12):E2282-E2287.
- (123) Undas A, Szuldrzynski K, Stepień E, Zalewski J, Godlewski J, Tracz W, Pasowicz M, Zmudka K. Reduced clot permeability and susceptibility to lysis in patients with acute coronary syndrome: effects of inflammation and oxidative stress. *Atherosclerosis* 2008 February;196(2):551-7.
- (124) Larsen MR, Trelle MB, Thingholm TE, Jensen ON. Analysis of posttranslational modifications of proteins by tandem mass spectrometry. *Biotechniques* 2006 June;40(6):790-8.
- (125) Puri R, Kataoka Y, Uno K, Nicholls SJ. The distinctive nature of atherosclerotic vascular disease in diabetes: pathophysiological and morphological insights. *Curr Diab Rep* 2012 June;12(3):280-5.

- (126) Ferreiro JL, Gomez-Hospital JA, Angiolillo DJ. Platelet abnormalities in diabetes mellitus. *Diab Vasc Dis Res* 2010 October;7(4):251-9.
- (127) Mercer BN, Morais S, Cubbon RM, Kearney MT. Diabetes mellitus and the heart. *Int J Clin Pract* 2012 July;66(7):640-7.
- (128) Price JF, Reynolds RM, Mitchell RJ, Williamson RM, Fowkes FG, Deary IJ, Lee AJ, Frier BM, Hayes PC, Strachan MW. The Edinburgh Type 2 Diabetes Study: study protocol. *BMC Endocr Disord* 2008;8:18.
- (129) Marioni RE, Strachan MW, Reynolds RM, Lowe GD, Mitchell RJ, Fowkes FG, Frier BM, Lee AJ, Butcher I, Rumley A, Murray GD, Deary IJ, Price JF. Association between raised inflammatory markers and cognitive decline in elderly people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study. *Diabetes* 2010 March;59(3):710-3.
- (130) Marioni RE, Deary IJ, Murray GD, Lowe GD, Strachan MW, Luciano M, Houlihan LM, Gow AJ, Harris SE, Rumley A, Stewart MC, Fowkes FG, Price JF. Genetic associations between fibrinogen and cognitive performance in three Scottish cohorts. *Behav Genet* 2011 September;41(5):691-9.
- (131) Meade TW, Chakrabarti R, Haines AP, North WR, Stirling Y. Characteristics affecting fibrinolytic activity and plasma fibrinogen concentrations. *Br Med J* 1979 January 20;1(6157):153-6.
- (132) Allan P, Uitte de WS, Abou-Saleh RH, Connell SD, Ariens RA. Evidence that fibrinogen gamma' directly interferes with protofibril growth: implications for fibrin structure and clot stiffness. *J Thromb Haemost* 2012 June;10(6):1072-80.
- (133) Abou-Saleh RH, Connell SD, Harrand R, Ajjan RA, Mosesson MW, Smith DA, Grant PJ, Ariens RA. Nanoscale probing reveals that reduced stiffness of clots from fibrinogen lacking 42 N-terminal Bbeta-chain residues is due to the formation of abnormal oligomers. *Biophys J* 2009 March 18;96(6):2415-27.
- (134) Syka JE, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc Natl Acad Sci U S A* 2004 June 29;101(26):9528-33.
- (135) Ng PC, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. *Nucleic Acids Res* 2003 July 1;31(13):3812-4.
- (136) Ramensky V, Bork P, Sunyaev S. Human non-synonymous SNPs: server and survey. *Nucleic Acids Res* 2002 September 1;30(17):3894-900.
- (137) Galanakis DK, Lane BP, Simon SR. Albumin modulates lateral assembly of fibrin polymers: evidence of enhanced fine fibril formation and of unique synergism with fibrinogen. *Biochemistry* 1987 April 21;26(8):2389-400.