Characterisation of the Fibrinogen RGD Sequence in Erythrocyte Binding and Clot Structure

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Intellectual Property and Publication Statements

The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Publications and Presentations

Oral presentation

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Poster Presentations

Effect of mutations in the fibrinogen $\alpha R_{95}G_{96}D_{97}$ sequence on clot structure and on the interaction of fibrinogen with red blood cells. Sabban A., Duval C., Guedes A.F., McPherson H.R., Macrae F., Saha S., Ajjan R., Santos N.C., Ariëns R.A.S., International Society of Thrombosis and Haemostasis Congress, Berlin, Germany 2017.

Effect of mutations in the fibrinogen $\alpha R_{95}G_{96}D_{97}$ sequence on clot structure and on the interaction of fibrinogen with red blood cells. Sabban A., Duval C., Guedes A.F., McPherson H.R., Macrae F., Boxall S., Saha S., Ajjan R., Santos N.C., Ariëns R.A.S. British Society of haemostasis and Thrombosis, Warwick, UK, 2017.

Publications

Ana Filipa Guedes, Filomena A Carvalho, Marco M Domingues, Fraser L Macrae 2, Helen R McPherson, Aliaa Sabban, Ivo C Martins, Cédric Duval, Nuno C Santos & Robert AS Ariëns. Impact of γ′γ′ fibrinogen interaction with red blood cells on fibrin clots. Nanomedicine (2018) 13(19), 2491–2505.

Abstract

Background: Fibrinogen is a large protein dimer that is composed of three polypeptide chains Aα, Bβ and γ and plays a vital role in haemostasis. Fibrinogen is suggested to be a risk factor for cardiovascular disease. The Aα-chain contains two RGD sequences, which provide the interaction sites of fibrinogen with integrins present on cells such as platelets and endothelial cells. New studies suggested that RBCs are not innocent bystanders in thrombus formation suggesting presence of polyhedral shaped RBCs inside the clot. An integrin-type receptor interaction between erythrocytes and fibrinogen was proposed but some controversy is still present regarding the possible receptor on the surface of the RBC involved in this interaction between fibrinogen and RBCs.

Hypothesis: Interaction between fibrinogen and RBCs is mediated by the RGD sequence in the coiled-coil of the Aα-chain of fibrinogen through $\alpha_{IIb}\beta_3$-like integrin receptor. Mutating this RGD site may inhibit the interaction between fibrinogen and RBCs and subsequently thrombosis.

Methods: Six recombinant fibrinogens variants (R95E, R95Q, G96V, D97K, D97N, and F98I) were generated by Site-Directed Mutagenesis. The integrity of the proteins were tested by SDS-PAGE and circular dichroism. Clots formed by variant fibrinogens were evaluated by clotability, turbidity and lysis, and laser scanning confocal microscopy. Interaction between fibrinogen and RBCs were studied by Plate-binding assay, solution-binding assay and flow cytometry. Involvement of the Aα-chain RGD sequence in binding between fibrinogen and platelets was tested by platelet spreading assay.

Results: Clot structure studies showed thinner, shorter fibres but no effect on fibrinolysis. Fibrinogen binding assays with both RBCs and platelets showed no effects of the mutations in the Aα-chain RGD sequence on cell binding.

Conclusion: Aα-95-98 RGD has no obvious effect on the binding between red blood cells and fibrinogen, or platelets and fibrinogen. Nonetheless, mutations at this site change clot structure and polymerisation profile.

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List of Abbreviations

°C	Degree Celsius
µg	Micrograms
µl	Microliters
µM	Micromole
µm	Micrometre
ACD	Acid Citrate-Dextrose
ADP	adenosine diphosphate
AFM	Atomic Force Microscopy
AGDV	Ala-Gly-Asp-Val
anh	Anhydrous
APC	Active Protein C
AP-FXIII	FXIII Activation Peptide
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartate
BCAM	Basal Cell Adhesion Molecule
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
CAD	Coronary Artery Disease
CAM	Cell Adhesion Molecules
CD Spectra	Circular Dichroism Spectra
cDNA	Complimentary DNA
CHO	Chinese Hamster Ovary
CO_2	Carbon Dioxide
C-terminal	Carboxyl End
Cys (C)	Cysteine
ddH_2O	Deionized water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide

DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
D-region	Distal region of fibrinogen
DVT	Deep Vein Thrombosis
ECM	Extracellular Matrix
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
E-region	Central region of fibrinogen
F	Forward primer
FBG	Fibrinogen
FBS	Foetal Bovine Serum
FII	Prothrombin
FIIa	Thrombin
FITC	Fluorescein Isothiocyanate
FIX	Factor IX
FIXa	Activated Factor IX
fl	Femtoliter
FpA	Fibrinopeptide A
FpB	Fibrinopeptide B
FSC	Forward Light Scatter
FV	Factor V
FVa	Activated Factor V
FVII	Factor VII
FVIIa	Activated Factor VII
FVIII	Factor VIII
FVIIIa	Activated Factor VIII
FX	Factor X
FXa	Activated Factor X
FXI	Factor XI
FXIa	Activated Factor XI
FXII	Factor XII
FXIIa	Activated Factor XII
FXIII	Factor XIII
FXIIIa	Activated Factor XIII

FXIII-A	FXIII A Subunit
FXIII-A_2B_2	Hetero-Tetrameric Zymogen of FXIII
FXIII-B	FXIII B Subunit
G	Relative Centrifugal Force
Gln (Q)	Glutamine
Glu (E)	Glutamate
Gly (G)	Glycine
GPIb-IX-V	Platelets Surface Receptor Glycoprotein Complex
GPVI	Platelets Immunoglobulin-like Receptor
H12	His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	High Fidelity
His (H)	Histidine
HRP	Horseradish Peroxidase
Hrs	Hours
Ht	Haematocrit
IAP	Integrin Associated Protein
ICAM-1	Intercellular Adhesion Molecule-1
ICAM-4	Integrin Cell Adhesion Molecule-4
IF-1	Anti-Fibrinogen Monoclonal Antibody, clone
Ig	Immunoglobulin
Ile (I)	Isoleucine
In	Inhibitor
ITS	Insulin Transferrin Sodium Selenite Supplement
KCl	Potassium Chloride
KDa	Kilo Dalton
LB	Luria-Bertani
LDS	Lithium Dodecyl Sulfate
L-His	L- Histidinol
LMWH	Low Molecular Weight Heparin
LN_2	Liquid Nitrogen
Lu	Lutheran
Lys (K)	Lysine
M	Molar

mAb	Monoclonal Antibodies
MES-hydrate	2-(N-Morpholino) Ethanesulfonic Acid Hydrate
MFI	Median Fluorescent Intensity
mg	Milligram
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Millilitres
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
MTB	Modified Tyrodes Buffer
Na_2HPO_4	Sodium Phosphate Dibasic
NaCl	Sodium Chloride
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NaH_2PO_4	Sodium Phosphate Monobasic
$NaHCO_3$	Sodium Carbonate
ng	Nanograms
nm	Nanometre
NO	Nitric Oxide
N-SDK	N-Terminal Disulfide Knot
N-terminal	Amino End
OD	Optical Density
OPD	1,2-Phenylenediamine Dihydrochloride
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PG1	Prostaglandin E1
PGE	Prostaglandins
PGH	Prostaglandin Endoperoxide
PGI2	Prostaglandin
Phe (F)	Phenylalanine
pMLP	Plasmid Major Late Promotor
PMSF	Phenylmethanesulfonyl fluoride
R	Reverse primer

RBC	Red blood cell
RGD	Arg-Gly-Asp
RGDC	Arg-Gly-Asp-Cys
RGDF	Arg-Gly-Asp-Phe
RGDS	Arg-Gly-Asp-Ser
rpm	Revolutions per minute
SDS-PAGE	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
sec	Second
Ser (S)	Serine
SLS	Scientific Laboratory Supplies Ltd
SSC	Side Light Scatter
TBS	Tris-Buffered Saline
TF	Tissue Factor
Tm	Melting Temperature
tPA	Tissue Plasminogen Activator
TxA2	Thromboxane A2
U	Enzyme Unit
UFH	Unfractionated Heparin
Val (V)	Valine
VCAM-1	Vascular Cell Adhesion Molecule-1
VTE	Venous Thromboembolism
vWF	von Willebrand Factor
WT	Wild Type
α	Alfa
α_2-PAI	α-2-Plasminogen Activator Inhibitor
α_2-PI	α-2-plasmin inhibitor
$\alpha_{IIb}\beta_3$	Alpha IIB Beta 3
β	Beta
γ	Gamma
εACA	ε-Aminocaproic acid-Aminohexanoic acid

Chapter 1 Introduction

1.1 Cardiovascular Disease and Thrombosis

Cardiovascular disease are one of the most common causes of morbidity and mortality worldwide. The tendency to develop cardiovascular disease is increased if the risk factors including obesity, diabetes, dyslipidaemia and hypertension are present from early childhood and adolescent life (Schwandt et al., 2009). The pathogenesis of cardiovascular disease is complex with intricate interactions between genetic, environmental and lifestyle factors. These factors affect the site and timing of onset, most seriously affected locations, type of initiation, in addition to the dynamics of progression of the disease and what type of cardiovascular outcome is produced (Messner and Bernhard, 2014). Complexity of cardiovascular disease is increased by the variation of the risk factors, type of cells, biological processes and physiochemical interactions that participate in the pathogenesis of the diseases (Messner and Bernhard, 2014).

1.1.1 Atherosclerosis

Atherosclerosis is a chronic systemic vascular disease, which is progressive and diffuse, involving the cerebro and cardiac-vasculature; heart and brain, as well as the peripheral circulation (Santos and Nasir, 2009). High mortality is associated with ischemic heart disease and cerebrovascular disease, which may be caused by acute thrombotic occlusions of the coronary or carotid arteries (Montecucco et al., 2009). Atherosclerosis and its complications, including the tendency to thrombosis, are caused by endothelial and vascular smooth muscles dysfunction (Creager et al., 2003). The disease is initiated by the accumulation of lipids, macrophages, foam cells, blood products, calcium deposits and carbohydrates within the sub-endothelial layer of the arteries' walls causing their thickening and rigidity (Zaman et al., 2000).

1.2 Thrombosis

Thrombosis is referred to as the pathological formation of clot (Gale, 2011). For the three main cardiovascular diseases; stroke, venous thromboembolism (VTE) and ischemic heart disease (acute coronary syndrome), thrombosis is the greatest common end-point of the disease (Raskob et al., 2014). Pulmonary

embolism (PE) is the main complication of deep vein thrombosis (DVT), when combined, they both are known as VTE (Budnik and Brill, 2018).

Differences between arterial and venous thrombosis are owed to the differences in blood flow between the two systems with higher shear rate for the arterial flow (Tangelder et al., 1988, Gale, 2011). Composition of arterial (platelets) and venous (fibrin and red blood cells) thrombi differ according to the different pathogenic mechanisms by which these two diseases occur. Although risk factors are different for arterial and venous thrombosis, some risk factors are common between both types such as obesity and high cholesterol (Gale, 2011). Hypercoagulability is a common pathogenic mechanism that is shared between arterial and venous thrombosis (Franchini and Mannucci, 2008). However, hypercoagulability is mainly associated as classical risk factor with venous thrombosis (Gale, 2011). A complex interplay between immune system, inflammation and lipids is more important for arterial than venous thrombosis (Previtali et al., 2011).

Wall shear stress is the haemodynamic force that is generated by the flowing blood (Resnick et al., 1993). Shear stress is a measure of the force that is required by a viscous liquid to produce a flow rate. For the arterial and venous circulations, this force is applied on the surfaces of different blood cells such as platelets and red blood cells (RBCs) and can cause alterations in clustering, exposure or structure of externally oriented constituents that are present in the cell membrane (Moake et al., 1988).

1.2.1 Arterial Thrombosis and Treatment

The arterial thrombus is primarily generated by platelet aggregation on ruptured atherosclerotic plaques (Gale, 2011), with little fibrin or red blood cells, which gives the thrombus a white appearance. Ischemic stroke, peripheral artery disease and myocardial infarction are atherothrombotic diseases, which are caused by arterial thrombosis (Franchini and Mannucci, 2008). Thrombus of patients with these arterial diseases in addition to abdominal aortic aneurism were found to demonstrate unfavourable fibrin clot structures, which are stiffer, more resistant to lysis, with shorter and thicker fibres, and containing smaller pores (Bridge et al., 2014, Collet et al., 2006). These clots are less permeable

and faster in fibrin polymerisation. The changes of clot structure were also found in the healthy first-degree relatives of the patients who are having altered clot structure, suggesting a genetic component to the alteration (Bridge et al., 2014, Mills et al., 2002). Arterial thrombosis may cause ischemic injuries. Thrombosis arises through platelet-mediated thrombi, which occurs after rupture or erosion of the atherosclerotic plaque. Stroke and cardiac ischaemia are considered as the two most severe outcomes of atherothrombosis (Previtali et al., 2011). Risk factors for arterial thrombosis include hyperlipidaemia, diabetes, hypertension, obesity and smoking (Franchini and Mannucci, 2008).

Aspirin (acetylsalicylic acid) is a well-known anti-platelet treatment of arterial thrombosis i.e. myocardial infarction and ischemic stroke (Patrono and Rocca, 2008, He et al., 2009). The inhibition of platelets occurs through acetylation of a specific amino acid in the active site of cyclooxygenase (COX); serine-530, even at very low doses of aspirin (30-50) μM (Roth and Majerus, 1975, He et al., 2009, Lucido et al., 2016). This inhibition of COX by acetylation reduces platelet aggregation potential by inhibiting thromboxane 2 irreversibly (TXA_2) (Ajjan et al., 2009). Another proposed mechanism by which aspirin is protective against arterial thrombosis is the inhibition of the production of thrombin, which might delay the growth of the clot in the narrowing artery (Szczeklik et al., 1992). Better fibrinolysis rate is thought to be another mechanism of the aspirin effect on thrombosis (Bjornsson et al., 1989). The latter two mechanisms may act on fibrinogen and prothrombin by the acetylation of their lysine residues (Williams et al., 1998). Aspirin was found to affect clot structure, formation and lysis, likely by acetylation of clotting factors such as fibrinogen, thrombin, tissue plasminogen activator (tPA) and factor XIII (FXIII) (Undas et al., 2007, Ajjan et al., 2009). Acetylation of fibrinogen by aspirin was studied by Ajjan et al *in vitro* using CHO cells expressing recombinant fibrinogen. The use of purified recombinant fibrinogen helped to eliminate the heterogeneity of the plasma-purified fibrinogen resulting from genetic factors and post-transitional modifications, as well as ensuring the absence of other clotting factors that are present in the *in vivo* environment (Ajjan et al., 2009). The authors found that clots formed by aspirin-treated recombinant fibrinogen had higher final turbidity and permeation coefficients reflecting thicker fibres and larger pores

respectively, which were confirmed by electron microscopy. The clots were found to have reduced rigidity and were less compact, which may lead to the shorter fibrinolysis time that was observed (Ajjan et al., 2009). The results of clot structure modulations were confirmed by aspirin-treated plasma-purified fibrinogen. This study identified the Aα-chain of fibrinogen as the site of acetylation on fibrinogen using an antibody against acetylated lysine residues (Ajjan et al., 2009).

Thienopyridine drugs ticlopidine and clopidogrel demonstrate antithrombotic effects by impairing platelet aggregation via inhibition of their response to adenosine diphosphate (ADP) (Mills et al., 1992). These drugs act specifically on ADP and on mediators that induce ADP secretion from the granules of the platelets such as thrombin. This specificity suggested that these drugs act on the ADP receptors on platelets instead of other receptors such as those for fibrinogen for example (Mills et al., 1992). Ticlopidine were approved as protective drugs against stroke in patients who are sensitive to aspirin. Similar to aspirin, Ticlopidine inhibits platelets response to ADP activation. (Mills et al., 1992). Anti $\alpha_{IIb}\beta_3$ integrin drugs including abciximab, eptifibatide and tirofiban, which are administered intravenously, are used for short term treatment of arterial thrombosis in combination with other treatment options (Cohen, 2009, Gale, 2011).

1.2.2 Venous Thrombosis and Treatment

The venous thrombus is primarily associated with fibrin- and red blood cell-rich clots (Gale, 2011). Venous thrombosis in contrast to the arterial thrombosis occurs on an apparently normal vein wall, where the shear rate and blood flow are low, forming a red blood cell-rich thrombus that is also called a red thrombus (Lowe, 2008). DVT PE, and VTE are the primary diseases caused by venous thrombosis (Franchini and Mannucci, 2008). Clots of patients of venous thrombosis have similarities to arterial clots in that they were denser and less permeable, with slower fibrinolysis compared to normal controls (Bridge et al., 2014, Undas et al., 2009b). However, interestingly, DVT clots showed slightly better properties of less dense, more permeable and faster fibrinolysis if the patients also had a PE. This may suggest that clot structure mechanism may

provide a mechanism for, or be a marker of the probability of formation of embolism in DVT patients (Bridge et al., 2014, Undas et al., 2009b).

After stroke and acute myocardial infarction, VTE is the most common thrombotic vascular disease. Pathogenesis of venous thrombosis is suggested to occur much more through mechanisms of a combination of hypercoagulability and stasis than through endothelial damage (Previtali et al., 2011). Risk factors for venous thrombosis include pregnancy, oestrogen use, cancer, immobilisation, surgery and fractures (Franchini and Mannucci, 2008).

Treatment of venous thrombosis is heparin associated with warfarin, the latter of which a is Vitamin K antagonist that is included in the treatment in the first 24 hours of the onset of thrombosis (Bauer, 2003). Heparin can be used in any of its two main forms; unfractionated heparin (UFH) and low molecular weight heparin (LMWH) (Dahlback, 2005). The production of LMWH is possible by chemical or enzymatic cleavage of UFH. LMWH is better in its pharmacokinetic properties, making laboratory monitoring unnecessary, and a single daily dose is enough to achieve haemostatic control. LMWH has longer half-life, better bioavailability and it is easier to administrate than UFH (Bauer, 2003, Dahlback, 2005). LMWHs produce less bleeding complications and immune-allergic thrombocytopenia than UFH so they are replacing many applications of UFH, although the latter is still in use (Bounameaux, 1998, Merli and Groce, 2010, Oduah et al., 2016). Reasons for this may include that UFH is less expensive than LMWH and studies are still ongoing regarding the efficacy and safety of the different types of heparin treatment (Bounameaux, 1998, Merli and Groce, 2010, Oduah et al., 2016). Heparin treatment can be stopped after a few days of concomitant treatment with warfarin, when the levels of functional vitamin K-dependent coagulation proteins reach the therapeutic levels of prothrombin time or International Normalised Ratio. Vitamin K antagonist can last for 3-6 months until the prothrombin time is fully back to normal (Dahlback, 2005, Bauer, 2003). Treatment of DVT is based on anticoagulants and vasoactive drugs to avoid the enlargement of the clot; this is in addition to physical procedures that modulate the flow of blood such as physical exercise, surgical thrombectomy, systemic thrombolysis and catheter directed thrombolysis (Luther et al., 2016, Hillegass et al., 2016, Scarvelis and Wells, 2006, Fleck et al., 2017).

1.2.3 Cancer

Thrombosis and coagulation abnormalities are strongly associated with malignant diseases; as evidenced by a high risk of thrombosis in patients with cancer (Falanga et al., 2015). The mechanism(s) by which cancer induces thrombosis involve expression of the tissue factor by tumour cells, in addition to the production of other prothrombotic molecules and complexes induced by the tumour such as microparticles, inflammatory cytokines tumour necrosis-α and interleukin 1-β, in addition to proangiogenic factors. Tumour cells also express adhesion molecules that bind to endothelial cells, platelets and leukocytes (Falanga et al., 2015).

Major defective haemostatic abnormalities occur in cancer patients including haemorrhage, coagulation and migratory thrombophlebitis. A study with fibrinogen-deficient mice indicated a direct role of fibrinogen in cancer pathophysiology and metastasis production (Palumbo et al., 2000). In the vasculature of cancer affected organs, tumor cell-associated emboli adhesion and survival are improved by fibrinogen. However, this does not include the growth of the metastasis, only its facilitation (Palumbo et al., 2000). On the other hand, fibrin is involved in the cancer metastasis through a variety of mechanisms including the stabilisation of tumour cells or their associated emboli adhesion to the vessel's wall, supporting tumour cells proliferation and migration in addition to the formation of stroma cells (Palumbo et al., 2000). Tumor embolism is a rare type of cancer spreading predominantly to the lung causing pulmonary embolism. This spreading is indicated by lymphatic, parenchymal and pleural metastasis. Embolism is difficult to be diagnosed because it is mostly sudden and causes death (Burchard and Carney, 1984, Laohachewin et al., 2014).

1.2.4 Haemostasis

Haemostasis involves a complex balance between coagulation and anticoagulation, which ensures normal blood flow while at the same time preventing excessive bleeding after injury (Guglietta and Rescigno, 2016). Blood is maintained in its fluid state by the anticoagulant luminal surface of the endothelium in the blood vessels, however, any damage of these vessels exposes the components of sub-endothelial matrix to the blood, activating the

haemostatic platelets and leading to fibrin formation, consequently initiating clot formation (Gale, 2011). Two main processes occur in physiologic conditions when an injury occurs to a blood vessel to stop bleeding (Guglietta and Rescigno, 2016). The first process is called primary haemostasis, which involves the aggregation of activated platelets to initiate the formation of a clot. The second process is called secondary haemostasis, which involves the activation of the coagulation cascade to form fibrin polymers (see below) (Guglietta and Rescigno, 2016). While this process of clot formation is important for physiologic control of blood loss, hyperactivity of both processes may lead to cardiovascular disease such as stroke and myocardial infarction by pathologically blocking blood vessels, which can lead to death (Renne et al., 2005).

1.2.5 Coagulation

Thrombin is generated by a series of plasma serine proteases that lead to the conversion of fibrinogen to fibrin as shown in Figure 1-1. Thrombin is also able to activate platelets (Renne et al., 2005). Thrombin is activated by the active form of Factor X (FX); through (FXa)-mediated processing of prothrombin into thrombin. FX itself is activated by two different mechanisms; called the intrinsic and extrinsic pathways (Budnik and Brill, 2018). The extrinsic pathway is triggered by the exposure of a transmembrane protein that presents in the vessel's sub-endothelial layer, but not the luminal surface of the endothelium and is called tissue factor (TF) (Budnik and Brill, 2018). TF is expressed on vascular cells surrounding the blood vessel, primarily fibroblasts, smooth muscle cells and other tissue cells. TF binds and activates FVIIa and the generated TF\FVIIa extrinsic tenase complex will activate FX into FXa, which in turn generates the conversion of prothrombin to thrombin as reviewed in (Butenas and Mann, 2002, Adams and Bird, 2009, Gailani and Broze, 1991). The generated thrombin itself undergoes amplification by activating FXI, FVIII (tenase complex) and FV (prothrombinase cpomlex) to form FXIa, FVIIIa and FVa respectively as reviewed in (Butenas and Mann, 2002, Adams and Bird, 2009, Gailani and Broze, 1991). The intrinsic pathway (blood-borne factors) is initiated by contact activation of FXII when the FXII zymogen interacts with negatively charged surfaces, which for example are provided by platelet phospholipids and released polyphosphates

in presence of calcium ions as reviewed in (Butenas and Mann, 2002, Adams and Bird, 2009). FXIIa in turn activates FXI to FXIa, which activates FIX. FIXa activates FX to convert prothrombin (FII) into thrombin (FIIa) in a higher amount than that produced by the extrinsic pathway by itself as reviewed in (Budnik and Brill, 2018, Renne et al., 2005, Butenas and Mann, 2002, Gailani and Broze, 1991). In summary, FX is activated by both intrinsic and extrinsic pathways via a series of enzymatic transformation steps (cascade), ending in the conversion of prothrombin to thrombin through several positive feedback loops, which converts fibrinogen to fibrin that is subsequently stabilised by FXIIIa-dependent crosslinking as reviewed in (Budnik and Brill, 2018, Renne et al., 2005, Adams and Bird, 2009).

The tenase and prothrombinase complexes both consist of the serine proteases, which are vitamin K-dependent coagulation factors, such as FII, FVII, FIX, and FX, in complex with cofactors VIIIa and Va respectively (Butenas and Mann, 2002, Adams and Bird, 2009). Vitamin K-dependent factors are inhibited by the vitamin antagonists that play a role as anticoagulants such as warfarin (Butenas and Mann, 2002, Adams and Bird, 2009).

Protein C is a blood coagulation protein inhibitor that also is a vitamin K-dependent. When activated, it proteolytically cleaves and inactivates procoagulant cofactors Va (FVa) and VIIIa (FVIIIa). The protein is called activated protein C in this form (APC) (Rosendaal et al., 1995). A mutation, which affects the activity of APC was found in the cleavage site of the protein on the factor V (Factor V Leiden) which is required for the activity of the APC and thus results in its resistance (Bertina et al., 1994, Rosendaal et al., 1995). The risk of deep vein thrombosis is higher in homozygous factor V Leiden mutated individuals than heterozygous individuals (Rosendaal et al., 1995).

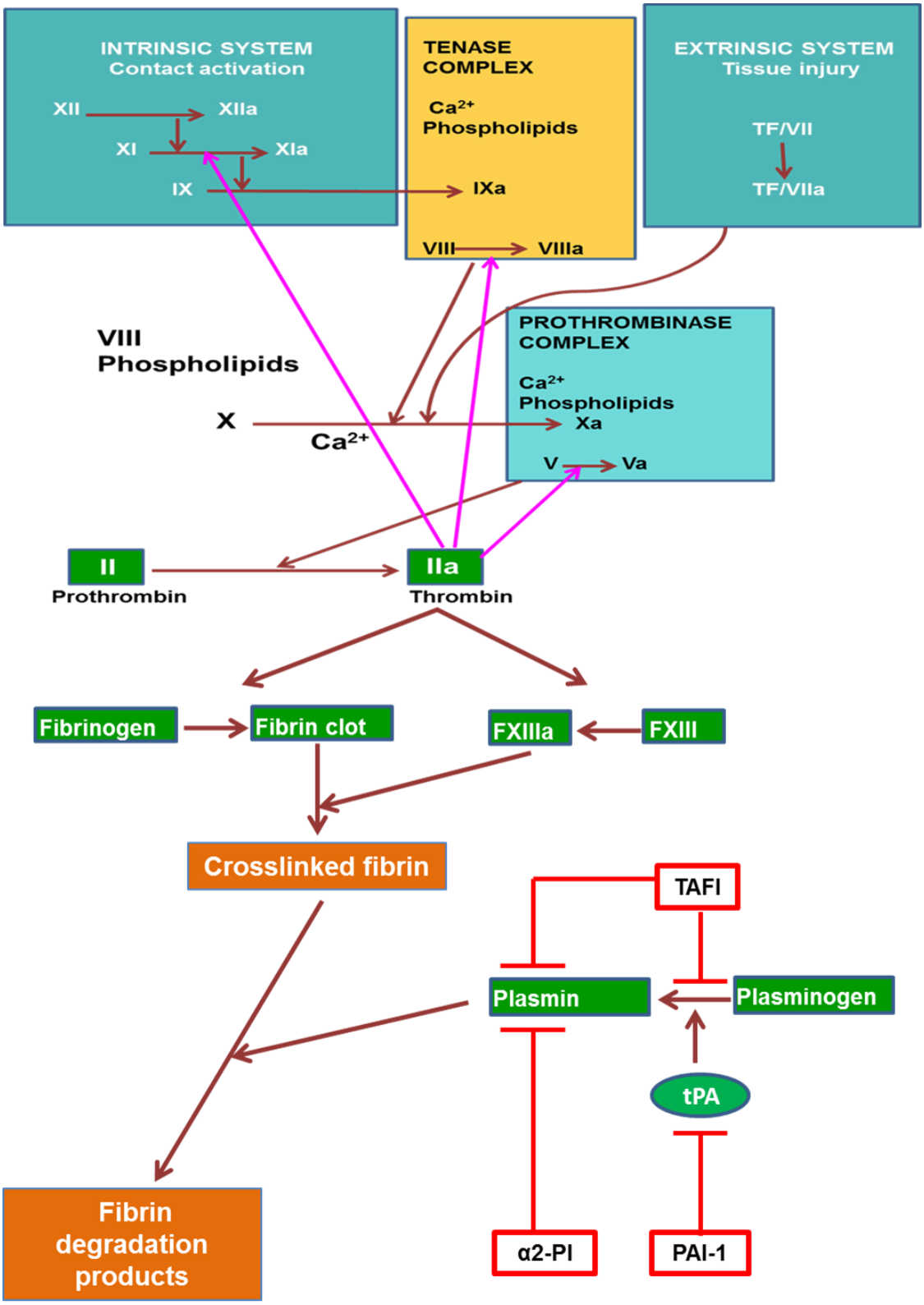


Figure 1-1 Schematic representation of the classical coagulation cascade. Generation of thrombin by the extrinsic and intrinsic pathways through the enzyme prothrombinase complex. Adapted from (Ajjan and Grant, 2006, Ajjan and Ariens, 2009).

1.2.6 Platelets

Platelets are synthesized in the bone marrow from megakaryocytes as anucleated cells, and function to assess the integrity of the vascular system while they are circulating (Ruggeri, 2002). They are the smallest cells in the body with an average diameter of 2-5 µm, 0.5 µm thickness and a mean cell volume of 10 femtoliter. Platelets are the second most abundant cell in the blood (after red blood cells) with a blood count of 150-450 10^9 platelets/L (Gremmel et al., 2016). Platelets circulate close to the endothelium at a rate of 85 million/ day and survive for 7-10 days (Walsh et al., 2015, Smyth, 2010). Platelets have the ability to distinguish between lesion areas that may progress to thrombosis and normal endothelial lining of the cells (Ruggeri, 2002). This function of platelets is called haemostasis in physiological conditions, but it can be a cause of thrombotic obstruction in diseased arteries. This may lead to tissue damage as a consequence of blockade of the blood flow (Ruggeri, 2002). The tendency of lesions to progress to thrombosis relies on factors that cause platelet activation such as exposure of collagen after rupture of unstable atherosclerotic plaques, coronary angioplasty causing altered vascular surfaces, inflammatory responses causing local endothelial cell dysfunction, and high shear stress in addition to genetic variations in the proteins that involved in the adhesion (Ruggeri, 2002).

Besides their well-known role in haemostasis, platelets also involved in other physiological functions such as immunity, inflammation and angiogenesis (Senis et al., 2009). Platelet aggregation is known to be the first mechanism involved in haemostasis (Du et al., 2014). The process starts with adhesion of platelets that are exposed to rapid blood flow by the help of the adhesive protein von Willebrand Factor (vWF) that immobilises on collagen (Ruggeri, 2003). Next, the platelet aggregate forms by the recruitment of other platelets to bind to the already adhered platelets resulting in the formation of a seal covering the wound (Du et al., 2014). A cross-linked fibrin network then forms around the aggregate plug resulting in its stabilisation. The adhesion and aggregation processes induce changes in the shape of the platelets causing clot retraction to occur at the site of injury (Du et al., 2014).

To keep platelets in a resting state in the healthy vasculature, inhibitors such as nitric oxide (NO) and prostaglandin (PGI_2) are released by the intact endothelium. An adequate balance of the way that platelets respond to these inhibitors is responsible to maintain the healthy state because any alteration in this balance would lead either to bleeding or pro-thrombotic phase (Atkinson et al., 2018).

At the site of injury, platelets adhere and aggregate to form a plug in the presence of a multimeric plasma protein; vWF, which is required for this process especially in arterial haemostasis and thrombosis under high shear stress. This clustering is through two membrane-surface receptor glycoproteins; GPIb-IX-V and GPVI complexes expressed by the platelets initiating their aggregation (Andrews et al., 2004, Chow et al., 1992, Moake et al., 1988). The interaction between platelet GPIb-IX-V and collagen is reversible and requires additional interaction between the platelets and extracellular matrix (ECM) through integrin receptors $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ to stabilise the platelet plug on the ECM (Nieswandt and Watson, 2003). Another glycoprotein receptor is immunoglobulin (Ig) receptor GPVI, which binds to collagen ensuring activation of platelets under high shear by inducing the release of ADP, thromboxane A_2 (TxA_2) and thrombin generation (Nieswandt and Watson, 2003). vWF is synthesised in the megakaryocytes and stored in platelet α-granules and also synthesised by and stored in endothelial cells (Moake et al., 1986).

Platelet involvement in clot retraction, aggregation and adhesion requires the presence of the $\alpha_{IIb}\beta_3$ surface receptor, which is found in high copy numbers on the platelet membrane compared to other receptors (Senis et al., 2014). Stimulation of the $\alpha_{IIb}\beta_3$ by inside-out signals by the released ADP from damaged RBCs and blood vessels or from activated platelet dense granules induces conformational changes in the integrin increasing its affinity to bind to ligands such as fibrinogen (Senis et al., 2014, Hollopeter et al., 2001, Gremmel et al., 2016). Binding of the integrin to fibrinogen forms intermediates that stimulate an outside-in signaling to induce platelet activation, aggregation, secretion, spreading and clot retraction (Senis et al., 2014, Hollopeter et al., 2001).

Platelet involvement in clot formation includes two separate steps, adhesion and aggregation as shown in Figure 1-2 (Ruggeri, 2000). Initial platelet cell contact (tethering) with the extravascular ECM and sub-endothelial constituents provides a substrate for the adhesion, which requires interaction of collagen or ECM to plasma vWF. The immobilised vWF then binds to glycoprotein receptors including $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ in synergetic action (Savage et al., 1998, Ruggeri, 2000). In summary, in flowing blood, platelets need to attach to substrate surfaces to immobilise them; this mechanism is applied to both adhesion and aggregation. Collagen, sub-endothelial matrix and ECM components play this role in adhesion by forming a substrate for the initial tethering of platelets, which is mediated by vWF and GPIbα. Previously adhered platelets play a substrate role for other platelets to join and adhere in aggregation, which is mediated by fibrinogen and $\alpha_{IIb}\beta_3$ (Ruggeri, 2000, Ruggeri et al., 1999, Savage et al., 1998).

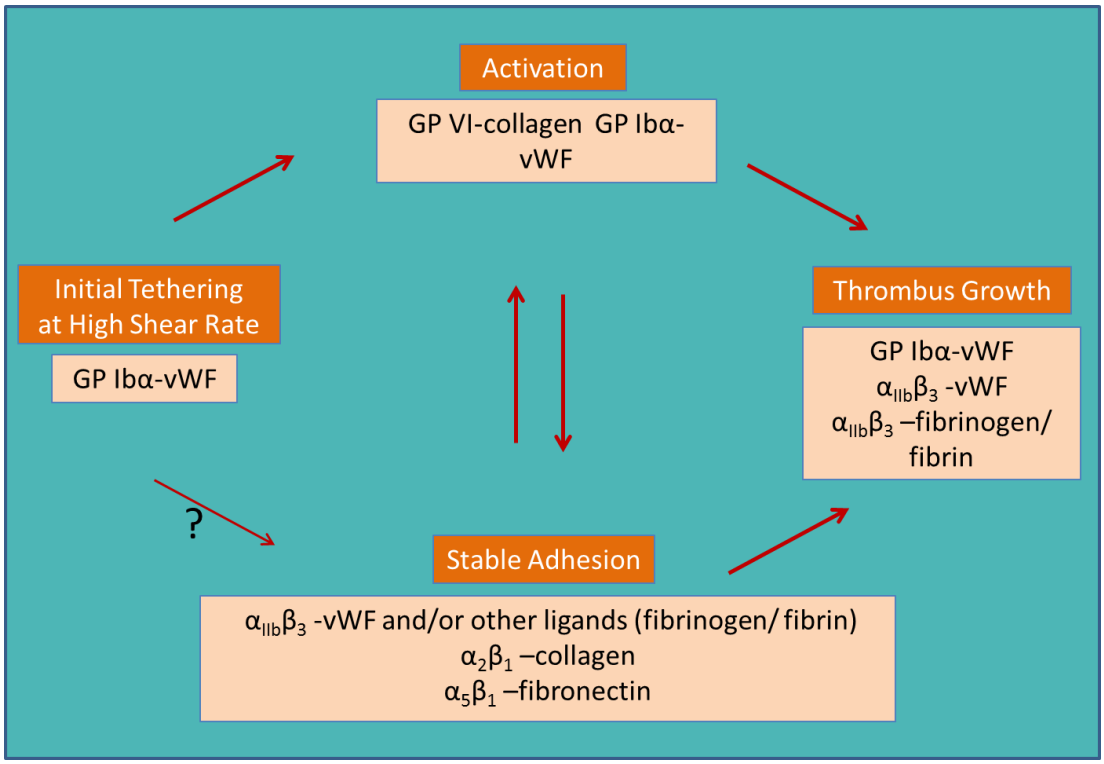


Figure 1-2 Schematic representation of the involvement of platelet adhesion and aggregation in thrombus formation. Only identified adhesive interactions are considered in the figure with no exclusion of other ligand-receptor pairs of antagonists involved in platelets activation and thrombus formation. Starting from the left, platelets tethering to initiate a reactive surface and stable adhesion may occur at low shear rate independently of the initiation. When thrombus formation occurs at high shear rate, the balance between stable adhesion and activation usually shifts to the activation. Adapted from (Ruggeri, 2000) and modified from (Savage et al., 1998).

Platelets have structures that help to distinguish them from other cells comprising a dense tubular system that is the smooth endoplasmic reticulum, an open canalicular system in addition to their secretory granules (Westmoreland et al., 2016). These granules are further classified into different types based on their appearance under electron microscope; dense granules containing ADP and serotonin, α-granules containing proteins and enzymes, and multi-vascular bodies and lysosomes (Westmoreland et al., 2016, Frojmovic and Milton, 1982). Integrity of these granules is very important for the role of platelets in haemostasis, because they release the bioactive molecules that are responsible for platelet function (Westmoreland et al., 2016). The α-granules play a role in platelets by storing proteins such as fibrinogen (synthesised outside the platelet) and vWF (synthesised inside the platelet) (Heijnen et al., 1998).

Platelets are activated by a variety of immobilised and soluble agonists at the site of the injury leading to recruitment of more platelets as a response of positive feedback mechanisms that strengthen the activation signals. The response to this activation induces granule secretion, platelet aggregation and shape changes (Li et al., 2003). Prostaglandins (PGE) have several physiological roles such as modulation of hormonal, neuro-hormonal and other stimuli in addition to their role as a pro-inflammatory response generator (Ricciotti and FitzGerald, 2011, Samuelsson et al., 1978). PGEs are synthesised in most mammalian tissues by unsaturated fatty acid; arachidonic acid via prostaglandin synthase, which is composed of two enzymes. The first is prostaglandin endoperoxide synthase, which converts arachidonic acid to prostaglandin endoperoxide (PGH). The second is prostaglandin endoperoxide E isomerase which converts PGH to PGE (Samuelsson et al., 1978). Thromboxane is a factor that induces platelet aggregation, which is derived from arachidonic acid and is an unstable intermediate agonist. Thromboxane plays a physiological role in haemostasis and a pathophysiological role in thromboembolic disease (Samuelsson et al., 1978, Hamberg et al., 1975). ADP is stored at high concentration in platelet dense granules and plays a role as platelet activation agonist. ADP is secreted by granules when platelets are stimulated (Koessler et al., 2018). ADP plays its role through three purinergic receptors, namely P2Y1, P2Y12 and P2X1. The first and last receptor stimulations are required for initiation of the platelet

aggregation, however; P2Y12 is supporting the action of P2Y1 by ensuring rapid calcium influx required for platelet aggregation (Koessler et al., 2018). Prostaglandin and thromboxane are able to expose platelet fibrinogen receptors when they are stimulated with ADP, further supporting platelet aggregation (Bennett et al., 1981).

1.2.6.1 Integrins

Cell adhesion molecules were first recognized by their ability to allow cell adherence to the surrounding ECM or allow cell-to-cell adhesion (Murray et al., 1999, Lam et al., 1987). Integrins were later found to allow interaction and communication of cells with the environment and with each other (Murray et al., 1999). Therefore, adhesion receptors are able to regulate a variety of cell functions such as gene expression, proliferation, migration, differentiation and apoptosis. This may include recognition of the cell surface receptor to specific regions in specific adhesive proteins (Lam et al., 1987, Murray et al., 1999). These cell receptors are composed of different groups of surface adhesion receptors, which may represent at least five families including adhesion molecules belonging to the immunoglobulin superfamily, integrins, CD44 family, selectins, and the cadherins (Hynes, 1987, Murray et al., 1999). The physiological and pathological importance of integrins is acutely apparent when they are affected by mutations resulting in defects of their function. Integrins and their adhesive ligands are involved in immune response, development, haemostasis and leukocyte trafficking (Hynes, 2002). Integrins can be used as a therapeutic target for many diseases such as thrombosis, cancer and inflammation by interfering with their function using antibodies or peptides (Hynes, 1992). Integrins are capable of recognizing a number of ECM ligands including fibrinogen, fibronectin, collagen, vWF and vitronectin by the recognition of the RGD sequences on these ligands (Cheresh, 1987).

Structure of Integrins

The integrin family of cell adhesion molecules consists of αβ heterodimers that are assembled into 24 different integrins formed by a non-covalent combination of different numbers and organisation of the two subunits (Hynes, 1992, Hynes, 2002). There are 8 β subunits of approximately 90-110 kDa molecular weight and 18 α subunits of approximately 120-180 kDa molecular weight. Integrins mainly recognise short peptide sequences of acidic amino acids (Hynes, 1992, Hynes, 2002). The α subunits of the RGD sequence's receptors are composed of α_{IIb}, α_V, α_5 and α_8 that form heterodimers with β_1 and β_3 subunits in different combinations and are able to recognise the RGD sequence in the ligands that are present in the ECM (Anderson et al., 2014). Each subunit consists of a large extra cellular domain in the N-terminal region, followed by a single-pass transmembrane section, and a short (most frequently) cytoplasmic domain at the C-terminal end of the protein (Zhu et al., 2008). Both subunits are formed by a ligand binding head connected to the transmembrane domain by "legs" that emerge from each subunit. The "legs" are divided into two levels; an upper and a lower level, which are connected by extremely bendy "knees" (Zhu et al., 2008). Integrins can be regulated either by variation in their expression, or by bidirectional signalling that includes inside-out signalling and outside-in signalling. The former includes conformational changes in the heterodimer that are induced by internal signals affecting the cytoplasmic tail of the integrin. This signal influences the affinity of the integrin for its ECM ligands [reviewed in (Anderson et al., 2014)]. In contrast, during outside-in signalling, the conformational changes are first induced by the binding of the integrin to the ECM ligand, after which they cluster to form large intracellular adhesion complexes [reviewed in (Anderson et al., 2014)] .

Integrin $\alpha_{IIb}\beta_3$ is a classic specific integrin that is important to control bleeding at the site of vascular wounds, in addition to its pathological role in thrombosis that may cause cardiovascular diseases. Glanzmann thrombasthenia is a genetic bleeding disorder that is caused by mutations in either subunit of $\alpha_{IIb}\beta_3$ and can be treated by inhibiting the integrin's binding ligands (Gkourogianni et al., 2018).

The specificity of the integrins vary, some receptors are specific for a single ligand whereas others are able to share more than one ligand (Pierschbacher and Ruoslahti, 1987). Using synthetic peptides, Pytela *et al*. suggested the presence of a receptor in platelets that can recognise the RGD sequence in fibrinogen, fibronectin and vitronectin and this receptor is $\alpha_{IIb}\beta_3$ (Pytela et al., 1986). However, these authors reported that this receptor is different from the fibroblast receptor which is capable to recognise the RGD sequence on fibronectin and vitronectin only, but not fibrinogen (Pytela et al., 1986). They also suggested that fibrinogen, fibronectin and vWF share the same binding site on platelets but not with vitronectin (Pytela et al., 1986).

1.3 Fibrinogen

Fibrinogen is a large dimeric protein that plays a vital role in haemostasis (Alexander et al., 2011, Lovely et al., 2011). Fibrinogen is synthesised by hepatocytes (Ritchie and Fuller, 1983) and assembled in the endoplasmic reticulum and Golgi organelles (Kudryk et al., 1982, Weisel and Litvinov, 2017). Fibrinogen is a 340 kDa glycoprotein, with a multitude of heterogeneity due to proteolytic degradation, alternative splicing, and post-translational modifications (Herrick et al., 1999). It is composed of three chains that are encoded individually by distinct genes each present on chromosome 4; the genes are transcribed in opposite directions and oriented in tandem. Aα (6 exons) and γ (10 exons) genes are transcribed in one direction, whereas the Bβ (8 exons) gene is transcribed in the opposite direction (Herrick et al., 1999). Fibrinogen is composed of a dimer in which the three polypeptide chains Aα, Bβ and γ comprise one half of the molecule and are joined together by disulfide bonds (Alexander et al., 2011, Lovely et al., 2011).

Fibrinogen polymerises to fibrin by the action of thrombin to form a meshwork like structure. During its growth, the clot is stabilised by crosslinking of fibrin fibres by the action of FXIIIa (Alexander et al., 2011, Lovely et al., 2011). Fibrinogen is suggested to be a risk factor for cardiovascular disease through a number of mechanisms, including its involvement in platelet aggregation by binding activated platelets via the $\alpha_{IIb}\beta_3$ receptor integrin. Other contributing factors

include the role of fibrinogen in plasma viscosity, its role in increasing fibrin formation as fibrinogen increases, and its inflammatory role as acute-phase protein (Stec et al., 2000). Plasma fibrin clot's viscoelastic properties and morphological properties (fibre length, diameter and porosity) were measured in premature CAD compared to healthy controls and were found to be stiffer and more resistance to fibrinolysis, in addition to being shorter with a higher number of fibres compared to controls (Collet et al., 2006).

The structural and functional properties of proteins can be determined by post-transitional modifications including phosphorylation, glycosylation, lipidation and oxidation (Wani et al., 2015). Identification of post-transitional amino acid modifications has become more widely available through advanced protein characterisation and sequencing. Moreover, post-transitional modifications of proteins are found to have a vital role in blood coagulation and anticoagulant systems (Hansson and Stenflo, 2005). Fibrinogen is susceptible to oxidative modification that accounts for about 20 times of the level of oxidative modification of albumin; the most abundant plasma protein. This high level of oxidation can be caused by the presence of calcium binding sites, which provide targets for iron binding oxidation in addition to the carbohydrate part of the molecule (Shacter et al., 1994, Gorobets et al., 2018). These oxidative modifications are able to alter fibrinogen functional properties such as lateral aggregation, thrombin-induced clot formation, platelet aggregation and fibrinolysis (Shacter et al., 1994, Gorobets et al., 2018).

1.3.1 Structure of Fibrinogen

Human fibrinogen is present as several variants with molecular weight ranging between 270-420 kDa as a result of degradation and splicing, however the most abundant type is the 340 kDa glycoprotein (Boehm et al., 2010). Fibrinogen's plasma concentrations range between 1-4 mg/ml in the healthy state, but increase up to 8 mg/ml in acute inflammation (Boehm et al., 2010). As mentioned above, fibrinogen is formed by three polypeptide chains comprising three non-identical chains arranged in two identical halves $(A\alpha, B\beta, \gamma)_2$ forming a dimer with each of the polypeptide chains comprising 610, 461 and 411 amino acid residues respectively (Henschen et al., 1983).

1.3.1.1 Amino acid structure

The three chains have different molecular weights, which are 67 kDa for Aα, 56 kDa for Bβ and 47 kDa for the γ chain. The Bβ and γ chains are connected to carbohydrate side chains (glycosylated) through N-glycosidic bonds at position Bβ-Asn364 and γ-Asn52, making them glycoproteins (Zhang and Redman, 1992, Brennan, 2015, Henschen et al., 1983). These carbohydrate side-chains are attached to asparagine residue in a sequence that presents only once in these two chains; asparagine-X-threonine or asparagine-X–serine (Henschen et al., 1983). However, this sequence is also found twice in the Aα-chain at position 296 and 400; asparagine-proline-serine and asparagine-valine-serine-proline. In spite of this presence of serine according to the sequence asparagine-X-serine in the Aα-chain, both positions contain a proline residue that is neighbouring the sequence. This presence of proline may be the reason of the absence of the carbohydrate side-chain from the Aα-chain, because there is no presence of an O-glycosidic bond in human fibrinogen (Henschen et al., 1983). The carbohydrate side-chain is composed of a number of sugars that bind to the amino acid via N-glycosidic bonds, including galactose, mannose, sialic acid and N-acetylglucosamine (Henschen et al., 1983). The amino-termini (N-termini) of the fibrinogen chains are grouped together in a dimeric structure in the centre of the molecule forming the E-region (50 kDa) where two coiled-coil structures emerge and extend towards two distal D-regions (80 kDa) that contain the carboxylic ends (C-termini) of the chains (Mutch et al., 2010, Zhang and Redman, 1992, Doolittle et al., 1977). The molecule looks under the electron microscope as a tri-nodular structure; the E-region and two D-regions connected together by the coiled-coil structure (Huang et al., 1993a, Zhang and Redman, 1992). This coiled-coil is a triple helix cord-like structure, made up of about 111 amino acids, which are enclosed by two cysteines (Cys-X-X-X-Cys) at each end. The tri-nodular domains are hydrophobic and joined by hydrogen bonding (Huang et al., 1993a, Zhang and Redman, 1992). The coiled-coil is formed by supercoiling of each one of the three chains via six disulfide bonds to produce α-helix-like structure (Fuss et al., 2001). The supercoil structure of each chain is a result of six disulfide bonds that also participate in the attachment of these chains to the central and lateral domains forming disulfide rings. The internal part of the

coiled-coil is hydrophobic whereas the external is polar according to the amino acid orientation (Fuss et al., 2001). Each Aα-chain extends, emerges and folds back from the corresponding D-region to form an αC-region consisting of Aα-chain amino acid residues 221-610 divided into two parts; one of them is a flexible αC-connector (amino acid residues 221-391), and the other is a compact αC-domain (amino acid residues 392-610) (Tsurupa et al., 2011). The structure of the fibrinogen molecule is shown in Figure 1-3.

1.3.1.2 Disulfide structure

The two halves of the fibrinogen molecules are joined together by three disulfide bonds that are symmetrical between the adjacent same chains at the cysteine (Cys) residues, two of them are between the adjacent γ-chains at positions 8 and 9 for each chain while the third is between the adjacent Aα at position 28 for each chain (Zhang and Redman, 1992). The N-terminal disulfide knot (N-SDK) is a 58 kDa fibrinogen fragment dimer that is formed by cyanogen bromide cleavage of fibrinogen and composed of the three N-terminal fragments of the fibrinogen chains. It contains 11 disulfide bonds out of the 29 disulfide bridges that are found in human fibrinogen to hold the two halves of the molecule together (Blomback, 1996, Herrick et al., 1999). Most of the other inter-chain disulfide bonds, which are not in the N-SDK are found in the C-terminal core fragment that is called fragment-D knot (Blomback, 1996).

The relationships between the structure, function and evolution of fibrinogen can be determined by studying the genetic amino acid sequences of the three chains of fibrinogen. Since a single change in one amino acid can result in functional abnormalities, this elucidates the relationships between a structural error and its (dys)functional effect (Henschen et al., 1983).

1.3.1.3 Calcium binding sites

Fibrinogen has both high affinity binding sites for calcium as well as low affinity sites, the latter of which are non-specific and originate from weak interactions associated with the net charge of fibrinogen (Marguerie et al., 1977). Polymerisation and crosslinking of fibrin is a calcium-dependent process (Yee et al., 1997).

When fibrinogen is exposed to heat, this causes prolongation of thrombin clotting time. The elongation can be overcome by binding of fibrinogen to calcium, which protects fibrinogen from heat denaturation (Ly and Godal, 1972). Calcium ions were found to protect fibrinogen fragment D against further attack and degradation by plasmin (Haverkate and Timan, 1977).

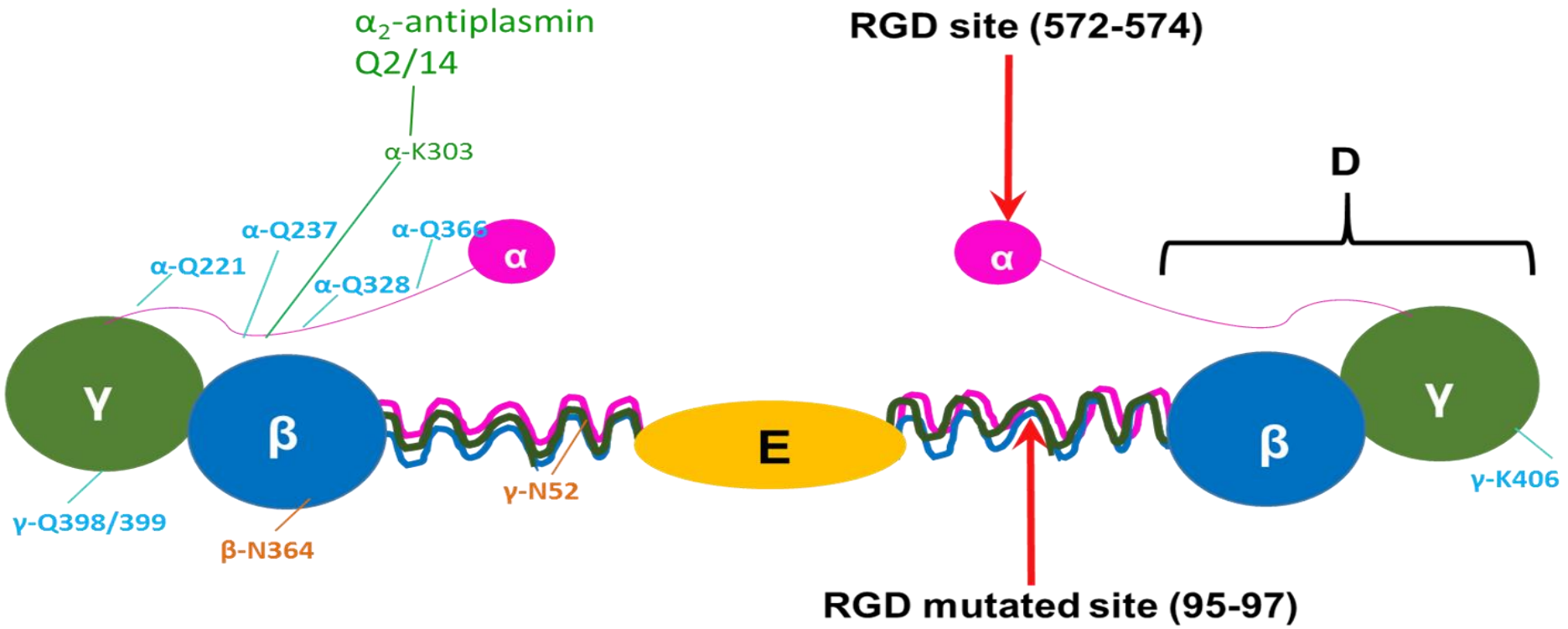


Figure 1-3 Schematic representation of fibrinogen structure. E-region (yellow) which contains the N-termini, two D-regions which contain the carboxyl-termini of Bβ-chain (**dark blue**) and γ-chain (**green**) and two αC domains which contain the carboxyl-termini of the α-chain that contains RGD (572-574). In the middle is the coiled-coil rope-like structure, which contains the RGD (95-97) sequence, which is of this project's interest. In **blue**: FXIII binding sites, **green** α-2antiplasmin binding sites, **orange** glycosylation sites. Adapted from (Undas and Ariens, 2011).

1.3.1.4 Gamma Chain Splice Variation

In fibrinogen, two different species of γ-chain are found which separate according to their electrophoretic properties; the first one is the original γ-chain, which is also named γA. The second is a natural occurring alternative splicing form of γ-chain that is called γ'-chain or γB. The difference in size and charge between the two chains is due to amino acid sequence differences that are present at the C-terminal of each chain (Wolfenstein-Todel and Mosesson, 1980, Fornace et al., 1984). Whereas γ' termini are larger, it has a leucine end in comparison to the valine end of the γA-chain (Wolfenstein-Todel and Mosesson, 1980). Fibrinogen γ' is produced by alternative splicing of the last intron of the γ-chain messenger (m) RNA. A unique amino acid sequence is produced for the γ'-chain that is highly negatively charged containing seven anionic residues, rich in glutamic and aspartic acids in addition to two sulfated tyrosines. (Fornace et al., 1984, Wolfenstein-Todel and Mosesson, 1981, Lord, 2007). These amino acid residues provide the γ'-chain with its electrophoretic heterogeneity from the γ-chain (Wolfenstein-Todel and Mosesson, 1981, Mosesson et al., 1972). It was found that γ'-chain accounts for around 11% of the total fibrinogen (Mosesson et al., 1972, Chung and Davie, 1984). Formation of this chain leads to the substitution of the last four amino acids (408-411) in the original γ-chain, which are ^{408}AGDV^{411}. This 4 amino acids sequence is replaced by a 20 amino acids sequence; ^{408}VRPEHPAETEYDSLYPEDDL^{427} in the γ'-chain. (Chung and Davie, 1984, Sabo et al., 2006). According to Lord 2007, approximately 10% of fibrinogen are heterodimers; each composed of a single γ-chain in one D-region and a single γ'-chain in the other D-region (Lord, 2007, Cooper et al., 2003). However, the lower the presence of γ'-chain and its ratio to total fibrinogen, the higher the risk of arterial thrombotic diseases. Because γ'-chain has thrombin binding sites, Lord had suggested that when thrombin binding sites to fibrin clot reduced, the risk of thrombosis increased (Lord, 2007). However, the same has been reported for venous thromboembolic disease in which a lower ratio of γ'-chain to total fibrinogen may increase the risk of DVT or thrombotic microangiopathy (Uitte de Willige et al., 2005, Mosesson et al., 2007). The controversy continued with Farrel 2014 who has demonstrated that γ'-chain is

related to arterial thrombosis, but not to venous thrombosis and discussed the controversial findings of this chain being thrombotic or antithrombotic (Farrell, 2014). The fibrinogen γ'-chain lacks the binding site that is essential for platelet adhesion and aggregation, it is crosslinked faster with FXIII producing clots that highly resistance to fibrinolysis and have an altered architecture (Lovely et al., 2007, Lovely et al., 2010). During clot structure functional analysis, turbidity and fibrinopeptides release showed a strong difference in the early polymerisation between the heterodimer γA/γ' and the normal variant γA/γA that led to altered fibrin structure. Clots formed by γA/γ' were found to have thinner fibres which are highly thrombotic, contained smaller pores and high branching (Cooper et al., 2003). It was found that both thrombin and γ' affect the protofibril packing. High concentrations of thrombin decrease protofibril packing but γA/γ' give lower protofibril packing than γA/γA at low concentrations of thrombin. Decreased protofibril number packed per fibre was found to decrease clot stiffness and alter clot structure (Domingues et al., 2016). There are some suggestions that the mRNA of the γ-chain is synthesised in the liver, bone marrow, lung and brain whereas the alternative splicing form of fibrinogen's γ-chain mRNA is synthesised in the liver only (Courtney et al., 1991, Haidaris and Courtney, 1990).

1.3.1.5 Alpha chain

The Aα-chain is the only non-glycosylated polypeptide chain of fibrinogen. It is the largest of the three chains and consists of 610 amino acids, with a molecular weight of 67 kDa (Doolittle et al., 1979, Zhang and Redman, 1992, Watt et al., 1979). Two thirds of the carboxyl ends of each half of the Aα-chain are known as αC-regions that extend and fold back from the coiled-coil fragments towards the central E-region. FXIII crosslinking sites are found in this region together with some plasmin cleavage sites (Rudchenko et al., 1996).

The fibrin(ogen) Aα-chain is heavily cross-linked by activated FXIII. Interactions between the αC region with the zymogen FXIII-A_2B_2 and the activated FXIII-A have been identified using truncations of recombinant αC region (233-425) of fibrinogen (Smith et al., 2011). A novel, high affinity interaction site for FXIII was identified within the αC region (371-425). FXIII-A was activated by thrombin and calcium, and a calcium dependent low affinity interaction of the activated FXIII-A

with αC region was also found (Smith et al., 2011). The binding site has been further studied and was further identified as a 14 amino acid peptide (Pep1) on the αC region (389-402) by chemical cross-linking derivatives of this site with recombinant activated FXIII-A_2 (Smith et al., 2013). Three individual lysine residues (Lys446, Lys275, Lys113) in the β sandwich of activated FXIII-A_2 were found that interact with Pep1 on the αC region, identifying residues 389-402 on the fibrinogen Aα-chain as a major site for FXIII substrate recognition (Smith et al., 2013). Finally, a central residue in the αC region that is involved in the interaction with FXIII-A_2 was identified as Glu396 (Smith et al., 2011, Smith et al., 2013).

The Aα-chain contains two RGD sequences, which provide the interaction sites of fibrinogen with integrins present on cells such as platelets and endothelial cells. One of the RGDs is close to the αC region in the C-terminal at amino acid residues 572-574 while the other is located towards the N-terminal at amino acid residues 95-97 (Cheresh et al., 1989a).

RGD Sequence

Surface integrin receptors on different types of cells such as platelets (membrane glycoprotein $\alpha_{IIb}\beta_3$ receptor, also known as GPIIbIIIa receptor complex) (Shattil et al., 1994), endothelial cells (endothelial cell adhesion receptor complex) (Cheresh, 1987) and human melanoma cells ($\alpha_5\beta_3$ receptor) (Felding-Habermann et al., 1992) can recognise fibrinogen through three different sites. Two of these sites are located on the Aα-chain, consist of the same three amino acids short sequence and can be differentiated from each other by a forth amino acid following them (Gailit et al., 1997). The first is RGDF (Arg-Gly-Asp-Phe), which is present in the coiled-coil region towards the N-terminal of the Aα-chain (Aα-95-98). The second is RGDS (Arg-Gly-Asp-Ser), which is present in the αC region towards the C-terminal of the Aα-chain (Aα-572-575) (Gailit et al., 1997, Doolittle et al., 1979). The third site is HHLGGAKQAGDV (His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val) (H_{12}), which is also known as dodecapeptide and is present at the C-terminal of the γA-chain (400-411) (Kloczewiak et al., 1984, Gailit et al., 1997, Lam et al., 1987).

Before the discovery of the integrin-binding RGD sequence, Pierschbacher *et al*, 1983 suggested the presence of specific binding sites on each of the two polypeptide chains of fibronectin responsible for its interaction with cells. The authors designed four synthetic peptides, each containing 29-30 amino acids as a part of the expected fragment that is involved in the cell interaction (Pierschbacher et al., 1983). A peptide that was composed of 30 amino acids and called peptide IV was found in the C-terminal of fibronectin that was responsible for the attachment of the original fragment to the cells (Pierschbacher et al., 1983). Next, Pierschbacher and Ruoslahti, 1984, produced a number of synthetic peptides with different sizes and compositions originating from peptide IV then tested their attachment to proteins coated to a plastic surface (Pierschbacher and Ruoslahti, 1984). It was found that all the synthetic peptide fragments of different lengths that bound to the fibroblast contained a tetra-peptide sequence of Arg-Gly-Asp-Cys (RGDC). The authors also showed that the effect of the peptides was decreased with the increase in size, which may cause a reduction in accessibility of the tetra-peptide to the coated proteins, or instability of its conformation (Pierschbacher and Ruoslahti, 1984).

A number of proteins including fibrinogen, fibronectin, vWF, vitronectin, thrombospondin, osteopontin and collagens (under some conditions) contain RGD sequences that are involved in their surface adhesion and interaction with different cells via specific integrins (Ruoslahti, 1996). Nevertheless, not all proteins that contain RGD sequences are necessarily involved in cell attachment, this might be because the environment of the surrounding amino acids of the RGD is not compatible with the cell integrin binding or the sequence might not be exposed on the surface of the protein (Ruoslahti, 1996).

Different binding sites on fibrinogen

Studies about the binding of fibrinogen with different cell types have been conducted for some time. Three known binding sites were found in fibrinogen; γ-400-411, Aα-RGDF and Aα-RGDS. A series of studies regarding the binding site of fibrinogen with ADP activated platelets via their integrin were performed. Using synthetic peptides, Kloczewiak *et al* 1984 were able to assign 12 amino acids

sequence (AGDV-containing dodecapeptide) which was found on γ-400-411 C-terminal of fibrinogen (γC-12) as its site of interaction with the platelet integrin receptor (Kloczewiak et al., 1984). This study was followed by another study by Hawiger *et al*, 1989 who also used synthetic peptides to demonstrate the binding site on fibrinogen with platelet integrin $\alpha_{IIb}\beta_3$, which was known at the time as GPIIbIIa. The authors suggested both RGD sequences on the Aα-chain; RGDF (95-98) and RGDS (572-575) as the sites of interaction with platelets in addition to the γ-chain site (Hawiger et al., 1989). However, in an earlier study from the same group, it was postulated that the γ-chain binding site have stronger interaction with the platelet integrin than the Aα-chain's sites without specifying which one of the two RGD sites (Hawiger et al., 1982). The cause of this weak interaction with platelets was later suggested to be that the position of the Aα-95-97 RGD is in the coiled-coil region, which might not be accessible for interaction with the platelet integrin. On the other hand, the Aα-572-574 RGD is not conserved in all species; making its role in the binding between fibrinogen and platelets arguable (Farrell et al., 1992).

The involvement of the RGDs in the fibrinogen binding was studied by Cheresh *et al*, 1989, in different cell types. They found that the $\alpha_V\beta_3$ integrin of the endothelial cells was able to bind the carboxylic end RGD in the Aα-572-574 of the fibrinogen, but not the N-terminal Aα-95-97 RGD or any other site on the fibrinogen (Cheresh et al., 1989b). However, in case of platelets, they confirm the role of γ-400-411 sequence on fibrinogen with the $\alpha_{IIb}\beta_3$ integrin of platelets in addition to its ability to recognise the Aα-572-574 RGD sequence (Cheresh et al., 1989b). This suggested that there is no competition between platelets and endothelial cells to bind fibrinogen since the former binds through the γ-chain mainly whereas the latter binds through the C-terminal Aα-chain. They indicated that the Aα-95-97 RGD does not have a role in cell adhesion although it was able to bind to cells when it was isolated from fibrinogen (Cheresh et al., 1989b). These results were partially agreed by Thiagarajan *et al*, 1996, who found that fibrinogen interaction with endothelial cells was via both the RGD sequences in the Aα-chain whereas there was no significant involvement of the γC-12 AGDV-containing dodecapeptide in fibrinogen adhesion to endothelial cells (Thiagarajan et al., 1996).

1.3.2 Secretion of Fibrinogen

Mature fibrinogen is secreted into the circulation after processing, glycosylation and assembly of each chain into the $A\alpha_2B\beta_2\gamma_2$ hexamer. Fibrinogen assembly is an extensively debated issue concerning the intermediates and pathways involved in this multistep process (Huang et al., 1993b). One model of fibrinogen biosynthesis and assembly is that the Bβ-chain is the first to appear as a part of dimeric structure attached to a membrane-bound polymerase, followed by fast assembly with pre-existing Aα and γ pools (Roy et al., 1991, Yu et al., 1984, Yu et al., 1983). This makes the Bβ-chain synthesis the rate-limiting step of the biosynthesis. By the completion of its synthesis, the complexes Bβ-γ and Bβ-α are released into the lumen of the rough endoplasmic reticulum (Roy et al., 1991, Yu et al., 1984, Yu et al., 1983). The second assumption is by Hartwig and Danishefsky, 1991 (Figure 1-4), which showed that γ-chain combined with either Aα, Bβ or γ chains separately in the absence of the other two chains to yield $\alpha\gamma_2$, $\beta\gamma_2$ or γ_3 according to their molecular weight (Hartwig and Danishefsky, 1991). This is supported by the suggestion that in each half, the γ-chain is assembled first in a triple coiled-coil strands, and then the Aα and Bβ chains displace two of the γ-chains (Doolittle, 1984). The results of the Hartwig and Danishefsky study agreed with the Doolittle assumption and suggested a modified mechanism that they have summarised in an outline figure that can be expressed as described in Figure 1-4 (Hartwig and Danishefsky, 1991). In this mechanism, it was suggested that γ-chain is firstly self-assembled to form a trimer (γ_3). The second step is formation of $\alpha\gamma_2$ trimer as a result of combination between the Aα and γ_3 releasing one γ-chain, however, this trimer do not join together (Hartwig and Danishefsky, 1991). The third step is similar to the second step, but the interaction is between the Bβ-chain with γ_3 to form $\beta\gamma_2$ with a release of one γ-chain as well. The fourth and final step is the assembly of the molecule, which can be done by two ways after the interaction between $\alpha\gamma_2$ and $\beta\gamma_2$ (Hartwig and Danishefsky, 1991). The first mechanism involves the production of αβγ and γ_3, but the molecular mass of produced coiled-coil in this step is greater than what expected for αβγ. Subsequently, the second mechanism may be the right model of fibrinogen assembly (Hartwig and Danishefsky, 1991). In this model, $\alpha\beta\gamma_2$ and 2γ-chains are produced by replacing one of the γ-chains either in the $\alpha\gamma_2$ by β of

$\beta\gamma_2$, or in the $\beta\gamma_2$ by α of $\alpha\gamma_2$. The last step is the formation of entire fibrinogen by the combination of two $\alpha\beta\gamma_2$ and release of 2γ-chains (Hartwig and Danishefsky, 1991).

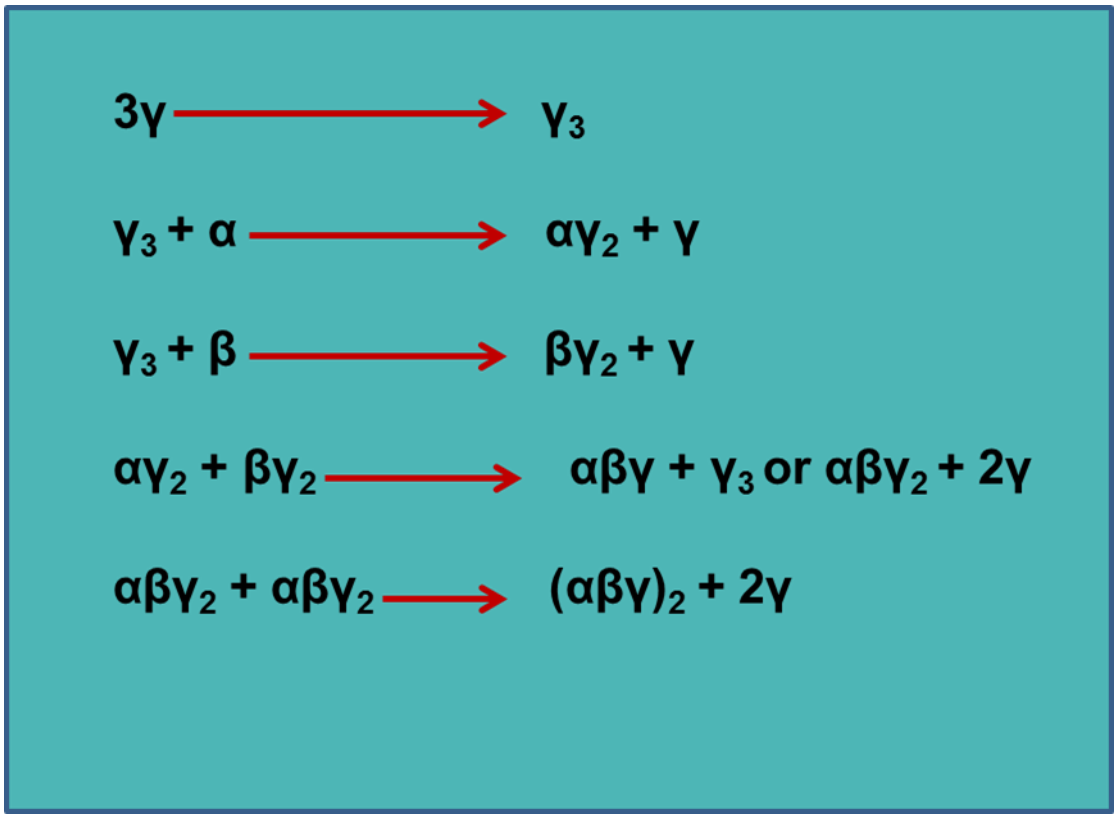


Figure 1-4 Schematic representation of an outline summary of fibrinogen chains assembly. The outline represents different combination of the three chains and ends by the formation of the fibrinogen dimer $(\alpha\beta\gamma)_2$. Adapted from (Hartwig and Danishefsky, 1991).

1.3.2.1 Fibrin Clot Formation

Clot formation is a two steps process that involves conversion of fibrinogen to fibrin. The first step involves the proteolytic cleavage of two fibrinopeptides A and two fibrinopeptides B from the N-termini of Aα and Bβ chains respectively in the E region of fibrinogen by thrombin (Weisel and Litvinov, 2017, Li et al., 2016). Thrombin is a potent and specific serine protease originating from proteolytic cleavage of prothrombin by FXa. The result of fibrinogen cleavage is the formation of a fibrin monomer that is 45 nm long and 4.5 nm thick. (Weisel and Litvinov, 2017, Li et al., 2016). Then, a second, non-enzymatic step involves a spontaneous self-assembly of fibrin monomers in a half-staggered way to form oligomers that extend to form two-stranded protofibrils (Li et al., 2016, Weisel and Litvinov, 2017, Weisel et al., 1993). The protofibrils then aggregate laterally as well as longitudinally producing ~130 nm thick fibres that branch to form the clot; a three-dimensional mesh-like structure with a gel-like texture. This clot is further matured and stabilised by FXIIIa crosslinking (Li et al., 2016, Weisel and Litvinov, 2017, Weisel et al., 1993).

1.3.2.2 Mechanisms Underpinning Protofibril Formation

Removal of fibrinopeptides A and B from the central E region by thrombin converts fibrinogen to fibrin by freeing two A and B knobs respectively in the process of clot formation as shown in Figure 1-5 (Guthold and Cho, 2011). Firstly, two fibrinopeptides A are removed to expose two A knobs (Gly-Pro-Arg) in the central region of Aα-chain that interact with complementary binding sites (a pockets) that are constitutively present in the distal γ-nodules in the D-region. This removal of the fibrinopeptides A is initiating the half-staggered assembly to form the two-stranded protofibrils; fibrin I (Ajjan et al., 2008, Guthold and Cho, 2011). Secondly, two fibrinopeptides B are removed by thrombin at a slower rate and expose two B knobs (His-Gly-Arg) on the Bβ-chain that interact with constitutively exposed complementary regions (b pockets) on the Bβ-chain initiating lateral aggregation; leading to fibrin II. These b pockets are found in the D-regions of the β-nodules (Ajjan et al., 2008, Guthold and Cho, 2011). Interactions of the charged knobs with their corresponding holes (A:a and B:b) are the key stages for protofibril formation, whereby the former (A:a) is the key

step in half staggered assembly (fibrin I) while the latter (B:b) is the key to protofibril assembly and lateral aggregation (fibrin II) (Li et al., 2016, Guthold and Cho, 2011). The delay in fibrinopeptide B release is important to elucidate its role in lateral aggregation by protofibril assembly and fibre formation (Weisel et al., 1993). At the same time of the release of fibrinopeptide B, the αC termini are released and become available for αC interactions that contribute to lateral aggregation of the protofibril (Mutch et al., 2010).

Adsorption of fibrinogen on different surfaces has been reported to affect its properties and reduce its accessibility to thrombin by decreasing the available fibrinopeptides for thrombin cleavage compared to fibrinogen in solution (Riedel et al., 2011). Fibrin monomers are further crosslinked by the action of FXIII in the presence of calcium and thrombin, a γ-dimer subunit is rapidly formed by the crosslinking between a lysyl donor group and a glutaminyl acceptor group (ε-γ-glutamyl-lysine crosslinks) in antiparallel fashion yielding the partially crosslinked fibrin (Fretto et al., 1978). A second, slower crosslinking occurs between Aα-chains yielding α-polymer subunits representing a fully stabilised crosslinked fibrin. No crosslinking occurs in the Bβ-chains (Fretto et al., 1978).

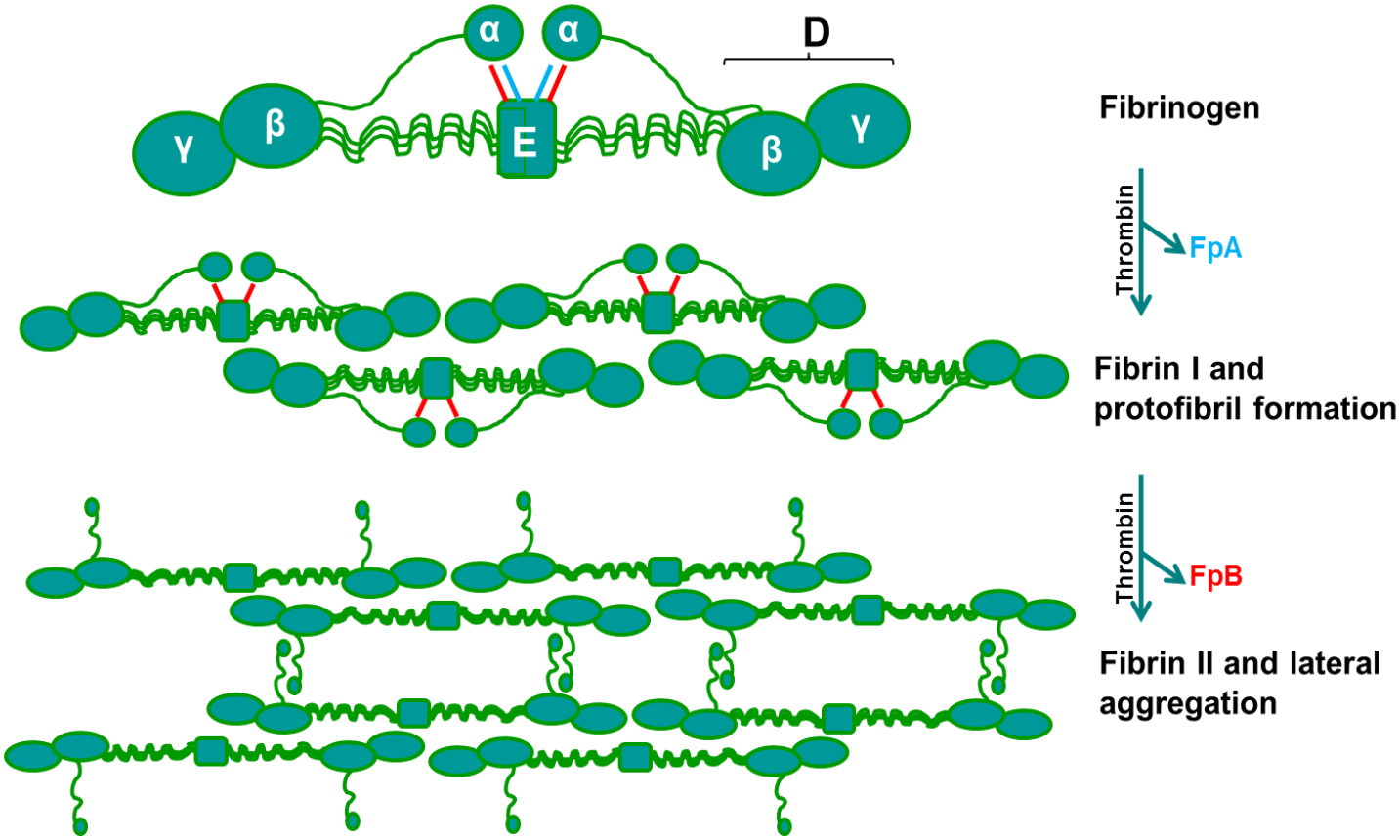


Figure 1-5 Schematic representation of fibrin formation from fibrinogen in presence of thrombin. First step, cleavage of fibrinopeptide A (FpA, blue) leads to protofibril formation. Second step cleavage of fibrinopeptide B (FpB, red) leads to release of αC domains and lateral aggregation. Adapted from (Undas and Ariens, 2011).

1.3.3 FXIII

FXIII is a pro-transglutaminase composed of two A, and two B subunits (FXIII-A and FXIII-B), and is found in the circulation as a hetero-tetrameric zymogen called FXIII-A_2B_2 (Byrnes et al., 2015). FXIII is activated by the cleavage of activation peptide (AP-FXIII) from the N-terminus of FXIII-A by thrombin, and then in the presence of calcium, FXIII-B detaches from the molecule releasing its inhibitory effect from the activated A-subunit (Muszbek et al., 2011). FXIII crosslinks fibrin Aα-chain at Q221, Q237, Q328, Q366 in addition to various lysine residues, whilst it crosslinks the γ-chain in a faster rate between residues Q398 and/or Q399 and K406 (Duval et al., 2014). FXIII crosslinking is able to alter clot structure rather than to simply stabilise the existing preformed network structure of fibrin (Duval et al., 2014, Hethershaw et al., 2014). Fibrin clot formation and structure were found to be influenced by FXIII crosslinking, which was studied using recombinant fibrinogen with mutations in the γ-chain crosslinking sites (Duval et al., 2014). Crosslinking of the Aα- and γ-chains of fibrin plays a role in regulation of the ultrastructure of the fibrin clot (Duval et al., 2014, Duval et al., 2016). Crosslinking of the γ-chain influenced fibre density, whereas crosslinking of the Aα-chain influenced fibrin polymerisation, polymerisation rate and fibrin fibre curvature by electron microscope (Duval et al., 2014). These changes may include increasing fibre density, decreasing fibre thickness and increasing the fibrin fibre resistance to fibrinolysis (Hethershaw et al., 2014, Duval et al., 2014). FXIII is able to crosslink fibrin to α_2-plasmin inhibitor (α_2-PI) to stabilise the fibrin clot and protect it against premature fibrinolysis (Sakata and Aoki, 1980, Duval et al., 2016).

1.3.4 Fibrinolysis

Plasmin, the active form of plasminogen is produced by proteolytic cleavage of plasminogen by the activity of either tissue plasminogen activator (tPA) or urinokinase plasminogen activator (uPA). Plasmin is found in the circulation in its inactive zymogen and when activated, it causes dissociation of thrombin or fibrin deposits in a process called fibrinolysis (Sakata and Aoki, 1980, Ajjan and Grant, 2006). The fibrinolytic system is composed of a series of enzymes that in

their active forms cleave fibrin into its degradation products. This process is the last control mechanism to restrict clot formation (Norris, 2003). The process can be inhibited by inhibiting either plasmin or plasminogen activators (Figure 1-6) (Sakata and Aoki, 1980, Ajjan and Grant, 2006).

tPA is a serine protease that produced by endothelial cells (Norris, 2003) whereas uPA is produced in urinary epithelial cells, macrophages, and monocytes. The affinity of plasminogen is higher for tPA compared with uPA (Camiolo et al., 1971, Chapin and Hajjar, 2015). Due to the presence of high concentrations of activator inhibitors, both enzymes have short half-lives in the circulation of 4-8 minutes, when they are removed by the liver (Chapin and Hajjar, 2015). Plasmin activation is more effective in the presence of fibrin compared to fibrinogen, which make fibrinogen less susceptible to lysis compared to fibrin (Camiolo et al., 1971, Chapin and Hajjar, 2015). This means that fibrin both acts as a stimulator for the activation of plasmin and as a plasmin substrate at the same time (Hoylaerts et al., 1982). The binding of plasmin to C-terminal lysine residues on fibrinogen catalyses the hydrolysis of fibrinogen by plasmin (Christensen, 1985). Continuous degradation of fibrin and generation of more C-terminal lysine residues on the degraded fibrin induces plasmin to control its own formation by a positive feedback loop, which is inhibited by the activity of activated Thrombin Activatable Fibrinolysis Inhibitor (TAFI) (Bouma and Mosnier, 2003). Regulation of fibrinolysis includes a number of other inhibitors, such as inhibitors of the plasminogen activator (PAI) of which there are four types; PAI-1, PAI-2, PAI-3 and protease nexin. Another inhibitor for fibrinolysis is α_2-antiplasmin, which binds to plasmin forming a stable irreversible complex (Norris, 2003, Wiman and Collen, 1978, Ajjan and Grant, 2006). FXIII crosslinks fibrin molecules and fibrin with other proteins in the clot such as α_2-antiplasmin, thereby increasing the resistance to fibrinolysis. Crosslinking of α_2-antiplasmin occurs between Lys303 on the Aα-chain of fibrin and Gln2 or Gln14 on α_2-antiplasmin (Kimura and Aoki, 1986, Fraser et al., 2011, Bridge et al., 2014).

Fibrinolysis rate is affected by changes in clot structure and architecture, with less dense clots, loosely packed fibres and larger pores sizes being lysed faster than denser clots with tightly packed fibres and smaller pores sizes (Bridge et

al., 2014). Although thin fibres are lysed faster than thick fibres, clots that are made up of more thin fibres lyse slower than clots made up of much thicker fibres (Collet et al., 2000, Bridge et al., 2014).

A risk factor of the association between diabetes and cardiovascular diseases may be via decreasing fibrinolysis and subsequently reducing clot lysis. The effect of diabetes on clot lysis is a result of changes involving inhibition of the process of fibrinolysis, increase of anti-fibrinolytic proteins, and modification of fibrin mesh structure (Ajjan et al., 2013). The mechanism of this effect occurs through the glycation of plasminogen, which decreases its activation and functional activity and subsequently, reduces fibrinolysis (Ajjan et al., 2013).

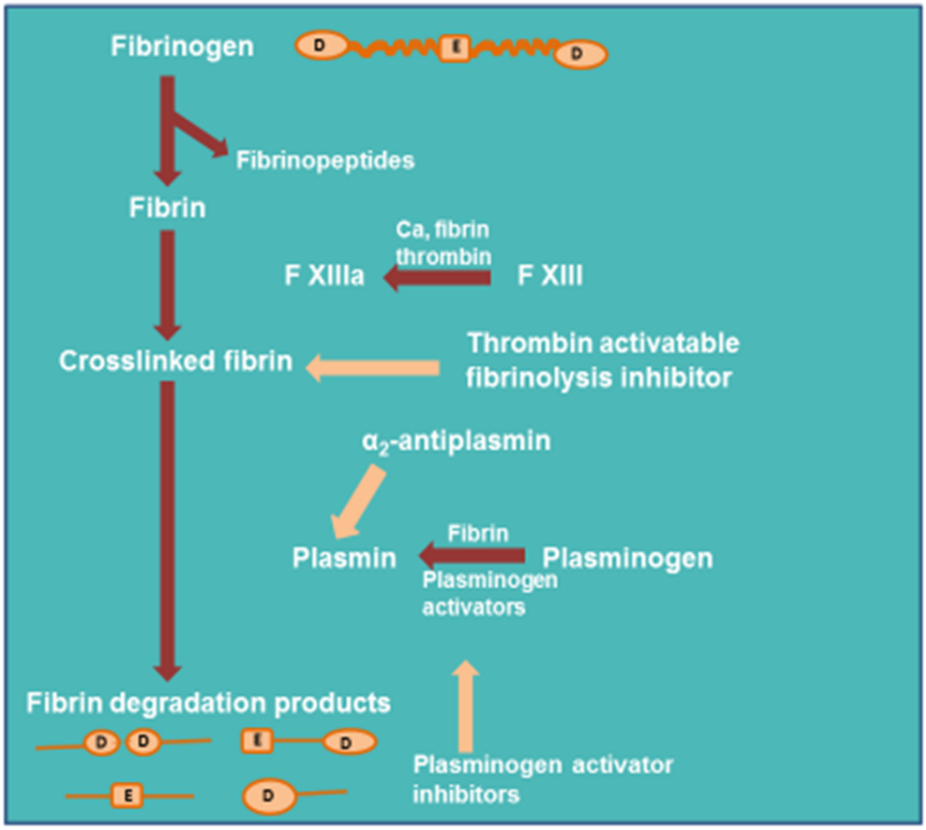


Figure 1-6 Schematic representation of fibrin polymerisation and fibrinolysis. Fibrinogen is converted to fibrin by the removal of fibrinopeptides in presence to thrombin to for a clot. FXIIIa stabilises the fibrin network by crosslinking. Plasminogen is converted to plasmin by activation on the surface of the fibrin causing clot proteolysis. Beige arrows represent the inhibitory control of the process. Adapted from (Weisel, 2005).

1.3.5 Other Functions of Fibrinogen

Fibrinogen is involved in a number of other functions including cell adhesion and inflammation; in addition to its role in several cardiovascular and metabolic diseases like atherosclerosis, hypertension and diabetes via the stimulation of erythrocytes hyperaggregation (de Almeida et al., 2009). Fibrinogen plays a role as acute-phase plasma protein in acute inflammation and infections. Changes in vascular permeability lead to rapid invasion of leukocytes followed by monocytes to the site of the injured vessels. A few hours later after the onset of infection, acute-phase plasma protein levels including fibrinogen are increased in the circulation as a sign of inflammation (Ritchie and Fuller, 1983). Fibrinogen structural properties support its role in proliferation and migration of some cell types in the body including platelets, endothelial, epithelial and fibroblast cells (Guadiz et al., 1997). Identification of extrahepatic tissues such as lungs, brain, bone marrow and non-hepatic epithelial cells that are able to synthesise fibrinogen has indicated that fibrinogen may be involved in functions other than haemostasis and thrombosis such as maintaining the structural integrity, as well as the cellular adhesive interactions in these tissues (Guadiz et al., 1997)

Fibrinogen deficiency presents with different clinical outcomes, which vary between qualitative and quantitative deficiencies. Qualitative deficiency is called dysfibrinogenaemia, whilst there are two types of quantitative deficiencies, hypo-fibrinogenaemia or a-fibrinogenaemia (Ariens, 2013). Yet the causes of the discrepancies in fibrinogen presentations, which vary in their symptoms between thrombosis and bleeding in addition to the possibility of being asymptomatic, are still uncertain. It has been suggested that some of the discrepancies in clinical outcomes may be the result of variations in clot structure, strength or stability. These variations provide the clot with instability that may lead either to thromboembolic or bleeding diseases (Ariens, 2013). A-fibrinogenaemia is a complete deficiency of circulating fibrinogen that is characterised by bleeding in tissues such as the umbilical cord at birth, soft tissues, genito-urinary tract, joints and central nervous system (Le Quellec et al., 2018). Additional symptoms may include spontaneous splenic rupture, poor wound healing, bone cysts, and tendency for venous and arterial thrombosis. Treatment of this disorder involves

fibrinogen replacement therapy (Le Quellec et al., 2018). Dysfibrinogenaemia and hypo-fibrinogenaemia are both associated with autosomal dominant inheritance. Hypo-fibrinogenaemia is largely an asymptomatic disorder that may be diagnosed incidentally due to abnormal coagulation tests, it is characterised by a functional and immuno-reactive proportional reduction of fibrinogen (Tiscia and Margaglione, 2018, de Moerloose et al., 2013). Dysfibrinogenaemia is characterised by a reduction of fibrinogen function with normal fibrinogen antigen concentration. Both disorders can cause miscarriages in pregnancy (Tiscia and Margaglione, 2018, de Moerloose et al., 2013).

1.4 Red Blood Cells

Human blood can be defined as a double-phase system that is composed of plasma, a low viscosity aqueous ionic solution, and a cellular fraction that occupies 0.45-0.5 of the volume of the blood (Picart et al., 1998). Of the cellular fraction, 0.99 is composed of RBCs, which are the most abundant cells of the blood, whereas white blood cells and platelets represent less than 0.01 of the cellular fraction (Picart et al., 1998). Thus, RBCs are a major determinant of blood rheological properties, which in turn affect the blood viscosity and fluidity (Baskurt and Meiselman, 2003, Lominadze et al., 2002). Blood viscosity is affected by haematocrit, plasma viscosity in addition to the two most important rheological properties of the RBCs; their deformability especially at high shear forces and their propensity to aggregate. RBCs are capable of aggregating in linear arrangements that are known as RBC rouleaux (Baskurt and Meiselman, 2003, Lominadze et al., 2002). Depending on the degree of shear forces affecting the cells, large plasma glycoproteins such as fibrinogen stimulate RBC aggregation, which under low shear is the main determinant of blood viscosity (Baskurt and Meiselman, 2003).

Human red blood cells originate in the bone marrow from haematopoietic stem cells that differentiate into erythroblasts and erythrocyte colony forming units (Pasini et al., 2006, Franco, 2012). Reticulocytes are secreted into the circulation after extrusion of nuclei from erythroblasts and degradation of endoplasmic reticulum; they then mature into anucleated RBCs capable of oxygen and carbon

dioxide transport between the blood and cells (Pasini et al., 2006, Franco, 2012). Mature RBCs survive in the blood for a mean lifespan of 115-120 days, after which they are removed by the reticuloendothelial system when they reach a specific age. The normal body is able to have a turnover pool of RBCs every three weeks to maintain an overall RBCs pool of 4-5 $X10^{12}$ cells/L (Franco, 2012, Giarratana et al., 2005). The average diameter of the mature RBC is 8 µm, however, the RBC is capable to pass through small capillaries of 3-4 micron due to its deformability (Aarts et al., 1983, Meiselman, 1981). The RBC membrane is made up of three main parts; a lipid bilayer membrane, proteins that extend across or embedded inside the membrane and a third part that is composed of proteins that do not enter the membrane and form the membrane skeleton (Boivin, 1988, de Oliveira and Saldanha, 2010). These proteins can be further divided according to their function as cytoskeletal proteins such as actin and spectrin, integral structural proteins such as glycophorins and band 3, and finally, anchoring proteins such as ankyrin and protein 4.2 (de Oliveira and Saldanha, 2010).

An overview of the three dimensional structure of the membrane skeleton of the RBC shows that the membrane might be composed of a dense convoluted network of spectrin filaments that are joined at junctional complexes to actin filaments (Nans et al., 2011). The thickness of the network together with its surface density is less by approximately twofold at the edges (54 nm) compared to the centre (110 nm) supporting their resting biconcave shape. This specialised structure of the RBC and its membrane helps to accommodate the high stress and facilitate its deformability (Nans et al., 2011). These membrane characteristics give RBCs the flexibility of regular changes in their shape in an extensive and reversible manner. This allows RBCs to pass through the microcirculation vessels that have a smaller diameter than the size of the RBCs under the effect of the intravascular fluid forces (Cines et al., 2014).

Cines *et al*, 2014 first reported on the presence of polyhedral shaped RBCs inside the clot, which they proposed are compressed erythrocytes. The name polyhedrocytes was given according to the shape nomenclature of the erythrocytes (Cines et al., 2014). Several explanations for this assumption were

suggested including that only RBCs are present in adequate amount in the blood to account for the large quantity of these polyhedral structures. Furthermore, partly compressed erythrocytes having an intermediary shape ranging between the biconcave and polyhedral shape were found (Cines et al., 2014). Moreover, normal biconcave erythrocytes were present outside and inside of the clot that formed by low platelet counts or low fibrin(ogen) concentrations with no presence of polyhedrocytes (Cines et al., 2014). These observations led to the conclusion that the polyhedral shape of erythrocytes are formed as a result of the platelet contraction on fibrin which caused the erythrocytes to compress. Both platelets and fibrin were found on the surface whereas the erythrocytes were packed in the centre of the clot (Cines et al., 2014). The polyhedral shape is formed by several flat surfaces that are connected by straight edges and can occupy a much smaller space than the normal biconcave shape of RBC (Ariens, 2014). This shape allows maximum cell-to-cell interaction in addition to lowest interstitial space occupation yielding stronger clots that are closely sealed by the packed polyhedrocytes. Polyhedrocytes are found in the center of the clot while normal biconcave shaped RBCs are located at the clot periphery as shown in Figure 1-7 (Ariens, 2014).

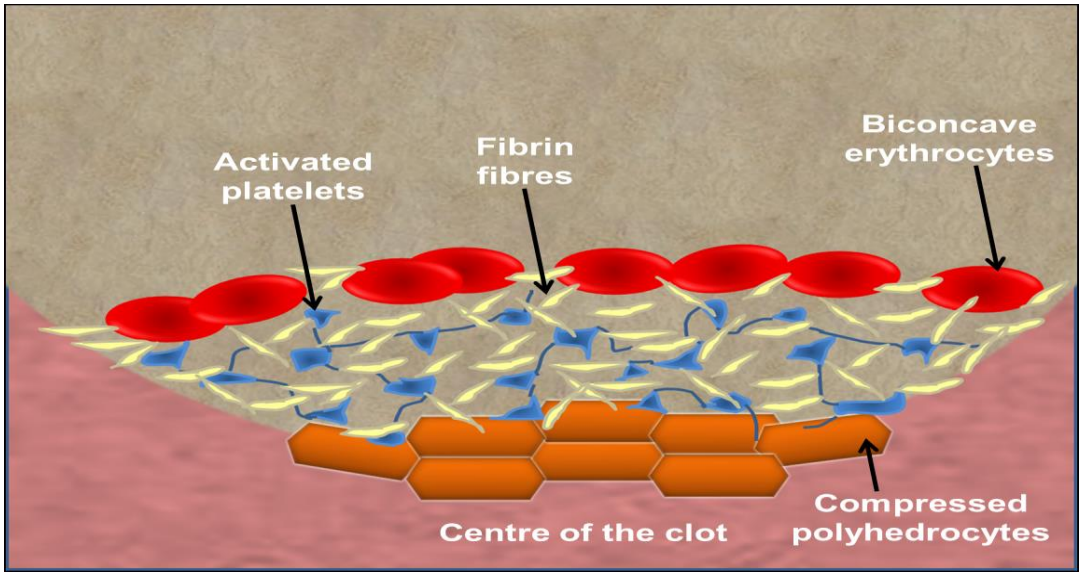


Figure 1-7 Schematic representation of the polyhedrocyte involvement in the clot. At the centre of the clot, contractile action of platelets on fibrin fibres lead to compression of the biconcave erythrocyte to polyhedrocytes producing a perfectly sealed clot. Adapted from (Ariens, 2014).

At low shear rate, erythrocytes sometimes aggregate, thereby increasing the viscosity of the blood whereas they disintegrate at high shear rate (Maeda et al., 1984). This aggregation is enhanced by plasma glycoproteins such as fibrinogen and immunoglobulins bridging the neighboring cells, however, negative charges on the surface of the RBCs may resist this aggregation and cause the cells to separate (Maeda et al., 1984). A balance between electrostatic repulsive and attractive forces at the cell surface may be crucial for RBC aggregation to occur. Aggregation requires the macromolecular bridging force to be greater than both the mechanical shearing and the electrostatic repulsive forces in order for aggregation to develop (Chien and Jan, 1973). Increased haematocrit can lead to increased viscosity. An increase in haematocrit enhances the residence time of circulating coagulation factors and platelets on the activated endothelium supporting clot formation (Andrews and Low, 1999).

Release of free extracellular haemoglobin into the blood because of RBC *in vivo* haemolysis comprises the pathogenic origin of haemolytic anaemias either of acquired or genetic cause. Haemolytic anaemia may lead to pro-thrombotic diseases of different severity (Litvinov and Weisel, 2017). Sickle cell disease is a well-characterised form of hereditary haemolytic anaemias that affects the RBC shape due to a recessive genetic mutation in haemoglobin. This mutation induces intracellular haemoglobin aggregation changing the RBC from biconcave to sickle shape (Tran et al., 2013). In this particular disease, the rheological and viscous properties of the blood are changed due to decreased deformability of the RBCs and increased membrane rigidity (Tran et al., 2013). Membrane stiffness of the RBCs is also increased in the diabetic patients possibly contributing to the increased risk of microvascular occlusion in this disease (Tran et al., 2013, Walton et al., 2015)

1.4.1 Red Blood Cell Receptors

Cell adhesion molecules (CAM) are a group of receptors that are present on the surface of the cells and are involved in their interaction with ECM or with other cells, but not to soluble molecules such as cytokines (Spring and Parsons, 2000). Mature RBCs are found to have some glycoproteins that either function as CAM or have similar functions. Several super-families of CAM are found in erythroid

cells of different ages include integrins, immunoglobulins, sialomucine in addition to selectin and cadherin families (Spring and Parsons, 2000). A number of membrane proteins on the RBC surface have been suggested to play a role as a target or receptor for soluble fibrinogen to interact with RBC. These receptors include (De Oliveira et al., 2012):

1. Integrin cell adhesion molecule-4 (ICAM-4)
2. Integrin associated protein (IAP) or CD47
3. Basal cell adhesion molecule (BCAM/LU)
4. CD147
5. CD44
6. CD58
7. Glycosphingolipids (gangliosides)

The adhesive role of RBCs is carried out by protein and glycolipid moieties, which are present on their surface. These molecules are composed of different structures comprising transmembrane proteins; glycosylphosphatidylinositol-anchored proteins, carbohydrates or lipid structures (Telen, 2005). Some examples of these adhesion molecules are presented in Table 1-1 showing a summary of some receptors and their related functions. CD44 is an adhesion receptor found on the RBC membrane and identified as a 80 kDa adhesion protein which is known as Inhibitor (In) Lutheran (Lu)-related p80 that is down-regulated by the inhibitor of Lutheran expression. The Indian antigen is found on the CD44, it is a blood group antigen that has two polymorphisms resulted from a single point mutation (Telen et al., 1983, Telen et al., 1996, Telen, 2005). IAP (CD47) is a 50 kDa protein that is composed of extracellular immunoglobulin binding domain and involves in several functions of $\alpha_V\beta_3$ and thrombospondin via the modulation of β_1 integrin (Wang et al., 1999, Green et al., 1999, Telen, 2005).

Table 1-1 Examples of adhesion molecules expressed on circulating erythrocytes (Telen, 2005)

Adhesion molecule and alternate name	Ligand/adhesive function
Indian [In(lu)-related p80, CD44]	Hyaluronan, possibly also fibronectin
Rh-related integrin-associated protein (IAP, CD47)	Thrombospodin
Lymphocyte-associated antigen-3 (LFA-3, CD58)	CD2
CD99, MIC2 gene product	Lymphocyte CD99 is necessary for formation of T-cell rosettes
JMH (semaphorin K1, SEMA7A, CD108	Possible role in adhesion of activated lymphocytes
Ok^a (neurothelin,, CD147)	Type IV collagen, fibronectin, laminin and other tissues
LW (ICAM-4, CD242)	Leukocyte integrin ($\alpha_4\beta_1$, $\alpha_4\beta_3$, $\alpha_V\beta_1$), platelet integrin ($\alpha_{IIb}\beta_3$), vascular integrin ($\alpha_V\beta_3$)
Lutheran (B-CAM/LU, CD239)	Laminin, possibly also integrin
Scianna (ERMAP)	Putative adhesive function
MER2 (CD151|)	Forms laminin-binding complexes with integrin
CD36 (reticulocytes only), platelet glycoprotein IV, Nak^a (platelets)	Thrombospondin (platelets)
VLA-4 (reticulocytes only), $\alpha_4\beta_1$ integrin (CD49d/CD29)	Thrombospondin, VCAM-1, fibronectin

1.4.2 Red Blood Cells in Thrombosis

As RBCs are the most abundant cells in the blood, they were classically thought to be passively involved in the process of thrombus formation, by being entrapped in the clot. However, new evidence from both laboratory or clinical studies support the idea that RBCs are not innocent bystanders in this process (Du et al., 2014). The effect of RBCs on clot formation and structure has not been extensively studied; this in spite of the fact that they have been suggested to have a contributing role in thrombosis and haemostasis (Gersh et al., 2009). This can be because their role in thrombus formation is uncontrolled and variable, and they interfere with many of the laboratory techniques that are used to investigate clot structure and function (Gersh et al., 2009).

The size of a clot can depend to a large degree on its content of the RBCs, however, not much is known about the role of RBCs in clot contraction (Cines et al., 2014). It has been reported recently that RBC contents directly affect the size of a venous thrombus, and that FXIIIa has a vital role in active RBC retention in these thrombi (Aleman et al., 2014). These findings indicate that RBCs may entail a novel therapeutic target to decrease venous thrombosis and increase clot lysis rates. One possibility to achieve this may be via the inhibition of FXIIIa, thus reducing the size of the clot, its mechanical stability and its ability to resist fibrinolysis (Aleman et al., 2014). It has been suggested that FXIIIa activity together with fibrin network density independently play a role in RBC retention in the clot. Nevertheless, FXIII does not directly crosslink RBCs, but induces their retention in the clot by crosslinking fibrin, especially the Aα-chain (Byrnes et al., 2015). While the fibrin network should be dense and stiff enough to enable RBCs to be retained, abnormal fibrin network density may not be the cause of RBCs extrusion from the clot by itself, but a defect in FXIII crosslinking would release the RBCs from the clot (Byrnes et al., 2015).

One of the early studies about the involvement of RBCs in thrombosis was done by Horne *et al*, 2005. In this study, an assay to study thrombin generation in presence of intact and lysed RBCs was introduced, because it was found that all routine analyses for blood coagulation rely on cell-depleted plasma. There was a concern about providing physiological conditions to the new assay so thrombin

generation was initiated by tissue factor (Horne et al., 2006). Endogenous thrombin potential was increased threefold by the physiological increase in haematocrit compared to its increase by the increase in platelets. Subsequently, in non-flowing blood, the sensitivity of thrombin generation was more affected by RBC concentration than by platelet concentrations (Horne et al., 2006).

A study by Lominadze *et al*, 2002 confirmed the presence of a non-specific binding of fibrinogen (Chien and Jan, 1973) with erythrocytes as well as a specific binding. A complete loss of the specific binding was found with the removal of calcium while a dose-dependent increase was shown in the presence of the cation. This suggested an integrin-type receptor interaction between erythrocytes and fibrinogen (Lominadze and Dean, 2002). This was confirmed by the use of an RGDS peptide antagonist that in a high concentration inhibits the fibrinogen-dependant erythrocyte aggregation as well as fibrinogen-erythrocyte specific binding (Lominadze and Dean, 2002). The high affinity of both fibrinogen and platelet binding argues against the involvement of the platelets in the interaction between fibrinogen and RBCs (Lominadze and Dean, 2002).

More recently, a study by Carvalho *et al*, 2010 compared the binding of fibrinogen to both platelets and erythrocytes using atomic force microscopy (AFM). The experiment was conducted on samples from healthy donors and Glanzmann thrombastenia (an $\alpha_{IIb}\beta_3$ integrin deficient) patients to evaluate the binding between fibrinogen and $\alpha_{IIb}\beta_3$ integrin both in RBCs and platelets (Carvalho et al., 2010). Because the activation of the integrin is calcium dependant, the influence of the presence of calcium ion was tested. To confirm if binding between fibrinogen and RBCs was through the $\alpha_{IIb}\beta_3$ integrin, the inhibitor of the integrin (eptifibatide) was used as well (Carvalho et al., 2010). It was found that in all cases both platelets and erythrocytes interaction to fibrinogen were inhibited (absence of calcium, absence of the $\alpha_{IIb}\beta_3$ integrin and presence of the inhibitor) but to a lesser extent in the erythrocytes, in addition to the presence of mutated β_3 gene in these patients (Carvalho et al., 2010). It was shown that fibrinogen has more affinity to platelets than to RBCs although they both were comparable in their rupture force and binding frequency to fibrinogen (Carvalho

et al., 2010). The presence of an integrin on the surface of the RBC was concluded, this integrin was suggested to be an $\alpha_{IIb}\beta_3$-like integrin that is related to the platelets integrin (Carvalho et al., 2010). In a following study, the same group focused on the effect of aging of erythrocytes by studying different subpopulations of the cells. It was found that, the interaction between fibrinogen and erythrocytes decreased in frequency, but not in strength by the aging of erythrocytes (Carvalho et al., 2011). This was because the cells were having the same morphology and rupture forces, but they required less fibrinogen concentrations for the younger aged erythrocytes. The decrease in fibrinogen binding to RBCs was related to the dynamic reduction of sialic acid in aged erythrocytes, suggesting a possible causal relationship between sialic acid content and fibrinogen binding (Carvalho et al., 2011). Another study excluded the involvement of $\alpha_{IIb}\beta_3$ integrin in the interaction between soluble fibrinogen and RBCs and suggested that it is age-dependant rather than integrin-dependent. The involvement of CD47 receptor as a recognised mediator in this interaction was suggested by using antibodies against this receptor to inhibit the fibrinogen-RBC interaction (De Oliveira et al., 2012).

1.4.3 Fibrinogen-RBC binding

The bridging of fibrinogen and its degradation products between neighbouring RBCs to support the erythrocytes aggregation was previously studied and demonstrated the presence of specific binding between the RBCs and fibrinogen (Zhang et al., 2008, Wagner et al., 2013, Maeda et al., 1987). It was suggested that fibrinogen and its derivatives may bind the RBCs on different sites avoiding a competition between these molecules on the binding of RBCs to induce rouleau formation (Zhang et al., 2008, Wagner et al., 2013, Maeda et al., 1987). Rouleau is a reversible aggregation of RBC in linear stacks of coin-like structures that is involved in bridging the cells and increasing viscosity (Zhang et al., 2008, Wagner et al., 2013, Maeda et al., 1987). Maeda *et al* found that the involvement of fibrinogen and its products in the rouleau formation was preserved in the whole molecule, which they called fragment X and fragment Y, the latter of which is composed of one D-domain with the E-domain (Maeda et al., 1987). However this ability of binding RBCs was lost in fragment D and fragment E, which

contains each region (D and E respectively) separately (Maeda et al., 1987). Another study has shown the presence of a specific binding between fibrinogen and erythrocytes that can be inhibited by using the integrin receptor antagonist RGDS peptides (Lominadze and Dean, 2002). The authors suggested that this interaction, which is involved in fibrinogen-induced erythrocyte aggregation could help in the production of therapeutic drugs against cardiovascular and metabolic diseases (Lominadze and Dean, 2002).

1.4.4 Involvement of RBCs in clot structure

Carr and Hardin, 1987 calculated the average pore diameter for the whole blood clot, by haemolysing the RBCs using water so that the haemoglobin and membrane fragments are easily flushed from the clot (Carr and Hardin, 1987). The authors then studied the residual fibrin gel to calculate the pore size. It was found that RBCs can affect the pore size of the clot, but not how the fibrin fibres structure were formed (Carr and Hardin, 1987). RBCs were found to increase the pore size to become large enough to allow the escape of some erythrocytes out of the clot. It was suggested that the increase in pore sizes is not a result of increasing the cell concentration, but by occupying a large space in the clot (Carr and Hardin, 1987). When RBCs leave the clot, their spaces become pores that cannot be filled by fibrin fibres (Carr and Hardin, 1987). Gersh *et al*, 2009 on the other hand found that addition of RBCs into fibrin clot was affected the clot structure significantly in a concentration-dependant manner as well as its mechanical properties. The authors suggested that this observation should be taken into account when assessing *in vivo* plasma clots (Gersh et al., 2009).

A role for RBC in affecting clot structure and mechanical stability was studied by Guedes *et al*, 2018 using two variants of fibrinogen, normal $\gamma_A\gamma_A$ and its alternative spliced variant $\gamma_A\gamma'$. It was found that incorporation of RBCs into the clot would change the behaviour of fibrinogen in the splice variant giving thinner fibres and stiffer clots with longer fibrinolysis time (Guedes et al., 2018b). The authors next investigated the effect of the homodimer $\gamma'\gamma'$ on the clot structure and interaction with the RBCs. Interestingly, $\gamma'\gamma'$ splice variant showed a stronger binding to the RBCs that was more frequent, compared with the $\gamma_A\gamma_A$ variant, suggesting that it would promote RBC aggregation (Guedes et al., 2018a). Clots

made by this splice variant demonstrate more viscosity in presence of RBCs, they are more branched having larger pores and denser fibres compared to the $\gamma_A\gamma_A$ as well as longer fibrinolysis. The effect of this variant on clot structure and fibrin polymerisation in presence of the RBCs make them more susceptible to embolism and induces erythrocyte hyper-aggregation (Guedes et al., 2018a).

RBCs were found previously to provide the clot with more stability by supressing tPA-induced fibrinolysis (Wohner et al., 2011). In addition to that, RBC rich clots have the ability to respond to eptifibatide inhibition via its specific receptor for fibrinogen (Wohner et al., 2011). Carvalho *et al*. compared the clot structure of the D97E and WT using SEM and found that there was no significant change in clot structure in the absence of RBCs. In the presence of RBCs, D97E clots looked more disorganised having more clusters entrapping the RBCs (Carvalho et al., 2018). The clots were loosely packed increasing the risk of embolism. Addition of the RBCs to the clot was found to significantly alter the fibre thickness in the clot, which apparently resulted from the interaction between fibrinogen and RBCs (Carvalho et al., 2018).

Based on the above-mentioned studies, it can be concluded that some controversy is still present regarding the interaction between fibrinogen and RBC, as well as regarding the possible receptor on the surface of the RBC involved in this interaction between fibrinogen and RBCs.

1.5 Aims and Hypothesis

1.5.1 Hypothesis

Conversion of fibrinogen to fibrin by thrombin is the principal step in clot formation followed by crosslinking of the fibrin network by FXIIIa to provide the clot with additional stability. Fibrinogen and consequently fibrin bind a variety of cells via ligand-receptor integrin complex such as $\alpha_{IIb}\beta_3$ for platelets, which gives the clot more strength. One of the key therapeutic targets in the treatment of cardiovascular diseases is blocking this interaction between platelets and fibrinogen. A new involvement of RBCs in clot formation has recently been suggested to involve integrin receptor binding to specific site on fibrinogen. This site could involve the RGD sequence in the coiled-coil of the Aα-chain of fibrinogen but the interaction between fibrinogen and RBCs is not well-characterised and its role in clot formation has not been clearly established yet.

The hypothesis of this project is that the interaction between fibrinogen and RBCs is mediated by the RGD sequence in the coiled-coil of the Aα-chain of fibrinogen through $\alpha_{IIb}\beta_3$-like integrin receptor, and that this interaction is responsible for stabilising the clot by RBC retention.

1.5.2 Aims

1.5.2.1 Overall Aims

To investigate the role of the fibrinogen Aα-chain $R_{95}G_{96}D_{97}$ sequence in regulating:

1. fibrinogen-RBC interaction
2. Fibrinogen-RBC binding site
3. Clot structure and function

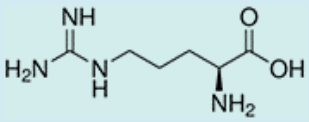
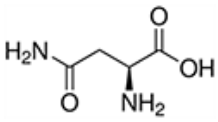
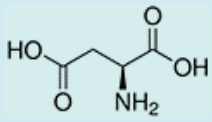
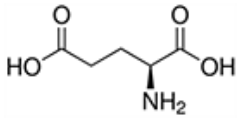
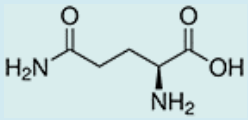
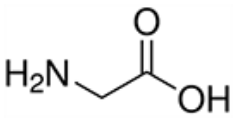
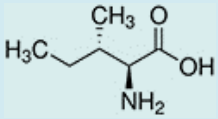
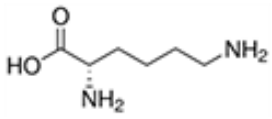
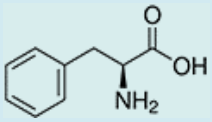
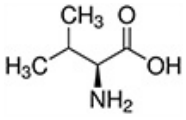
1.5.2.2 Specific objectives:

1. Production of six mutations (R95E, R95Q, G96V, D97K, D97N, F98I) in the RGD sequence of the Aα-chain of fibrinogen. New amino acids with neutral side chains (Q95 and N97) will be used to

replace the original amino acids that have charged side chains (positive charge of R95 and negative charge of D97). New amino acids with oppositely charged side chains, which are negative E95 and positive K97, will also be used to replace the original charged side chains in R95 and D97. A similar mutation to G96V in fibronectin was found to decrease the interaction between fibroblasts and fibronectin. F98I is a naturally occurring mutant from a patient with dysfibrinogenaemia just next to the RGD. Original and mutated amino acid structures are shown in Table 1-2.

2. The effect of the mutations on the interaction between fibrinogen and RBCs will be investigated by establishing several new methods; a plate binding assay, a solution-based interaction assay, a flow cytometric assay and clot retraction.
3. Effects of the mutations on fibrinogen and fibrin clot structure and function will be investigated using circular dichroism spectra, clot turbidity and lysis assays, clotability assay, laser scanning confocal microscopy and scanning electron microscopy.
4. The effect of the mutations on the interaction between platelets and fibrinogen will be investigated using platelet spreading assays.

Table 1-2 Original and mutated amino acid structure .Structure are taken form Sigma-Aldrich (Meric) website (https://www.sigmaaldrich.com/life-science/metabolomics/learning-center/amino-acid-reference-chart.html).

Amino Acid	Structure
Arginine (R)	
Asparagine (N)	
Aspartic Acid (D)	
Glutamic Acid (E)	
Glutamine (Q)	
Glycine (G)	
Isoleucine (I)	
Lysine (K)	
Phenylalanine (F)	
Valine (V)	

Chapter 2 Material and Methods

2.1 Site-Directed Mutagenesis

2.1.1 Plasmid Design

The cDNAs of the three fibrinogen chains were previously sub-cloned into plasmid p284, into the multiple cloning site that is located downstream from the adenovirus 2 major late promotor (MLP). The pMLP-α plasmid was constructed by S. Lord by insertion of a pVuII/SspI fibrinogen Aα cDNA fragments into SmaI-cleaved p284. The vector also contains the SV40 polyadenylation promotor and enhancer site as shown in Figure 2-1 (Binnie et al., 1993).

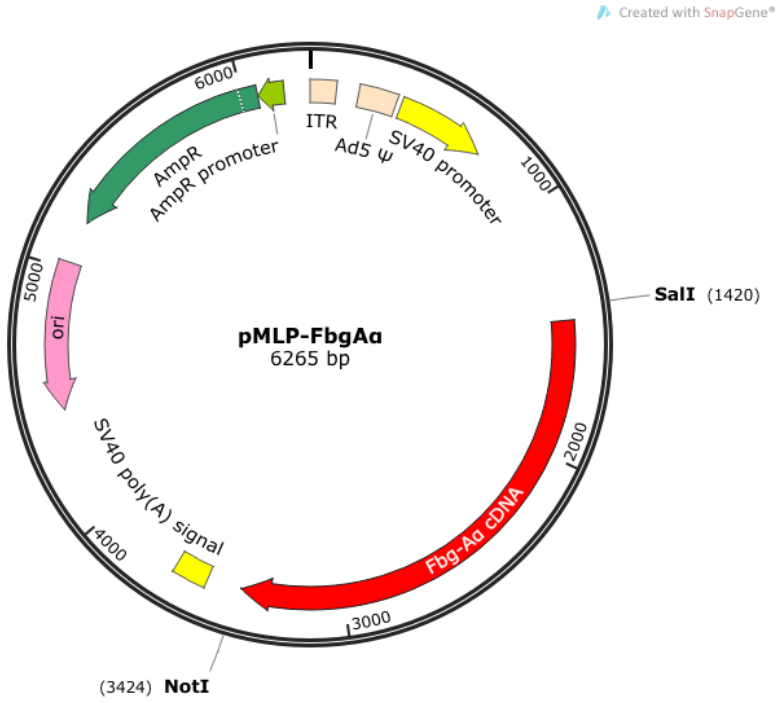


Figure 2-1 Representation of pMLP-Aα map. The figure is showing the pMLP vector of Aα-chain gene of fibrinogen which has a length of 6265 bp. It is composed of Ampicillin resistance (AmpR) promotor, origin of replication, SV40 promotor, Fgb-Aα is inserted between SalI and NotI restriction sites. The map was kindly provided by Dr Cédric Duval.

Chinese hamster Ovary (CHO) cells already transfected with Bβ- and γ-plasmids (βγ-CHO) of fibrinogen chains were used in this project. These cells, resistant to Geneticin G418, were co-transfected by the mutated pMLP-Aα plasmid together with the selection gene pMSV-his to allow the use of L-Histidinol as a selection marker for transfection.

2.1.2 Primer Design

For the site-directed mutagenesis, primers were designed using QuickChange® Primer Design Program (Agilent Technologies, Stockport, UK) as follow in Table 2-1, and ordered from Invitrogen (Paisley, UK)

Table 2-1 Primer sequences of the proposed mutations. F is the forward primer, R is the reverse primer for each mutation, F2 and R2 represent a second set of primers designed for R95Q and D97N. Highlighted letters represent the mutated codon. R95Q primers introduce a mutation from AGA (R) to CAA (Q), R95E primers introduce a mutation from AGA (R) to GAA (E), D97N primers introduce a mutation from GAT (D) to AAT (N), D97K primers introduce a mutation from GAT (D) to AAA (K), F98I primers introduce a mutation from TTT (D) to ATT (I).

Mutation	Primer
R95Q	F1: CGTTGACCACTAATATAATGGAAATTTTGCAAGGCGATTTTTCCTCAGCC
	R1: GGCTGAGGAAAAATCGCCTTGCAAAATTTCCATTATATTAGTGGTCAACG
	F2: CGTTGACCACTAATATAATGGAAATTTTGCAAGGCGATTTTTC
	R2: GAAAAATCGCCTTGCAAAATTTCCATTATATTAGTGGTCAACG
R95E	F: CGTTGACCACTAATATAATGGAAATTTTGGAAGGCGATTTTTCCTCAGCC
	R: GGCTGAGGAAAAATCGCCTTCCAAAATTTCCATTATATTAGTGGTCAACG
G96V	F: TAATATAATGGAAATTTTGAGAGTCGATTTTTCCTCAGCCAATAACC
	R: GGTTATTGGCTGAGGAAAAATCGACTCTCAAAATTTCCATTATATTA
D97N	F1: TATAATGGAAATTTTGAGAGGCAATTTTTCCTCAGCCAATAACCG
	R1: CGGTTATTGGCTGAGGAAAAATTGCCTCTCAAAATTTCCATTATA
	F2: CGTTGACCACTAATATAATGGAAATTTTGAGAGGCGATTTTTCC
	R2: GGAAAAATCGCCTCTCAAAATTTCCATTATATTAGTGGTCAACG
D97K	F: AATATAATGGAAATTTTGAGAGGCAAATTTTCCTCAGCCAATAACCGTGAT
	R: ATCACGGTTATTGGCTGAGGAAAATTTGCCTCTCAAAATTTCCATTATATT
F98I	F: GGAAATTTTGAGAGGCGATATTTCCTCAGCCAATAACCG
	R: CGGTTATTGGCTGAGGAAATATCGCCTCTCAAAATTTCC

2.1.3 Mutagenesis reaction

MATERIALS

- 100% Dimethyl Sulfoxide (DMSO) (New England BioLabs, Ipswich Massachusetts, USA).
- Chinese hamster Ovary (CHO) cells were kindly provided by Dr Susan Lord, University of North Carolina, USA.
- DpnI Restriction Enzyme (10 U/µl) (ThermoFisher Scientific, Paisley, UK).
- Nuclease-free water ddH_2O (Sigma-Aldrich, Dorset, UK).
- PCR machine Veriti® 96-Well Thermal Cycler, Applied Biosystems™ (ThermoFisher Scientific).
- PfuUltra High-Fidelity DNA polymerase (Agilent Technologies).
- Phusion High-Fidelity DNA polymerase (New England BioLabs).
- QuikChangeII Site-Directed Muatgenesis Kit (Agilent Technologies).
- Thermo Molecular Biology PCR tubes and Domed Caps (ThermoFisher Scientific).
- Water Bath (Grant, Shepreth, UK).

For each mutant, the reaction mixtures using *Phusion* High-Fidelity DNA Polymerase were prepared as shown in Table 2-2. The generation of the mutations occurred after different attempts, each mutant was produced under different conditions. The table is showing the successful combination of the reaction mixtures that were used in producing the mutations. Before the use of this enzyme and in the first few optimisation experiments, PfuUltra High-Fidelity DNA polymerase was used, but each attempt was unsuccessful.

Table 2-2 PCR mix for Site-Directed Mutagenesis using *Phusion* High-Fidelity DNA Polymerase.

Reaction	1	2	3	4	5	6	7
Template DNA	20 ng	20 ng	10 ng	10 ng	20 ng	20 ng	20 ng
5X Phusion HF Buffer	1X						
10 mM dNTPs	200 µM						
10 µM primers (F+R) mix	0.5 µM	0.5 µM	0.5 µM	0.5 µM	0.5 µM	0.5 µM	0.5 µM
100% DMSO	6%	12%	6%	12%	5%	3%	5%
***Phusion* DNA Polymerase**	2U					3U	
Nuclease-free water (ddH_2O)	Up to 50 µl						

The reaction mixtures in the PCR tubes were transferred to the PCR machine in a 98°C pre-heated block. The PCR reaction steps are shown in Table 2-3.

Table 2-3 PCR thermal cycling conditions.

Step	Temperature (°C)	Time (sec)
Initial Denaturation	98	30
18 cycles	Denaturation 98 Annealing 60 Extension 72	30 30 210
Final Extension	72	600
Hold	4	∞

Following the PCR, the tubes were transferred on ice and 1 µl of *DpnI* restriction enzyme (10 U/µl) was added directly to each tube and mixed by pipetting up and down, the tubes were then transferred to a 37°C water bath for 90 minutes (min).

2.1.4 Transformation

MATERIALS

- D-(−)-α-Aminobenzylpenicillin, Ampicillin (Sigma-Aldrich).
- Falcon Tubes (Sigma-Aldrich).
- Gallenkamp Economy Size 2 Incubator with Fan (GoIndustry DoveBid, Leeds, UK).
- Inoculation Loops (Sarstedt AG & Co.KG, Nümbrecht, Germany).
- LB-medium: 1% w/v Tryptone (Lab M Limited, Heywood, Lancashire, UK), 0.5% w/vg Yeast Extract, 1% w/v Sodium Chloride (NaCl), 500ml ddH_2O, autoclaved.
- Luria Broth (LB)-Agar: 3% w/v Tryptone, 0.5% w/v Yeast Extract (Lab M Ltd), 1% w/v NaCl 1.5% w/v Bacteriology Agar (Lab M Ltd), 300 ml ddH_2O, autoclaved (Classic Prestige Medical, Coventry, UK) then cooled to 50°C, Ampicillin was added to 100 µg/ml final concentration, and the Agar was poured into petri Dishes.
- NaCl (ThermoFisher Scientific).
- NanoDrop (ND-1000 Spectrophotometer v3.10, ThermoFisher Scientific).
- New Brunswick Scientific Model G-25 Incubator Shaker (Global Medical Instrumentation GMI, Ramsey, Minnesota, USA).
- PureYield™ Plasmid MaxiPrep System Kit (Promega Corporation, Madison, WI, USA).
- QIAprep® Spin MiniPrep Kit (QIAGEN®, Hilden, Germany).
- ROTANTA 460R Centrifuge (Hettich Zentrifugen, Tuttlingen, Germany).
- Super Optimal Broth with Catabolite Repression (SOC-medium) (Sigma-Aldrich).
- Water (Molecular Biology Reagent, Sigma-Aldrich).
- X320 Petri Dish NUNC with Lid Vented (100X15) mm Sterile (ThermoFisher Scientific).
- XL10-Gold Ultra-Competent Cells (Agilent Technologies).
- XL1-Blue Super-Competent Cells (Agilent Technologies).

A total of 50 µl of XL10-Gold Ultra-Competent Cells (XL1-Blue Super-Competent Cells in the first optimisation experiments) were transferred into a pre-chilled 50 ml falcon tube and 1 µl of the *Dpn-I*-treated DNA was added, they were then incubated on ice for 30 min. The bacteria were then heat shocked by transferring them to 42°C water bath for 45 sec and transferred on ice for 2 min, this step is required to insert the plasmid into the bacteria. 500 µl of SOC-medium were then added to the transformed bacteria and incubated at 37°C, in the G-25 Incubator Shaker at 225 rpm for 1 hr in order to start the bacterial growth. The bacteria were then inoculated using sterile inoculation loops in four different volumes (50 µl, 100 µl, 150 µl, 200 µl) on LB-Agar with ampicillin dishes, and incubated overnight in a 37°C incubator for the bacteria to grow if they had been successfully transformed with the DNA constructs including ampicillin resistance.

2.1.5 Plasmid DNA extraction

Five different single colonies of bacteria were randomly selected from the plates, and each colony was streaked on a separate square on the master plate as shown in Figure 2-2 using a sterile loop. For each colony, the same loop was stirred in 5 ml of LB-Medium containing Ampicillin (100 µg/ml), in a 50 ml falcon for overnight growth culture. The master plate was incubated overnight at 37°C, and the overnight growth culture tubes were incubated in the G25 Incubator Shaker at 225 rpm, 37°C.

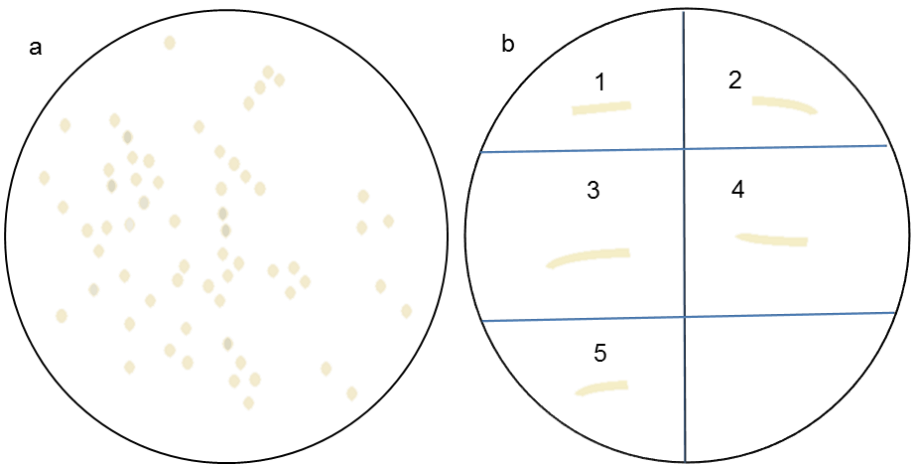


Figure 2-2 A schematic representation of the a) bacterial culture on the plate. b) master plate for plasmid extraction.

The next day, the overnight cultures were centrifuged at 6,800 g for 10 min at room temperature using ROTANTA 460R centrifuge. For each colony, the plasmid DNA was extracted from the bacteria using QIAprep® Spin MiniPrep Kit following manufacturer's instructions. The eluted DNA was quantified by NanoDrop and stored at -20°C for later transfection.

The DNA was sent for sequencing to the Medical Research Council Protein Phosphorylation and Ubiquitination Unit (MRC PPU) for DNA sequencing and services at the University of Dundee, Scotland. The sequences were then analysed using BioEdit™ (BioEdit Sequence Alignment Editor, Copyright© 1997-2013 Tom Hall, Ibis Biosciences, Carlsbad, CA, USA) and Serial Cloner™ (v2.6, Softonic Corporate, New York, USA) programmes. The obtained DNA sequences were subsequently aligned using ClustalW2 (The European Bioinformatics Institute (EMBL-EBI), Hinxton, Cambridge, UK) to the expected sequences, and similarities/differences in sequences were investigated.

2.1.6 Preparation of large quantities of plasmid DNA

For each mutation, the colony containing the correct sequence match (excluding silent mutations) of mutated DNA was transferred from its master plate (see above). Using a sterile loop, a small amount of bacteria was taken and stirred into a 50 ml falcon tube containing 5 ml LB-Medium, Ampicillin (100 µg/ml) then incubated for 1 hr in G25 Incubator Shaker at 225 rpm, 37°C. Following the incubation, the cultured bacteria were distributed equally between two of 1 L flasks containing 250 ml LB-Medium and Ampicillin (100 µg/ml), for overnight culture in the G25 Incubator Shaker at 225 rpm, 37°C.

PureYield™ Plasmid MaxiPrep System Kit was used to produce DNA from the cultured bacteria, following manufacturer's instructions. The eluted DNA was quantified using the NanoDrop, stored and sequenced as previously described.

2.2 Protein Production

2.2.1 Cell Transfection

MATERIALS

- 0.1% PBS/EDTA: PBS, 10% v/v Ethylene Diamine Tetra Acetic Acid EDTA (Sigma-Aldrich).
- 0.2 µm Filters (ThermoFisher Sientific).
- 1 ml Cryotubes (ThermoFisher Scientific).
- 1.5 ml Micro-Centrifuge Tubes, Eppendorf (ThermoFisher Scientific).
- 10 cm Dishes, Cell Culture Dish 100 mm x 20 mm Style (Corning®, New York, USA).
- 10 ml Disposable Plastic Syringe without Needle (Terumo Europe N.V., Leuven, Belgium).
- 10% Freezing Medium: 10% v/v DMSO (Sigma-Aldrich), 90% v/v FBS, filter sterilised.
- 10% v/v filter sterilised Glycerol (ThermoFisher Sientific).
- 2.5 M $CaCl_2$ (Sigma-Aldrich).
- 2X HEPES Buffer 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid (0.021% w/v Na_2HPO_4 (Sigma-Aldrich), 1.19% w/v HEPES (Sigma-Aldrich), 1.64% w/v NaCl (ThermoFisher Sientific), pH 7.05, in ddH_2O, filter sterilised).
- 5 cm X400 TC Dish Nunclon, 58 mm x 15 mm (ThermoFisher Sientific).
- 5 ml Costar Strippette Disposable Plastic Serogical Pipette (Corning®).
- Antibiotic Antimycotic Solution (100x), Stabilised with 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml, filter-sterile, BioReagent (Sigma-Aldrich).
- CKX41 Microscope (Olympus, Hamburg, Germany).
- Corning® Costar® TC-Treated 24-Well Plates (Corning®).
- DULBECCO'S Phosphate Buffered Saline (PBS sterile) pH 7.4 PBS (Invitrogen).
- Fisherbrand™ Electric Pipet Controller (ThermoFisher Sientific).
- Gilson Pipette (Gilson, Dunstable , UK).

- Growth Medium / Antibiotic: Growth Medium, 1% v/v Antibiotic-Antimycotic.
- Growth Medium: Dulbecco's Modified Eagle Medium “DMEM” F12 Ham (Invitrogen), 5% v/v Foetal Bovine Serum FBS, (ThermoFisher Sientific), 5% v/v Nu Serum (Scientific Laboratory Supplies Ltd (SLS), Nottingham, UK).
- IncuSafe, IR Sensor, Safe Cell Incubator (Sanyo, Japan).
- Integra Vacusafe Aspirator (Integra Biosciences Ltd, Thatcham, UK).
- L-Histidinol (L-His) Selection Medium: Growth Medium, 250 µM filter sterile L-His (SLS).
- Lipofectamine® 2000 (Invitrogen).
- Pasteur Pipette Rubber Bulb (Sigma-Aldrich).
- Pasteur Pipettes, short form 150 mm (Kimble Chase Life Science and Research Products, LLC., Meiningen, Germany).
- Pipetman Diamond Tips-Autoclavable TowerPack™ (Gilson).
- Reduced Serum Medium, Gibco™ Opti-MEM™ (modification of Eagle's Minimum Essential Media, ThermoFisher Sientific).
- ROTANTA 35 centrifuge, (Hettich Zentrifugen).
- Selection Medium: Growth Medium, 0.4% v/v selection marker G418/ Neomycin (Invitrogen).
- T75 Flask (Corning®).
- TipOne® Filter Tips (Star Lab, Hamburg, Germany).
- Trypsin-*EDTA*: 1X Trypsin-EDTA (Sigma-Aldrich).

Cell Culture Passaging

A 1 ml vial of βγ-CHO cell line was taken from liquid nitrogen (LN_2), rapidly defrosted by incubation in a 37°C water bath for one minute. The 1 ml cell content of the vial was added to 9 ml of selection medium and centrifuged at 800 g for 4 min. The pellet was then re-suspended in 10 ml of selection medium and incubated in a T75 flask at 37°C, 5% CO_2 IncuSafe Incubator (this is the incubation condition for all the subsequent culture work) until confluency was reached. To split the cells, medium was aspirated using glass Pasteur pipettes and Vacusafe aspirator; the cells were gently washed by adding 10 ml

PBS/EDTA, followed by aspirating the medium. Next, 1 ml of Trypsin-EDTA was added for a few minutes to detach the cells and 9 ml of selection medium were added to deactivate the trypsin and re-suspend the cells. 1 ml of the re-suspended cells was added to T75 flask containing 9 ml growth medium (1:10) and incubated at 37°C for freezing or further passaging as required.

cDNA Transfection

To prepare cells for transfection, re-suspended cells were seeded in 1:10 concentration into 3 of 10 cm petri dishes each containing 9 ml of selection medium by adding 1 ml of re-suspended cells in each dish. The cells were then incubated overnight to reach 30% confluency to be ready for Calcium Phosphate transfection which was used for R95Q, D97N and F98I mutations. Another type of transfection was used later in this project for the other mutations, which was the Lipofectamin 2000 method. For this method of transfection, 1 ml of re-suspended cells was seeded in 1:5 concentrations into 3 of 5 cm Petri dishes each, containing 4 ml of selection medium. The cells were then incubated overnight to reach confluency.

Calcium Phosphate Method

The next morning, the medium was replaced with 10 ml of fresh growth medium and incubated for 2 hours. The DNA reaction mixture was prepared an hour before transfection as per the following protocol in Table 2-4.

Table 2-4 Transfection reaction mix.

Reagent	Transfection A	Transfection B
cDNA pMLP-α	10 µg	20 µg
Selection vector pMSV-His (1:10 of DNA concentration)	1 µg	2 µg
$CaCl_2$ filter sterilised	0.25 M	0.25 M
Filter sterilised ddH_2O	Up to 500 µl	Up to 500 µl
2X HEPES buffer	500 µL	500 µL

Two different concentrations of plasmid DNA were used (10 µg, 20 µg) for each mutation to increase the chances of obtaining single clones with the new plasmid. For each transfection, 500 µl of HEPES buffer pH 7.05 were added into a 50 ml falcon tube, air bubbles were induced using a 5 ml Strippette and Electric Pipette Controller whilst the DNA reaction mixture was added drop-wise using a Gilson pipette. The transfection mixture was then left to stand at room temperature for 30 min. The DNA preparation was then added dropwise across one of the incubated petri dishes for each DNA concentration and incubated for 4 hrs at 37°C incubator.

The next step was glycerol shock for the mutated plasmid to enter the cells. The media were gently removed from the two transfected petri dishes, in addition to a one control dish that was not transfected, 2 ml of 10% v/v filter sterilised Glycerol were added to each dish and incubated for 3 min. Glycerol was removed and the dishes were washed quickly for three times with 5 ml PBS. Finally, 10 ml of growth medium were added, and petri dishes were incubated for two days at 37°C incubator.

Lipofectamine® 2000 Method

For the last group of mutations (R95E, G96V, D97K), a new transfection method with higher efficiency was used. In this method, 11 µg of DNA was transfected in 5 cm confluent dishes of CHO cells with 1:1 v/v ratio of Lipofectamine in a final volume of 500 µl. Two reaction Eppendorf tubes were prepared, each one containing either mutated DNA plasmid or Lipofectamine in final volume of 250 µl in Opti-MEM™ medium, and then incubated separately at RT for 5 min. Subsequently, the two tubes were combined together and incubated for 15 min at RT. During the incubation, media was changed to fresh growth media for the plate, which was going to be transfected. After the incubation period, DNA/Lipofectamine mixture was added dropwise to the plate and incubated for 6 hrs in a 37°C incubator. After 6 hrs, the medium was changed to fresh growth medium from the transfected plate and a control plate. Unlike the Calcium Phosphate method, cells were incubated at 37°C incubator for one day only.

Clone Selection

At this stage, both methods of transfection were treated the same. The cells were split at different concentrations (1:50, 1:100, 1:150, and 1:200) in duplicates using growth medium, and incubated for 2 days. Two controls (un-transfected) were split 1:50 and 1:200. The medium was then changed to L-His selection medium, and every two days, until sparse clones appeared. All dishes were checked under a CKX41 microscope for suitable clones. 24 single clones were selected that were large, healthy and as far apart from other clones as possible. The selected clones were then transferred into 24-well plate; each well containing 500 µl of growth medium/antibiotic for its corresponding clone. The cells were transferred using glass cloning cylinders, glass Pasteur pipette and rubber bulb after they were detached by Trypsin/EDTA. The 24-well plates were incubated until 60-70% confluency was reached. At this stage, the medium was harvested for ELISA, to measure the amount of fibrinogen produced by each clone, and the cells were trypsinised to be transferred into two of 5 cm dishes containing 4.5 ml growth medium/antibiotic per clone, and incubated until confluency. When ELISA results were available, only the highest 8-10 fibrinogen producing clones were kept. At confluency, each of the two dishes per clone

were trypsinised by 0.5 ml of Trypsin/EDTA, one for freezing (see below), whilst the other was re-suspended in 4.5 of growth medium/antibiotic and split in 1:10 into a 10 cm dish and incubated until confluency was reached again. When the cells in the petri dishes were confluent again, their media were changed to serum-free media with antibiotic Antimycotic for a week. The media were then harvested for a second ELISA in order to select the highest producing clone for the fibrinogen expression stage. The following five highest producing clones were retained in LN_2 as a backup whereas the other clones were discarded.

Cell Freezing

The other 5 cm dish was re-suspended in 9.5 of growth medium/antibiotic then centrifuged at 800 g, room temperature for 4 min using ROTANTA 35 centrifuge, the pellet was then re-suspended in 1 ml of 10% freezing medium and transferred into 1 ml Cryotubes. The tubes were incubated overnight at -80°C then transferred to liquid nitrogen.

2.2.2 Fibrinogen ELISA

MATERIALS

- Blocking Buffer: 1% w/v Bovine Serum Albumin (BSA) (Sigma-Aldrich).
- Buffer A: Coating Buffer; 0.039% w/v of Sodium Phosphate Monobasic Dihydrate $NaH_2PO_4.2H_2O$ (Sigma-Aldrich), 0.1.335% w/v of Sodium Phosphate Dibasic Dihydrate $Na_2HPO_4.2H_2O$ (Sigma-Aldrich), 0.8474% w/v of NaCl in ddH_2O, pH 7.2.
- Buffer B: Buffer A, 0.2% v/v Tween20, 2.075% w/v NaCl (ThermoFisher Scientific), pH 7.2.
- Buffer C: Citrate Phosphate Buffer; 0.73% w/v Citric Acid.H_2O (Sigma-Aldrich), 1.187% w/v $Na_2HPO_4.2H_2O$, in ddH_2O, pH 5.0.
- Buffer E (stop buffer): 1.5 M Sulfuric Acid (Sigma-Aldrich).
- Coating Fibrinogen Antibody: 3 µl of Polyclonal Rabbit Anti-Human Fibrinogen Antibody (DAKO, Agilent Technologies), 12 ml of Coating Buffer A (1:4000) dilution.

- Detecting Fibrinogen Horseradish Peroxidase HRP-Antibodies: 1 µl Goat Polyclonal to Fibrinogen HRP (Abcam, Biotech, Cambridge, UK), 16 ml buffer B.
- Dynex, MRX TC Revelation Microplate reader (LABEQUIP LTD, Ontario, Canada).
- F96 Maxisorp Nunc-Immuno Plate NUNC (Sigma-Aldrich).
- OPD solution: 4 tablets of 1,2-Phenylenediamine Dihydrochloride OPD (2 mg, DAKO, Agilent Technologies), 5 µl of 30% Hydrogen Peroxide (Sigma-Aldrich) in12 ml buffer C.
- Plastic Film Thermo Scientific™ Sealing Tapes (ThermoFisher Scientific).
- Stuart Microplate Shaker/ SSM5 Shaker (Bibby Scientific Limited (Group HQ), Staffordshire, UK).

A 96-well Maxisorp plate was coated by adding 100 µl of fibrinogen antibody (1:4000) to each well, then covered by a plastic film and incubated overnight in the cold room. In all the following steps, unless stated otherwise, the plate was covered and incubated at room temperature on a shaker at 400 rpm for 60 min. Next day, the antibodies were removed and the plate was blocked by adding 150 µl of 1% w/v BSA blocking buffer to each well and incubated. During the incubation period, a standard curve was prepared by diluting recombinant fibrinogen standard 100 µg/ml in buffer B as follows: 1 µg/ml, 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml, 31.25 ng/ml, 15.63 ng/ml, 7.81 ng/ml, 3.91 ng/ml, 1.95 ng/ml and blank. At the end of the incubation, the blocking buffer was removed and the plate was washed 3 times with 200 µl per well buffer B (this is how all the subsequent washes were performed). Total of 100 µl of each standard or neat media samples were added to the wells, in duplicate, incubated again for 120 min. Following the incubation, the standards and samples were removed and the plate was washed before 100 µl per well of the HRP-labelled antibodies were added, and incubated. The HRP-antibodies were removed afterwards and the plate was washed. Total of 100 µl per well of OPD solution were then added for a few minutes while waiting for the yellow colour to develop. The reaction was stopped by adding 100 µl per well of buffer E just before the plate was read using Dynex, Microplate reader at wavelength λ=490 nm. The

fibrinogen concentration of each sample was plotted against the standard curve using Microsoft Excel 2013.

2.2.3 Fibrinogen Expression

MATERIALS

- 0.1 M PMSF: 1.74% w/v of Phenyl Methane Sulfonyl Fluoride PMSF (Sigma-Aldrich) in 99.7% Isopropanol (Sigma-Aldrich).
- 850 cm^2 Roller Bottle Vented Cap (Corning®).
- Beads: 4% w/v Cytodex Microcarrier Beads - Cytodex1, 131-220 µm, (SLS) in 10 mM PBS, autoclaved.
- CK2 Microscope (Olympus).
- Growth Medium with Antibiotic: 2% v/v Antibiotic-Antimycotic in Growth Medium.
- ITS Medium: DMEM F12, 2% v/v Antibiotic-Antimycotic, 0.001% w/v filter-sterile Insulin Transferrin Sodium Selenite Supplement ITS (Roche Diagnostics, Burgess Hill, UK), 0.004% w/v filter-sterile Aprotinin (Sigma-Aldrich).
- Jencons Wheaton R2P Roller Cultural Apparatus Incubator (ThermoFisher Scientific).
- Serum Free Medium/ Antibiotic: 2% v/v Antibiotic-Antimycotic in DMEM F12 Ham.
- Supplement Free Medium: DMEM F12 Ham.
- Whatman Filter Paper Grade 2 (ThermoFisher Scientific).

According to the fibrinogen concentration data obtained with the ELISA from the serum-free medium incubation, the highest expressing clone was taken from the liquid nitrogen, defrosted and seeded in a T75 flask in growth medium with antibiotic to confluency (as per section 2.2.1). When confluent, the cells were split into threeT75 flasks; two were used for freezing new stock samples of this highest producing clone into 6 frozen vials as stated above. The third flask was used for further splitting into 4 other flasks to proceed with mutated fibrinogen expression. The cells were subsequently split into fourty of 10 cm petri dishes

and incubated until confluency was reached. Each two dishes (20 ml cells re-suspension) were combined into one roller bottle that contained 200 ml growth medium with antibiotic. The cells were incubated in the roller bottles incubator at 37°C, 5% CO_2 for two days, these incubation conditions were used throughout the expression stage in the roller bottles. The medium was changed every 2 days by replacing 100 ml of current medium by the same amount of fresh growth medium with antibiotic. During this period, beads were prepared in PBS and autoclaved to be ready when needed. When the cells became confluent, PBS was aspirated from the precipitated autoclaved beads; and they were washed twice by 500 ml of supplement free medium, mixed and left to sediment again. After the washes, 500 ml of growth medium with antibiotic was added to the beads and mixed to give a suspension of beads in medium. For each roller bottle, 100 ml of medium was discarded and replaced by 50 ml of the beads suspension and 50 ml growth medium with antibiotic, and incubated for three days. Following the incubation period, the cells were checked for their adherence to the beads and for confluency. Once confluency was reached, cells were washed from the serum present in the growth medium by adding 50 ml of serum free medium with antibiotic, the bottle was laid down and rolled slowly for a full turn. The medium was then discarded again and replaced by 100 ml of serum free medium with antibiotic and incubated overnight to ensure the full removal of serum. The serum free medium was replaced by 200 ml of ITS medium, incubated for two days. The next step was starting the media collection every two days by replacing a 100 ml of ITS medium in the roller bottle by a fresh 100 ml of the same medium. The collected medium was filtered and 1 ml aliquot was reserved for ELISA before adding PMSF to 0.15 mM final concentration. The media and the ELISA aliquots were stored at -40°C until the media were used for fibrinogen precipitation and purification. The ELISA aliquots were used to perform ELISA every two weeks to monitor the fibrinogen production.

2.2.4 Fibrinogen Precipitation

MATERIALS

- 1 L Nalgene Centrifuge Bottles (ThermoFisher Scientific).
- Avanti J26S x PI Centrifuge (Beckman Coulter, High Wycombe, UK).
- Cling Film (The Lab Warehouse, Grays, UK).
- JA 25.50 rotor of Avanti J26S x PI Centrifuge (Beckman Coulter).
- Jenway 1000 Stirrer (Keison Products, Chelmsford, UK).
- JLA 8.100 Rotor of Avanti J26S x PI Centrifuge (Beckman Coulter).
- Pellet Cocktail: 0.066% w/v ε-Amino Caproic Acid-Aminohexanoic acid (εACA) (SLS), 0.079% w/v Benzamidine Hydrochloride Hydrate (SLS), 5 µM Pepstatin A (SLS), 5 µM Leupiptin (SLS), 100 µM PMSF, 996.098 U/ml Soybean Trypsin Inhibitor, (SLS), 1 mM EDTA (Sigma-Aldrich), 300 mM NaCl (ThermoFisher Scientific), 200 mM Tris-Base, pH 7.4 (Sigma-Aldrich), in ddH_2O.
- Protease Inhibitor Cocktail: 2.18% w/v εACA, 2.62% w/v Benzamidine, 1 µM Pepstatin A, 35 µM Leupiptin, 3.5 mM PMSF, 700 mM MES-Hydrate 2-(N-Morpholin Ethanesulfonic Acid Hydrate buffer pH 5.6 (SLS) .
- Saturated Ammonium Sulphate: 76% w/v Ammonium Sulphate (ThermoFisher Scientific) in ddH_2O.

When adequate amount of expression medium was available, precipitation was started, 6 L of collected medium was defrosted and poured equally between two of 5 L beakers, 100 ml of protease inhibitors cocktail, which were prepared on ice were added to each beaker and stirred at 500 rpm in the cold room. While the medium and proteases were stirring, 2 L of saturated ammonium sulphate were added for each 3 L of medium slowly by filtration; foaming was avoided by slowing down the stirring during the addition of ammonium sulphate. Ammonium sulphate was added slowly at 4°C to prevent denaturation of fibrinogen by creating an uneven pH around the protein. The beaker was then covered by cling film and incubated in the cold room overnight with no stirring. Next day, one of the 5 L of medium was distributed between 6 of 1 L Nalgene centrifuge bottles,

placed in pre-cooled JLA 8.100 rotor of the Beckman Coulter centrifuge and centrifuged at 14,500 g, 4°C, maximum acceleration with no break for deceleration, for 45 min. After the first run, the medium was discarded and the same steps were repeated by transferring the remaining 5 L of medium in the same bottles. After the second run, the pellets were dissolved in 2.8 ml of pellet protease inhibitor cocktail per bottle and kept on ice for 30 min in a 50 ml Nalgene centrifuge tube. They were then placed in a pre-cooled JA 25.50 rotor and spun at 43,000 g, 4°C, maximum acceleration and deceleration, for 30 min. The supernatant was collected in 5 ml aliquots and stored at -80°C until purification.

2.2.5 Fibrinogen Purification

MATERIALS

- 1 M $CaCl_2$ (Sigma-Aldrich).
- 10 ml Disposable Plastic Syringe without Needle (Terumo Europe N.V.).
- AKTA AVANT Chromatography System (GE Healthcare Life Sciences, Buckinghamshire, UK).
- Anti-Fibrinogen monoclonal antibodies (mAb), Clone IF-1 (10 mg; Kamiya BioMedical Co, Tukwila, WA, USA) / CnBr activated Sepharose 4B (1.5 mg; VWR; Lutterworth, UK).
- EDTA (Sigma-Aldrich).
- Elution Buffer B1: 20 mM Tris, 300 mM NaCl, 5 mM EDTA, pH 7.4.
- Equilibration Buffer A1: 20 mM Tris, 300 mM NaCl, 1 mM $CaCl_2$, pH 7.4.
- NaCl (ThermoFisher Scientific).
- Tris-Base (Sigma-Aldrich).
- Tube roller (Star Lab).
- Wash Buffer I A2: 20 mM Tris, 1 M NaCl, 1 mM $CaCl_2$, pH 7.4.
- Wash Buffer II A3: 5 mM Na-Acetate (Sigma-Aldrich), 300 mM NaCl, 1 mM $CaCl_2$, pH 6.0.
- XK16/20 column (GE Healthcare Life Sciences).

Fibrinogen was purified by affinity chromatography using an AKTA AVANT and IF-1 Anti-Fibrinogen calcium-dependant monoclonal antibodies. 5 ml of the supernatant, which were collected from each precipitation were defrosted each time and 10 mM final concentration of $CaCl_2$ was added, and incubated on a roller at room temperature for 10 min just prior to the injection using a syringe that is compatible to the injected volume. Following sample loading with 1 column volume (each column volume equals 7.438 ml), the column was washed using 5X column volumes of washing buffer A2 then A3, and the fibrinogen was eluted from the column using 7X column volumes of EDTA containing buffer B1. Finally, the column was equilibrated with 5X column volumes of buffer A1. The eluates of the fibrinogen peak fraction collector in 2 ml aliquots were chosen by using 1.51 extension co-efficient and collected, pooled and stored at -80°C until dialysis.

2.2.6 Dialysis

MATERIALS

- 10X Dialysis Buffer: 500 mM Tris pH 7.4, 1 M NaCl, pH 7.4.
- 100,000 MWCO, 5-20 ml Pierce™ Protein Concentrator (ThermoFisher Scientific).
- NanoDrop (ND-1000 Spectrophotometer V3.10, ThermoFisher Scientific).
- D-tube Dialyzer Maxi, MWCO 12-14 kDa (Merck Chemicals GmbH, Darmstadt, Germany).

The eluted fibrinogen was defrosted, pooled and kept cool on ice in the cold room. The concentration started by adding the fibrinogen in two of Pierce™ Protein concentrators, spun at 3,000 g for 30 min. The fibrinogen was refilled in each concentrator after each spin, the amount of fibrinogen added and the time of spin varied according to how fast the elution buffer was passed through the membrane filtering the fibrinogen. This process was continued until the remaining fibrinogen in the concentrators was approximately 1 ml; at that point, the fibrinogen was transferred into D-tube Dialyzer in cold room. Fibrinogen was stirred at low speed 50 rpm for 1 hr in a 500 ml dialysis buffer, the dialysis buffer was then replaced by a fresh 1 L dialysis buffer, and incubated overnight while

still stirring at low speed in the cold room. Next morning, the dialysis buffer, was replaced again with a fresh 500 ml dialysis buffer, and stirred again for 1 hr. The concentration of fibrinogen was read at absorbance 280 nm using the NanoDrop (extinction coefficient 15.1). The purified fibrinogens were then aliquoted and stored at -80°C ready for functional analysis (Duval et al., 2014).

2.3 SDS-PAGE

MATERIALS

- 1X Running Buffer: 5% v/v of 20X NuPage MES SDS Running Buffer, pH 7.3 (Invitrogen), 0.25% v/v NuPage Antioxidant (Invitrogen), ddH_2O up to 500 ml.
- G:Box (SYNGENE, Cambridge, UK).
- Gel Code® Blue Stain Reagent (ThermoFisher Scientific).
- Ladder: Precision Plus Protein Standards (Bio Rad, Hercules, CA, USA).
- Lid: X-Cell superlock (ThermoFisher Scientific).
- NuPage 4-12% Bis-Tris Gel (Invitrogen).
- NuPage Lithium dodecyl sulfate (LDS) Sample Buffer 4X, pH 8.4 (Novex life cell technologies, Carlsbad, USA).
- PowerPac™ Basic 300 Power Pac 300 V Power Supply (Bio Rad).
- Sample Reducing Buffer 10X (Novex).
- Stuart Microplate Shaker/ SSM5 Shaker (Bibby Scientific Limited).
- Tank: Mini-Cell (Novex).

The sample reduction reaction buffer was prepared by adding 2.5 µg fibrinogen to 1X of (LDS) Sample buffer, 1X of Sample Reducing buffer final concentrations, made up to 25 µl by ddH_2O. The samples were vortexed, heated at 95°C for 10 min then transferred on ice before they were loaded (25 µl per well) into the gel. To identify the produced bands, 5 µl of the Ladder (5-250 KDa) were loaded as a reference well. Running buffer was added to the tank and run at 140 Volt for 90 min. Next, the gel was washed by ddH_2O and incubated in the coomassie blue stain on a shaker at 60 rpm, room temperature for 1 hr, followed by washing 4 times by ddH_2O on a shaker at 60 rpm, room temperature for 15 min each.

After the last wash, fresh ddH_2O was added and the gel was incubated overnight in the cold room. The gel was imaged using G:Box.

2.4 Fibrinogen Clotability Assay

MATERIALS

- 1 M $CaCl_2$ (Sigma-Aldrich).
- 250 U/ml α-Thrombin (Sigma-Aldrich).
- Activation Mix: 0.1 U/ml Thrombin, 5 mM $CaCl_2$ in 1 X TBS.
- Eppendorf™ MiniSpin™ Microcentrifuges (Sigma-Aldrich).
- NanoDrop (ND-1000 Spectrophotometer V3.10, ThermoFisher Scientific).

This method is relying on volumes rather than concentrations. To test the clotability of the produced mutants, 20 µl of each recombinant fibrinogen was added to TBS at final volume of 50 µl, and measured using NanoDrop 3 times each at Abs=1 mg/ml to exclude the thrombin and Calcium when blanking them, λ=280 nm. Next, 20 µl of activation mix was added to the fibrinogen and incubated at 37°C water bath for 2 hrs, a blank control reading was also made by adding the activation mix to 50 µl TBS. After incubation, the samples were centrifuged at 13,000 g for 1 hr in a micro-centrifuge to pellet the coagulated fibrin. The supernatants were transferred to fresh tubes and measured using NanoDropp again 5 times to measure the non-coagulated protein remaining in the supernatant. The results were analysed using Excel 2013 and GraphPad Prism 7.0 using one way ANOVA multiple comparison using WT as a control column and Dunnett's test for p-value.

2.5 Turbidity and Lysis Analysis

MATERIALS

- 1 M $CaCl_2$ (Sigma-Aldrich).
- 1X Tris-Buffered Saline (TBS): 50 mM Tris-Base, 100 mM NaCl, pH 7.4.
- 11 µM Human Glu-plasminogen (Enzyme Research \laboratories Ltd, Swansea, UK).

- 140,000 pM Tissue Plasminogen Activator (tPA) (Pathway Diagnostics, Dorking, UK).
- 250 U/ml α-Thrombin (Sigma-Aldrich).
- 384-Well Plate (Greiner bio-one, Stonehouse, UK).
- Activation Mix for Lysis: 25 mM $CaCl_2$, 1.25 U/ml α-Thrombin, 500 pM tPA (Pathway Diagnostics) in 10 µl final volume TBS.
- Bio-Tek ELX808 Microtiter Plate Reader (Labtech International; Ringmer, UK).
- Lysis Mix: 1.2 µM Human Glu-plasminogen (Enzyme Research \laboratories Ltd) in 1X TBS, pH 7.4 in 10 µl final volume TBS.
- Master Mix for turbidity: 10 mM $CaCl_2$, 0.2 U/ml Human α-Thrombin (Sigma-Aldrich), in 50 ul TBS for 96 well-plate and 25 ul TBS for 348 well-plate final volume, pH 7.4.
- NaCl (ThermoFisher Scientific).
- Tris-Base (Sigma-Aldrich).

2.5.1 Turbidity Using a 348-Well Microtiter Plate

For R95Q, D97N, F98I, 25 µl of fibrinogen were added in triplicates to 348 well-plate and combined with 25 µl of Master Mix just before the reading, to achieve final concentrations of 0.5 mg/ml fibrinogen, 5 mM $CaCl_2$ and 0.1 U/ml α-Thrombin in final volume of 50 µl. Absorbency was measured at 340 nm, every 12 sec for 2 hrs at 37°C, using microtiter plate reader (Duval et al., 2014). The results were analysed using Excel 2013 and GraphPad Prism 7.0 using one way ANOVA.

For the new mutants (F95E, G96V, D97K), where the amount of protein was less than other mutants, 25 µl of fibrinogen was combined with 25 µl Master Mix to achieve final concentrations of 0.2 mg/ml fibrinogen, 5 mM $CaCl_2$ and 0.1 U/ml Thrombin in final volume of 50 µl. The plate was read in the same conditions as above, and the results were analysed using Excel 2013 and GraphPad Prism 7.0 using one way ANOVA multiple comparison using WT as a control column and Dunnett's test for p-value.

2.5.2 Lysis Using a 348-Well Microtiter Plate

In each well of the 348-well microtiter plate, 30 µl of fibrinogen were added to the final concentration of 0.5 mg/ml, and then 10 µl of Lysis Mix was added to the final concentration 0.24 µM plasminogen. Next, 10 µl of the Activation Mix for Lysis were added just before reading the plates with 10 sec gaps between each column to the final concentration of 5 mM $CaCl_2$, 0.25 U/ml α-Thrombin, 100 pM tPA. Absorbency was measured at 340 nm, every 12 sec for 2 hrs at 37°C, using a microtiter plate reader (Duval et al., 2014). The results were analysed using Excel 2013 and GraphPad Prism 7.0 using one way ANOVA multiple comparison using WT as a control column and Dunnett's test for p-value.

2.6 Circular Dichroism Spectroscopy

MATERIALS

- 1 mm Path-Length Cuvette (Applied Photophysics, Surrey, UK).
- 1 X TBS: 50 mM Tris-Base, 100 mM NaCl, pH 7.4.
- Chirascan Plus Spectropolarimeter (Applied Photophysics).
- NaCl (ThermoFisher Scientific).
- Tris-Base (Sigma-Aldrich).

To study the effects of the mutations on the secondary structure of the proteins, Circular Dichroism spectroscopy (CD-spectra) technique was used. To perform the experiment, 0.2 mg/ml final concentration in 200 µl final volume TBS was transferred into a cuvette and the far-UV spectroscopy was recorded for 190-260 nm spectra using a Chirascan Plus Spectropolarimeter. The results were analysed using CDNN (Circular Dichroism analysis using neutral Network) analysis software. CD spectra was performed with the help of Dr Nasir Khan, School of Molecular and Cellular Biology, University of Leeds.

2.7 Labelling Fibrinogen

MATERIALS

- AlexaFluor® Antibody Labelling Kit (Molecular probes® by life technologies, Paisley, UK).
- NanoDrop (ND-1000 Spectrophotometer V3.10, ThermoFisher Scientific).
- 10 mM PBS (One tablet of PBS dissolved in 200 ml of deionized water yields 0.01 M Phosphate Buffer, 0.0027 M Potassium Chloride (KCl) and 0.137 M NaCl, pH 7.4, at 25°C. Sigma-Aldrich).

Mutated fibrinogen was fluorescently labelled with AlexaFluor^{488} dye according to the manufacturer's instructions. 100 µl of each mutant was dialysed overnight in PBS to purify them from any ammonium that may remain from the ammonium sulphate precipitation. The concentration of the produced labelled protein was measured by a NanoDrop at 280 nm (protein) and 494 nm (dye) using the formula provided by the manufacturer. The degree of labelling was calculated using the formula provided by the manufacturer.

2.8 Laser Scanning Confocal Microscopy

MATERIALS

- 1 M $CaCl_2$ (Sigma-Aldrich).
- 1X TBS.
- 250 U/ml α-Thrombin (Sigma-Aldrich).
- AlexaFluor^{488}-labelled fibrinogen.
- Scanning Confocal Electron Microscopy LSM700 inverted Zeiss (Zeiss, Jena, Germany).
- Uncoated 15 µ-slide VI 0.4 (Ibidi GmbH, Martinsried, Munich, Germany).

The clot for confocal microscopy was prepared in 40 µl per each channel of the slide final volume in TBS by adding fibrinogen to the final concentration of 0.5 mg/ml, AlexaFluor^{488}-labelled fibrinogen to the final concentration of 25 µg/ml, $CaCl_2$ to the final concentration of 5 mM and just before transferring the clot to

its corresponding channel of the slide, thrombin was added to the final concentration of 0.1 U/ml, mixed quickly by pipetting. The 40 µl of clot mix were then transferred into the corresponding channel of the slide, while care was taken to avoid the presence of bubbles. The slide was kept in a humidity chamber at room temperature overnight to prevent dehydration of the clot. The clot was then imaged using a X40/1.3 Oil objective, at wavelength 494 nm emission and 519 excitation for AlexaFluor^{488}. Z-stacks were taken at 20.3 µm thick for 30 slices. The images were then analysed for fibre density, using imageJ-win64.exe (Fiji) and quantified using macro plugins of the programme New Clot Density PC1024 batch and the results were analysed using Excel 2013 and GraphPad Prism 7.0 using one way ANOVA multiple comparison using WT as a control column and Dunnett's test for p-value.

2.9 Blood Preparation

MATERIALS

- 1% v/v of 0.5 M EDTA (Sigma-Aldrich).
- 10 ml Disposable Plastic Syringe without Needle (Terumo Europe N.V.).
- 2.5 ml Disposable Plastic Syringe without Needle (Terumo Europe N.V.).
- Real-time PCR (Transnetyx, Cordova, USA).
- Surflu Winged Infusion Set (Terumo Europe N.V.).

2.9.1 Human Blood

Blood was taken by venepuncture (median cubital vein) from healthy volunteers, after informed consent under the ethical approval from the School of Medicine Research Ethics Committee, reference number HSLTLM12045 (Appendix B). Before obtaining the blood, the first 2.5 ml of blood were discarded using 2.5 ml syringe and winged needle. Using 10 ml syringe and the same needle, 10 ml of fresh blood were collected in 1% v/v EDTA.

2.9.2 Murine Blood

Murine blood was kindly provided by Dr Cédric Duval. All procedures were performed according to accepted standards of humane animal care, approved by the ethical review committee of the University of Leeds, and conducted under license from the United Kingdom Home Office. All animals were maintained in individually ventilated cages, at 21°C 50-70% humidity, light/dark cycle 12/12 hrs on standard diet ad libitum. $FGA^{-/-}$ mice (Suh et al., 1995), on C57BL/6J background, were generated from $FGA^{-/-}$ (male) x $FGA^{+/-}$ (female) crossing, and genotypes were determined using real-time PCR. Animals were bled from the inferior vena cava, under terminal anaesthesia, with 1% v/v EDTA, and terminated by cervical dislocation. Three mice were used in the plate-binding assay method; 30 weeks old male, 21 and 28 weeks old females.

2.9.3 Isolation of Red Blood Cells

MATERIALS

- 10 mM PBS (One tablet of PBS dissolved in 200 ml of deionized water at 25°C. Sigma-Aldrich).
- Jouan CR3i centrifuge (DJB Labcare Limited, Buckinghamshire, UK).

Blood was centrifuged at 1,040 g 4°C for 10 min. To wash the RBCs, a mark was drawn on the top of the plasma layer. The plasma was then removed, discarded and replaced by the same amount of PBS. The tube was then inverted gently 10 times to mix the blood and PBS before centrifuging the blood at 1,040 g, 4°C for 5 min, this washing step was repeated again for 3 more times. After the last wash, the supernatant was discarded and the produced RBCs were used in the different experiments. When the supernatant was discarded in each wash, a very small amount from the surface of the RBCs was discarded as well to ensure the elimination of white blood cells and other cells and that only RBCs were collected. Murine blood was isolated using the same method in a micro-centrifuge. EDTA anticoagulation, centrifuge speeds and number of washes were taken from Carvalho *et al.* (2010).

2.10 Fibrinogen-Red Blood Cell Plate-Binding Assay

MATERIALS

- 0.5% v/v DiD Vybrant™ Cell-Labelling Solution (Molecular Probes Europe BV, Leiden, The Netherlands). Mouse Anti-Human CD51/CD61 (Integrin alpha v beta 3 $\alpha_v\beta_3$) purified antibodies 100 µg (OriGene Technologies, Inc., Rockville, USA).
- 10 mM PBS (One tablet of PBS dissolved in 200 ml of deionized water at 25°C. Sigma-Aldrich).
- F96 Maxisorp Nunc-Immuno Plate NUNC (Sigma-Aldrich).
- Fibrinogen, Plasminogen Depleted, Human Plasma (Calbiochem, San Diego, USA).
- Mouse anti-Melanoma Human cells Monoclonal Antibody Integrin alpha V α_V (NKI-M9) 200 µg (Santa Cruz Biotechnology, Inc., Dallas, USA).
- Normal Mouse IgG unconjugated, affinity purified isotope control immunoglobulin 200 µg (Santa Cruz Biotechnology, Inc.).
- Plastic Film Thermo Scientific™ Sealing Tapes (ThermoFisher Scientific).
- Stuart Microplate Shaker/ SSM5 Shaker (Bibby Scientific Limited).

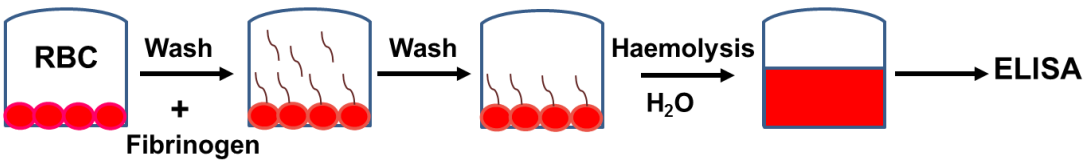


Figure 2-3 A schematic representation of the steps of fibrinogen-RBCs plate binding assay. Coating with RBCs, adding fibrinogen, haemolysing, then storage for ELISA.

Commercial fibrinogen was used in the optimisation steps. Washed RBCs were diluted to 5% haematocrit (Ht) in PBS. First, 100 µl/ well of the 5% RBCs were coated in a Maxisorp plate and incubated on a shaker at 400 rpm, room temperature for 1 hr. By the end of the incubation, the plate was washed gently 3 times by PBS then drained on tissue between washes, to prevent hard tapping of the plate which may get rid of RBCs. To test the interaction of the RBC with

fibrinogen, 100 µl per well of 20 µg/ml of fibrinogen were incubated for 1 hr, on a shaker at 400 rpm, room temperature, and then washed as above. Next, the cells were haemolysed by adding 100 µl per well of ddH_2O incubated for a few minutes on the shaker. The plate was then stored at -80°C overnight for future quantitation of RBC-bound fibrinogen by ELISA, as described in section 2.2.2 and the results were analysed using Excel 2013 and GraphPad Prism 7.0 using one way ANOVA multiple comparison using WT as a control column and Dunnett's test for p-value. A schematic representation of the method is shown in Figure 2-3. DiD Vybrant™ Cell-Labelling Solution was used in the optimisation steps of the experiment.

The same experiment was done to determine the effect of some inhibitory antibodies on the binding between fibrinogen and RBCs. To each 20 µg/ml of WT fibrinogen, 10 µg/ml final concentration of either IgG, α_V, $\alpha_V\beta_3$ antibodies were added in 100 µl total volume in PBS.

2.11 Fibrinogen-Red Blood Cell Binding Assay in Solution

MATERIALS

- 1% w/v (BSA) (Sigma-Aldrich).
- 10 mM PBS (One tablet of PBS dissolved in 200 ml of deionized water at 25°C. Sigma-Aldrich).
- Coating Fibrinogen Antibody: 3 µl of Polyclonal Rabbit Anti-Human Fibrinogen Antibody (DAKO, Agilent Technologies), 12 ml of Coating Buffer A (1:4000) dilution.
- Dynex, MRX TC Revelation Microplate reader (LABEQUIP LTD).
- Eptifibatide (Tocris, Abingdon, UK).
- F96 Maxisorp Nunc-Immuno Plate NUNC (Sigma-Aldrich).
- Mouse Anti-Human CD51/CD61 (Integrin alpha v beta 3 $\alpha_v\beta_3$) purified antibodies 100 µg (OriGene Technologies, Inc., Rockville, USA).
- Mouse anti-Melanoma Human cells Monoclonal Antibody Integrin alpha V α_V (NKI-M9) 200 µg (Santa Cruz Biotechnology, Inc., Dallas, USA).

- Normal Mouse IgG unconjugated, affinity purified isotope control immunoglobulin 200 µg (Santa Cruz Biotechnology, Inc.).
- Plastic Film Thermo Scientific™ Sealing Tapes (ThermoFisher Scientific).
- Stuart Microplate Shaker/ SSM5 Shaker (Bibby Scientific Limited).
- Tube roller (Star Lab).

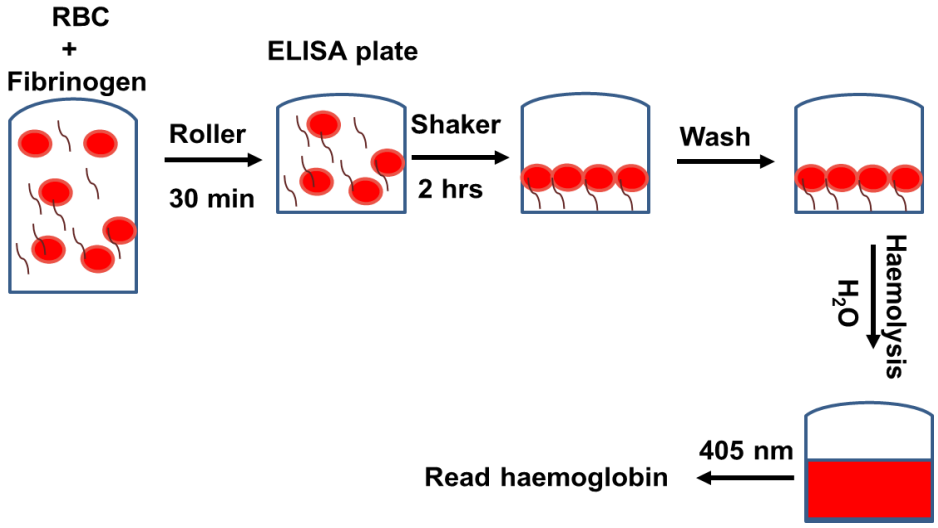


Figure 2-4 A schematic representation of the steps of fibrinogen-RBCs binding assay in solution. Mixing fibrinogen and RBCs in solution, transfer to ELISA plate, haemolysis then read fibrinogen released at 405 nm.

A Maxisorp plate was coated with 100 µl Fibrinogen Antibodies (1:4,000) on a shaker at room temperature, 400 rpm for 1 hr then blocked by 300 µl of 1% BSA overnight in the cold room. The next day, fibrinogen and RBCs were mixed together in PBS as follows, 20 µg/ml of fibrinogen were incubated with 5% Ht of RBCs to 300 µl total volume on a roller at room temperature for 30 min. The plate was washed once using the ELISA washing buffer before adding 100 µl of the fibrinogen-RBC mix in duplicates and incubating on a shaker at 400 rpm, room temperature, for 2 hrs. The plate was then washed 3 times by PBS, drained gently, before adding 100 µl ddH_2O per well to haemolyse the RBCs. The plate was incubated on the shaker at 400 rpm room temperature for a few minutes for the haemolysis. The produced haemoglobin was then measured using endpoint Dynex, Microplate reader at 405 nm filter. The results were analysed using Excel 2013 and GraphPad Prism 7.0 using one way ANOVA multiple comparison using

WT as a control column and Dunnett's test for p-value. A schematic representation of the method is shown in Figure 2-4.

The same experiment was done to determine the effect of some inhibitory antibodies in addtiton to the $\alpha_{IIb}\beta_3$ integrin inhibitor eptifibatide on the binding between fibrinogen and RBCs. To each 20 µg/ml of WT fibrinogen, 10 µg/ml final concentration of either IgG, α_V, $\alpha_V\beta_3$ antibodies or 5 µg/ml of eptifibatide were added together with 5% RBC in 300 µl total volume in PBS.

2.12 Flow Cytometry

MATERIALS

- 10 mM PBS (One tablet of PBS dissolved in 200 ml of deionized water at 25°C. Sigma-Aldrich).
- BD LSR Fortessa™ Cell Analyser from BD Bioscinces (Becton, Dickinson U.K. Limited).
- CD235a (Glycoprotein A)- phycoerythrin (PE) Antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).
- Eppendorf™ MiniSpin™ Microcentrifuges (Sigma-Aldrich).
- FacsDiva™ software from BD Bioscinces (Becton, Dickinson U.K. Limited).
- Fibrinogen From Human Plasma, AlexaFluor^{488} Conjugate (ThermoFisher Scientific).
- Haemocytometer (Optik Labor, Neubauer Improved, Lancing, UK), Depth 0.100 mm and 0.0025 mm^2.
- Mouse Anti-Human CD51/CD61 ($\alpha_v\beta_3$) purified 100 µg (OriGene Technologies, Inc.).
- Mouse anti-Melanoma Human cells Monoclonal Antibody Integrin alpha V α_V (NKI-M9) 200 µg (Santa Cruz Biotechnology, Inc.).
- Normal Mouse IgG unconjugated, affinity purified isotope control immunoglobulin 200 µg (Santa Cruz Biotechnology, Inc.).
- Tube roller (Star Lab).

Red blood cells were counted using a haemocytometer. To prepare the samples, $1x10^4$ RBC/ml were added to 5 µg/ml final concentration of AlexaFluor^{488}-labelled fibrinogen to the final volume of 400 µl in PBS. 20 µl of CD235a antibodies (RBC marker) were then added to the cell-protein mixture and incubated on the roller for 30 min at RT in foil wrapped tubes. Next, cells were washed by adding 400 µl PBS, mixed gently then centrifuged for 5 min at 1,000 g on a micro-centrifuge. The supernatant was discarded and the cells re-suspended in 400 µl PBS, mixed gently again, and then taken to the flow cytometer and analysed using FacsDiva™ software.

RBC-bound fibrinogen was quantified on the basis of fluorescence of the phycoerythrin (PE)-conjugated CD235a antibodies and AlexaFluor^{488}-labelled fibrinogen. Fluorescence was detected using a 488 nm laser and 530/30 nm band pass (bp) filter for AlexaFluor^{488}, and 575/26 nm bp filter for PE. Several controls were used; PE-labelled RBCs an AlexaFluor^{488}-labelled commercial fibrinogen separately, and compensated for bleed-over from one channel into the other. Bleed-over compensation was taken out according to the controls. Labelled RBCs and fibrinogen were separated according to the forward FSC and side SSC scatter. The results were analysed using Excel 2013 and GraphPad Prism 7.0 using one way ANOVA multiple comparison using WT as a control column and Dunnett's test for p-value. A schematic representation of the gating strategy is shown in Figure 2-5.

The experiment was performed using the same inhibitory antibodies as in the solution assay to study the binding site of fibrinogen on the RBC.

Flow cytometry experiments were performed with the help of Dr Sally Boxall, Bio-imaging and Flow Cytometry Facility Manager, Faculty of Biological Sciences, University of Leeds.

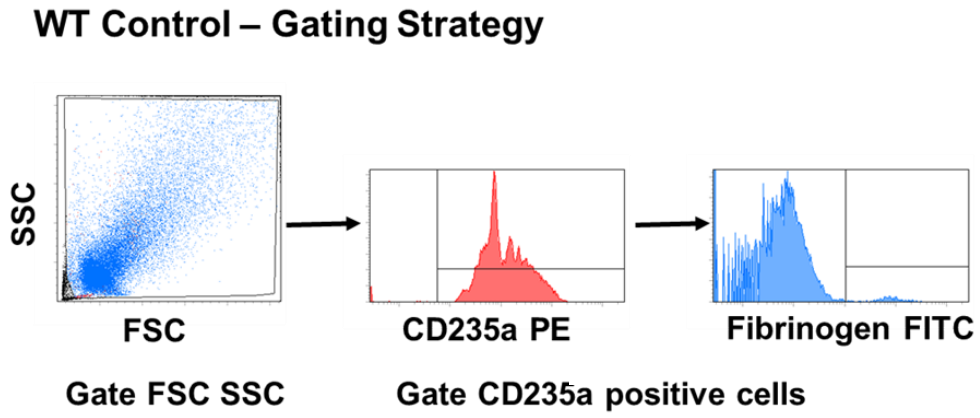


Figure 2-5 A schematic representation of the gating strategy of the flow cytometric experiment of the fibrinogen and RBCs interaction. FSC and SSC gate, gated on RBCs population then CD235a labelled then to fibrinogen labelled. % of positive labelling for PE and FITC was measured.

2.13 Platelet Spreading Assay

2.13.1 Isolation of Human Platelets

MATERIAL

- 0.3 Citric Acid: Citric Acid Monohydrate (Sigma-Aldrich).
- 10 ml Disposable Plastic Syringe without Needle (Terumo Europe N.V.).
- 2.5 ml Disposable Plastic Syringe without Needle (Terumo Europe N.V.).
- 50 ng/ml Prostaglandin E1 (PG E1) (Sigma-Aldrich).
- Acid Citrate-Dextrose ACD: 113.8 mM D-Glucose Anhydrous (ThermoFisher Scientific), 29.9 mM Tri-Na Citrate dehydrate (Sigma-Aldrich), 72.6 mM NaCl, 2.9 mM Citric Acid Monohydrate (Sigma-Aldrich).
- Modified Tyrodes Buffer MTB: 15 mM NaCl, 5 mM HEPES Sodium Salt (Sigma-Aldrich), 0.55 mM NaH_2PO_4 anh. (Sigma-Aldrich), 7 mM $NaHCO_3$ anh. (ThermoFisher Scientific), 2.7 mM KCl, 0.5 mM magnesium chloride $MgCl_2$ (ThermoFisher Scientific), 5.6 D-Glucose Anh.
- Wash Buffer: (0.036 M Citric Acid Monohydrate (Sigma-Aldrich), 0.01 M EDTA (Sigma-Aldrich), 0.005 M Glucose (ThermoFisher Scientific), 0.005 M KCl (Sigma-Aldrich), 0.09 M NaCl, 2.9 mM Citric Acid.
- Z1 Coulter® Particle Counter (Beckman Coulter).

Platelets were kindly provided by the laboratory of Prof Khalid Naseem using method that is adapted and based on (Vargas et al., 1982). All buffers were incubated at 37°C before use. Venous blood was collected into 1:5 ACD containing tube using the same phlebotomy protocol as section 2.9.1. Blood was centrifuged at 200 g, 20°C for 20 min. Next, platelet rich plasma was transferred into a clean 12 ml falcon tube, and then PG E1 was added to platelets in a final concentration of 50 ng/ml to prevent platelet activation. platelet rich plasma containing PG E1 was centrifuged at 800 g, 20°C for 12 min. After centrifugation, platelet poor plasma supernatant was removed and the tube was left to drain. The pellet was gently re-suspended in 1 ml of MTB and counted using the Z1 coulter.

2.13.2 Staining of Actin Fibres in Spread Platelets for Fluorescent Microscope

MATERIALS

- 0.2% Triton-X 100 in water.
- 10 mM PBS (One tablet of PBS dissolved in 200 ml of deionized water at 25°C. Sigma-Aldrich).
- 200 U/ml AlexaFluor^{488}-Phalloidin (Molecular Probes™ Invitrogen detection technologies).
- 5 mg/ml Fatty Acid Free BSA 10 G (Sigma-Aldrich): was boiled for 10 min to denature the protein and cooled down before used.
- Corning® Costar® TC-Treated 24-Well Plates (Corning®).
- Cover glass 13 mm round thickness of 0.13-0.17 mm (VWR Lutterworth, UK).
- Fisherbrand Microscopic Slides T/F Ground 1.0-1.2 mm (ThermoFisher Scientific).
- Paraformaldehyde, 4% in PBS (ThermoFisher Scientific).
- Vectashield Mounting Medium for Fluorescence (Vector Laboratories, Inc., Burlingame, USA).
- Zeiss AX10 Fluorescent Microscope (Zeiss).

The experiment was started by coating coverslips with 200 µl of 100 µg/ml fibrinogen on the centre of each cover slip, which were placed in the wells of a 24-well plate. The plate was incubated in a humidity chamber at 4°C overnight. Next morning, the coverslips were washed 3 times by 300 µl of PBS. Washing steps were repeated between every step of the experiment. The coverslips were blocked by adding 300 µl per well of denatured BSA and incubated at room temperature for 1 hr. Next, 300 µl per well of 5 x 10^6 Cell/ml washed platelets were added and incubated at 37°C for 45 min then fixed by 300 µl per well of 4% Paraformaldehyde at room temperature for 10 min. Subsequently, platelets were permeabilised with 300 µl per well of 0.2% Triton-X100 at room temperature for 5 min, before 1 unit (5 µl) of Phalloidin in 300 µl per well of PBS were added to each well, and incubated at room temperature, in the dark for 1 hr. The coverslips were mounted on slides by adding a tiny drop of mounting medium on the slide then the coverslip was transferred on the slide with the platelets facing the slide. Care was taken not to introduce bubbles. The slides were left in the dark for about 1 hr to dry, then stored in the cold room to be imaged the next day. The slides were imaged by 63x oil lens of Zeiss fluorescent microscope, at wavelength 495-518 nm, using Zen Software and analysed using ImageJ by applying automatic threshold to calculate Platelet count/ field of view (1790.52X1341.60 micron) and percent surface coverage. The results were analysed using Excel 2013 and GraphPad Prism 7.0 using one way ANOVA multiple comparison using WT as a control column and Dunnett's test for p-value.

Chapter 3 Recombinant Fibrinogen Generation

3.1 Introduction

In this project, six different mutants were produced in the coiled-coil region of the Aα-chain of fibrinogen. The initial concept of the choice of the new residues to be introduced relied on changing the charge of the side-chain of the original residue, whilst ensuring that the new residue has a similar size. The main residues R95 (positive) and D97 (negative) were changed following this concept to neutral amino acids (Q95 and N97) and oppositely charged amino acids (negative E95 and positive K97). F98I was chosen because it is a naturally occurring mutant that was described previously in a patient and it is located immediately after the RGD sequence (Riedelova-Reicheltova et al., 2014). G96V was not in the initial plan of the project, but was produced because it was described to have an effect on the interaction between fibronectin and fibroblast (Pierschbacher and Ruoslahti, 1984).

The process of generating the mutated fibrinogen was started by Site-Directed Mutagenesis which is a fundamental tool that has been used in modern recombinant DNA technology and which introduces a specific new nucleotide substitution in the gene sequence and its coded protein (Ho et al., 1989). A big part of this project was the production of mutated fibrinogen; this was achieved by transfecting the mutated expression vector pMLP-Aα in CHO cells. The Aα expression vector was constructed by the insertion of a *pVuII/SspI* Aα cDNA fragment from p549.12 into p284 cleaved with *SmaI*, the vector was sequenced to confirm the absence of other accidental mutations (Binnie et al., 1993). The cells were simultaneously co-transfected with the selection plasmid pMSV-His. CHO cells have the advantage of expressing and secreting a significantly constant level of recombinant fibrinogen for a long period in serum free medium (Kim et al., 2012). This fibrinogen is found to be stable for a few days at 37°C (Binnie et al., 1993). L-histidinol selection is dependent on the fact that removal of an essential amino acid (histidine) would stop the growth of the cells and subsequently causes their death. Addition of a single gene coding for an enzyme (histidinol dehydrogenase) able to metabolise a precursor into the lacking amino acid that is able to penetrate the cell membrane is the basis of this selection strategy (Hartman and Mulligan, 1988). In a medium that is lacking the essential

amino acid histidine, but contains its precursor L-histidinol, cells can grow and survive if they are able to metabolise it to histidine by the action of histidinol dehydrogenase, an enzyme that catalyses the terminal reaction of histidine biosynthesis in bacteria and is a product of the *hisD* gene (Hartman and Mulligan, 1988). Only cells successfully transfected with pMSV-His can survive in the presence of L-histidinol.

After successful transfection, protein was expressed in CHO cells then precipitated by ammonium sulfate. Protein solubility increases by the addition of salt, which is called salting-in, however this solubility decreases to the extent of precipitation by increasing the salt concentration above the saturation level, which is called salting-out (Wingfield, 2001). The use of salt saturation of ammonium sulfate in precipitating proteins is dependent on the concentration and nature of the protein, in addition to the pH and temperature of the solution. 5-10% oversaturating the salt would precipitate about 90% of the protein (King, 1972). However, salting out does not result in a homogenous protein lysate, the produced protein will require some more steps to be purified. Next, proteins were purified using antibody affinity chromatography in which the column was packed with immobilised calcium-dependent IF1 monoclonal antibody, which is the solid phase that allows the separation of the contaminants (Cuatrecasas, 1970, Coelho et al., 2012). When proteins passed through the column, fibrinogen which has high affinity for the antibodies, in the presence of calcium, binds whereas other proteins will flow through. Fibrinogen was later eluted by EDTA which chelate calcium (Cuatrecasas, 1970, Coelho et al., 2012).

This chapter will cover the production of six mutated fibrinogen starting from primer design through transfection, expression, purification and finally the confirmation of purity and integrity of the produced fibrinogen.

3.2 Results

3.2.1 Generation of Mutants by Site-Directed Mutagenesis

Mutagenesis for R95Q and D97N was repeated a number of times (12 attempts) in order to get bacterial colonies after the transformation step. This was achieved by changing the conditions in the reaction one at a time in each repeat (Table 3-1, Table 3-3) and changing the PCR run conditions (Table 3-2, Table 3-4). Mutagenesis for the new mutants (R95E and D97K) was started from the last optimised conditions stage of the R95Q and D97N that successfully generated the mutated colonies. However, more optimisations were again required to produce these new mutants, because with some conditions, colonies were produced but they did not have the mutations as stated in Table 3-6 which shows the reaction conditions and Table 3-4, demonstrating PCR runs accompanying each condition. In contrast, G96V produced the mutated colonies from the first attempt (Table 3-6). Work on F98I was started at the roller bottle of fibrinogen expression stage, because the previous stages (mutagenesis and transfection) were performed by a visiting collaborator (Miss Ana Filippa Guedes, University of Lisbon).

Mutagenesis was performed using QuickChange II Site-Directed Mutagenesis Kit, as described in Table 3-1.

R95Q

This was the first mutant produced as shown in Table 3-1. First, the *PfuUltra* High-Fidelity DNA polymerase was used following the Site-Directed Mutagenesis protocol, employing different amounts of template DNA (10, 20, 40 ng) and XL1-Blue Super-Competent Cells, but no colonies were produced. A few changes to the protocol were undertaken to optimise the method. The focus in these attempts was by changing the PCR conditions (Table 3-2). Extension time was decreased to reduce copy error, whereas PCR cycle number was increased to 18 cycles since 16 cycles is recommended by the Site-Directed Mutagenesis protocol for a single amino acid change, but this failed to produce results. Increasing the number of cycles to 18 was suggested to increase the yield by

enhancing the chance for the mutated cDNA to be replicated, which is still within the optimum number of cycles that preserve the enzyme and other reaction constituent efficiency. However, these changes did not affect the reaction and no colonies were produced.

Table 3-1 Site-Directed Mutagenesis first set of attempts for R95Q and D97N using QuickChange II Site-Directed Mutagenesis Kit. Each total reaction is 50 µl, reaction steps and mixtures that were used are summarised in separate table.

Conditions	**Attempts**			
	1	**2**	**3**	**4**
Mutation	**R95Q, D97N**	**R95Q, D97N**	**R95Q, D97N**	**R95Q, D97N**
Template DNA	10, 20 ng	20, 40 ng	20, 40 ng	20, 40 ng
Reaction Buffer	1X			
dNTP	200 µM			
Primers (F+R) each	125 ng			
***PfuUltra* High-Fidelity DNA polymerase**	2.5U			
PCR	PCR reaction A for 16 cycles	PCR reaction B for 18 cycles	PCR reaction A for 16 cycles	PCR reaction A for 18 cycles
DpnI (1 hr at 37°c)	10 U/µl			
Transformation	50µl of XL1-Blue Super-Competent Cells			
DpnI treated DNA	1 µl		4 µl	
Results	No colonies			

Table 3-2 PCR steps that were used in the first set of attempts for R95Q and D97N.

PCR Steps	PCR Reaction A	PCR Reaction B
Initial Denaturation	95°C for 30 sec	95°C for 30 sec
Cycle Denaturation	95°C for 30 sec	95°C for 30 sec
Cycle Annealing	55°C for 60 sec	55°C for 60 sec
Cycle Extension	68°C for 435 sec	68°C for 390 sec

Following successive failures to obtain colonies, the next step was to change the enzyme to *Phusion* High-Fidelity DNA Polymerase, in addition to various changes among the different attempts in the PCR mixture (Table 3-3), as well as reaction conditions (Table 3-4). These changes included using different amounts of template DNA ranging from low (5 ng) to high (40 ng). The bacteria which were used for transformation were replaced by XL10 Gold Ultra-Competent Cells, which provide the highest efficiency available for the *E. coli* strains for transformation of ligated and large DNA.

Table 3-3 Site-Directed Mutagenesis second set of attempts for R95Q and D97N using *Phusion*® High-Fidelity DNA Polymerase Mutagenesis Kit. Each total reaction is 50 µl, PCR reactions steps that were used are summarised in separate table.

Conditions	Attempts							
	5	6	7	8	9	10	11	12
Mutation	R95Q	R95Q	R95Q	R95Q D97N	R95Q	D97N	D97N	D97N
Template DNA	5 ng	20,40 ng	10,40 ng	10,40 ng	20,40 ng	10,20,40 ng	10,20 ng	10,20 ng
Phusion Buffer (HF)	1X							
GC buffer	-	1X	-	-	-	-	-	-
dNTP	200 µM							
Primers (F+R) each	0.5 µM	0.5 µM	0.5 µM	0.5 µM New	0.5 µM	0.5 µM	0.5 µM	0.5 µM Old
DMSO	-	-	6%	2,12%	6%	2, 6%	6%	6, 12%
Phusion Polymerase	1U	1U	1U	1U	2U	2U	2U	2U
PCR	C for 25 cycles	D for 35 cycles	E reaction for 18 cycles					
DpnI (10U/µl)	10 U/µl							
Parental DNA Digestion Time	1 hr						1:30 hrs	1:30 hrs
Transformation	50µl of XL1-Blue Super-Competent Cells	XL10 Gold Ultra-Competent Cells						
DpnI treated DNA	1 µl							
Results	No colonies				Presence of mutated colonies	Colonies, no mutation		Presence of mutated colonies

Table 3-4 PCR steps. These PCR conditions were used in the second set of attempts for R95Q and D97N in addition to all attempts of R95E, G96V and D97K.

Step	PCR Reaction C	PCR Reaction D	PCR Reaction E
Initial Denaturation	98°C for 30sec	98°C for 30sec	98°C for 30sec
Cycle Denaturation	98°C for 10sec	98°C for 10sec	98°C for 30sec
Cycle Annealing	72°C for 30sec	72°C for 105sec	60°C for 30sec
Cycle Extension	72°C for 30sec	-	72°C for 210sec
Final Extension	72°C for 420sec	72°C for 600sec	72°C for 600sec

In order to improve the efficiency of the mutagenesis reaction, some additives were used according to the manufacturer's instructions. One of the additives was DMSO and the other was the GC buffer, the later buffer was used only once replacing the HF buffer. Both additives were to improve the amplification and reaction performance for the difficult templates with high GC or secondary structure contents.

During the optimisation process, one major change was undertaken that was using new primers after evaluating the primers physical properties. The primers of R95Q (forward and reverse) were analysed using *OligoEvaluator*™, an online software by Sigma-Aldrich used to assess the presence of secondary structures, which make amplification difficult. Based on the results of the primer properties analysis by the *OligoEvaluator*™ which are shown in Table 3-5, new shorter primers were designed. Both the original forward and reverse primers of R95Q mutant had moderate secondary structures and 40% GC contents and no primer dimer. The new primers had weak secondary structures and 34.9% GC contents, which was less than the original. Melting temperatures (Tm) for the modified (77.9°C) primers were a slightly less than the original ones (82.2°C). Taking into account all analysis, the data would suggest that the secondary structure could

be the factor that had affected the primer efficiency and prevented the successful insertion of the mutation in the DNA.

The final change in the optimisation process was by increasing the *Phusion* High-Fidelity DNA Polymerase up to 2U as recommended by the protocol depending on the primer length and complexity. Finally, this time, colonies were produced; and by sequencing, the correct mutation was found in the extracted DNA plasmid.

D97N

The generation of this mutant started for the first few attempts with the *PfuUltra* High-Fidelity DNA polymerase Site-Directed Mutagenesis protocol, in line with R95Q and showed the same negative results (Table 3-1).

When the R95Q primers were evaluated, D97N primers were evaluated as well for their physical properties using the *OligoEvaluator*™ and the results are shown in Table 3-5. In case of D97N both forward and reverse original primers had strong secondary structures, which were improved to moderate secondary structures in both new primers. The GC content of the new primers were slightly higher (36.4%) than the original ones (35.6%). Original reverse D97N was the only primer to have primer dimer. Again, Tm for each modified primer was a slightly less than the original ones.

The generation of D97N was temporarily stopped, whilst R95Q was being generated, then resumed after the successful generation of R95Q mutant. The same successful conditions of the last attempt of R95Q mutant were used as described in Table 3-3 and Table 3-4. The results were presence of colonies; nonetheless, the DNA sequencing showed that the cDNA was not mutated yet. The experiment was repeated again with only one change, which was increasing the *Dpn*I digestion time to 1:30 hrs, in order to allow longer time for the enzyme to digest the parent DNA. At the end of the experiment, colonies were present, but the DNA sequencing indicated that the mutations were not present yet.

Table 3-5 Oligo Evaluator Results by Sigma. Representation of the some of elements that included in the primers' assessment by Oligo Evaluator website for the presence of secondary structure.

Sequence Type	Oligo Type	Sequence	Base Count	Tm (°C)	GC%	Primer Dimer	Secondary Structure
Original	**R95Q-F**	CGTTGACCACTAATATAATGGAAATTTTGCAAGGCGATTTTTCCTCAGCC	A = 14, U = 0, G = 9, C = 11, T = 16, I = 0, Total = 50	82.6	40	No	Moderate
Modified	**R95Q-F2**	CGTTGACCACTAATATAATGGAAATTTTGCAAGGCGATTTTTC	A = 13, U = 0, G = 8, C = 7, T = 15, I = 0, Total = 43	77.9	34.9	No	Weak
Original	**R95Q-R**	GGCTGAGGAAAAATCGCCTTGCAAAATTTCCATTATATTAGTGGTCAACG	A = 16, U = 0, G = 11, C = 9, T = 14, I = 0, Total = 50	82.6	40	No	Moderate
Modified	**R95Q-R2**	GAAAAATCGCCTTGCAAAATTTCCATTATATTAGTGGTCAACG	A = 15, U = 0, G = 7, C = 8, T = 13, I = 0, Total = 43	77.9	34.9	No	Weak
Original	**D97N-F**	TATAATGGAAATTTTGAGAGGCAATTTTTCCTCAGCCAATAACCG	A = 15, U = 0, G = 8, C = 8, T = 14, I = 0, Total = 45	79.1	35.6	No	Strong
Modified	**D97N-F2**	CGTTGACCACTAATATAATGGAAATTTTGAGAGGCGATTTTTCC	A = 13, U = 0, G = 9, C = 7, T = 15, I = 0, Total = 44	77.9	36.4	No	Moderate
Original	**D97N-R**	CGGTTATTGGCTGAGGAAAAATTGCCTCTCAAAATTTCCATTATA	A = 14, U = 0, G = 8, C = 8, T = 15, I = 0, Total = 45	79.1	35.6	Yes	Strong
Modified	**D97N-R2**	GGAAAAATCGCCTCTCAAAATTTCCATTATATTAGTGGTCAACG	A = 15, U = 0, G = 7, C = 9, T = 13, I = 0, Total = 44	77.9	36.4	No	Moderate

The original primers were used again with some changes this time, 2U of the *Phusion* High-Fidelity DNA Polymerase was used instead if 1U of the enzyme as in the previous attempts of the original primers. Moreover, DMSO was used to overcome the secondary structure and even the primer dimer; these conditions combined successfully produced D97N mutated colonies.

R95E

The mutagenesis process for this variant started following the successful generation of R95Q and D97N. Representation of the attempts can be found in Table 3-6 and PCR conditions are shown in Table 3-4.

A few attempts were performed for R95E mutant using 10 ng and 20 ng of template DNA and different concentrations of DMSO, but the results were either no colonies or colonies where the mutation was not present, as shown in Table 3-6. Finally mutated colonies were present in the fourth attempt, when 10% DMSO was used.

D97K

This mutant was generated in line with the R95E mutant, but it did not produce colonies for the conditions used for R95E mutant, as shown in Table 3-6 (PCR conditions are shown in Table 3-4).

One more attempt for D97K was performed by increasing the amount of *Phusion* High-Fidelity DNA Polymerase up to 3U. This change was successful and generated D97K bacterial colonies.

G96V

Only one attempt was enough to produce the successful mutated colonies of G96V mutation using 20 ng DNA, 6% DMSO and 2U *Phusion* polymerase as shown in Table 3-6 (PCR conditions are shown in Table 3-4).

Table 3-6 Site-Directed Mutagenesis attempts for R95E, G96V and D97K using *Phusion*® High-Fidelity DNA Polymerase Mutagenesis Kit. Each total reaction is 50 µl, PCR reactions steps that were used are summarised in separate table.

Conditions	Attempts					
	1	2	3	4	5	6
Mutation	R95E D97K	R95E D97K	R95E D97K	R95E D97K	D97K	G96V
Template DNA	20 ng	10,20 ng	10,20 ng	20 ng	20 ng	20 ng
Phusion Buffer (HF)	1X					
dNTP	200 µM					
Primers (F+R) each	0.5 µM	0.5 µM	0.5 µM	0.5 µM	0.5 µM	0.5 µM
DMSO	12%	6%	6%	8, 10%	6, 10%	6%
Phusion Polymerase	2U	2U	2U	2U	3U	2U
PCR	E reaction for 18 cycles					
DpnI (10U/µl)	10 U/µl					
Parental DNA Digestion Time	1:30 hrs					
Transformation	XL10 Gold Ultra-competent Cells					
DpnI treated DNA	1 µl		3 µl		1 µl	
Results	Colonies, no mutation	No colonies		Colonies R95E 10% DMSO	Presence of mutated colonies	

3.2.2 Sequencing

The next step after mutagenesis of all the constructs was DNA extraction and full mutated Aα-chain cDNA sequencing. The converted amino-acid sequences of the five mutants are shown in the next few tables. Amino acids numbering is for the mature protein, and starts at position 20 (A, red) where the pro-peptide is cleaved and the mature fibrinogen starts. Full DNA sequence is presented in Appendix A.

For R95E, the original amino acid Arginine, which is positive (R, AGA), was replaced by a negative Glutamate (E, GAA) (Table 3-7).

Table 3-7 Representatitive part from the sequencing alignment shows the results of R95E using the serial cloner programme. The sequencing shows replacement of R (Arginine) by E (Glutamate), highlighted in green. Red A is where the mature fibrinogen starts.

```
WT        1  MFSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVRGPRVVERHQSACKDSDWPFCSDEDW  60
R95E      1  MFSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVRGPRVVERHQSACKDSDWPFCSDEDW  60

WT       61  NYKCPSGCRMKGLIDEVNQDFTNRINKLKNSLFEYQKNNKDSHSLTTNIMEILRGDFSSA  120
R95E     61  NYKCPSGCRMKGLIDEVNQDFTNRINKLKNSLFEYQKNNKDSHSLTTNIMEILEGDFSSA  120

WT      121  NNRDNTYNRVSEDLRSRIEVLKRKVIEKVQHIQLLQKNVRAQLVDMKRLEVDIDIKIRSC  180
R95E    121  NNRDNTYNRVSEDLRSRIEVLKRKVIEKVQHIQLLQKNVRAQLVDMKRLEVDIDIKIRSC  180
```

For R95Q, the original amino acid Arginine, which is positive (R, AGA), was replaced by a neutral Glutamine (R, CAA) (Table 3-8).

Table 3-8 Representatitive part from the sequencing shows the results of R95Q using the serial cloner programme. The sequencing shows replacement of R (Arginine) by Q (Glutamine), highlighted in green. Red A is where the mature fibrinogen starts.

```
WT        1  MFSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVRGPRVVERHQSACKDSDWPFCSDEDW  60
R95Q      1  MFSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVRGPRVVERHQSACKDSDWPFCSDEDW  60

WT       61  NYKCPSGCRMKGLIDEVNQDFTNRINKLKNSLFEYQKNNKDSHSLTTNIMEILRGDFSSA  120
R95Q     61  NYKCPSGCRMKGLIDEVNQDFTNRINKLKNSLFEYQKNNKDSHSLTTNIMEILQGDFSSA  120

WT      121  NNRDNTYNRVSEDLRSRIEVLKRKVIEKVQHIQLLQKNVRAQLVDMKRLEVDIDIKIRSC  180
R95Q    121  NNRDNTYNRVSEDLRSRIEVLKRKVIEKVQHIQLLQKNVRAQLVDMKRLEVDIDIKIRSC  180
```

For G96V, the original amino acid, Glycine (G, GGC) was replaced by a Valine (V, GTC) (Table 3-9). Both amino acids are neutral and this mutant was chosen based on the literature (Pierschbacher and Ruoslahti, 1984).

Table 3-9 Representatitive part from the sequencing shows the results of G96V using the serial cloner programme. The sequencing shows replacement of G (Glycine) by V (Valine), highlighted in green. Red A is where the mature fibrinogen starts.

WT	**1**	MFSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVRGPRVVERHQSACKDSDWPFCSDEDW	**60**
R95Q	**1**	MFSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVRGPRVVERHQSACKDSDWPFCSDEDW	**60**
WT	**61**	NYKCPSGCRMKGLIDEVNQDFTNRINKLKNSLFEYQKNNKDSHSLTTNIMEILRGDFSSA	**120**
R95Q	**61**	NYKCPSGCRMKGLIDEVNQDFTNRINKLKNSLFEYQKNNKDSHSLTTNIMEILRVDFSSA	**120**
WT	**121**	NNRDNTYNRVSEDLRSRIEVLKRKVIEKVQHIQLLQKNVRAQLVDMKRLEVDIDIKIRSC	**180**
R95Q	**121**	NNRDNTYNRVSEDLRSRIEVLKRKVIEKVQHIQLLQKNVRAQLVDMKRLEVDIDIKIRSC	**180**

For D97K, the original amino acid Aspartate, which is negative (D, GAT), was replaced by a positive Lysine (K, AAA) (Table 3-10).

Table 3-10 Representatitive part from the sequencing shows the results of D97K using the serial cloner programme. The sequencing shows replacement of D (Aspartate) by K (Lysine), highlighted in green. Red A is where the mature fibrinogen starts.

WT	1	MFSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVRGPRVVERHQSACKDSDWPFCSDEDW	60
D97K	1	MFSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVRGPRVVERHQSACKDSDWPFCSDEDW	60
WT	61	NYKCPSGCRMKGLIDEVNQDFTNRINKLKNSLFEYQKNNKDSHSLTTNIMEILRGDFSSA	120
D97K	61	NYKCPSGCRMKGLIDEVNQDFTNRINKLKNSLFEYQKNNKDSHSLTTNIMEILRGKFSSA	120
WT	121	NNRDNTYNRVSEDLRSRIEVLKRKVIEKVQHIQLLQKNVRAQLVDMKRLEVDIDIKIRSC	180
D97K	121	NNRDNTYNRVSEDLRSRIEVLKRKVIEKVQHIQLLQKNVRAQLVDMKRLEVDIDIKIRSC	180

For D97N, the original amino acid Aspartate (D, GAT), which is negative, was replaced by a neutral Asparagine (N, AAT) (Table 3-11).

Table 3-11 Representatitive part from the sequencing shows the results of D97N using the serial cloner programme. The sequencing shows replacement of D (Aspartate) by N (Asparagine), highlighted in green. Red A is where the mature fibrinogen starts.

WT	**1**	MFSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVRGPRVVERHQSACKDSDWPFCSDEDW	**60**
D97N	**1**	MFSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVRGPRVVERHQSACKDSDWPFCSDEDW	**60**
WT	**61**	NYKCPSGCRMKGLIDEVNQDFTNRINKLKNSLFEYQKNNKDSHSLTTNIMEILRGDFSSA	**120**
D97N	**61**	NYKCPSGCRMKGLIDEVNQDFTNRINKLKNSLFEYQKNNKDSHSLTTNIMEILRGNFSSA	**120**
WT	**121**	NNRDNTYNRVSEDLRSRIEVLKRKVIEKVQHIQLLQKNVRAQLVDMKRLEVDIDIKIRSC	**180**
D97N	**121**	NNRDNTYNRVSEDLRSRIEVLKRKVIEKVQHIQLLQKNVRAQLVDMKRLEVDIDIKIRSC	**180**

3.3 Protein Production

3.3.1 Transfection

Two different transfection methods were used in this project, because the initial protein yield was low. The first method was Calcium-Phosphate transfection (10 or 20 µg of cDNA), which relies on shocking the cells with 10% glycerol to introduce the new plasmid. The second method involves Lipofectamine 2000 (11 µg of cDNA), which relies on coating the plasmid in a liposome-like structure to facilitate its entry into cells. In both cases, transfection of cDNA was performed in CHO cells (containing fibrinogen Bβ- and γ-chains), that were grown in G418 selection medium before transfection. The successfully transfected cells were selected by addition of the L-histidinol selection marker. Twenty-four clones that originated from single cells were plated in 24-well plates for each condition of Calcium-Phosphate method (R95Q, D97N) and up to 48 for Lipofectamine method (R95E, G96V, D97K). At 70-100% confluency, the cells were split into two 5 cm dishes, the supernatants were collected for ELISA and the highest fibrinogen producing clones, relative to their confluency (Figure 3-1, Figure 3-2, Figure 3-3, Figure 3-4, Figure 3-5), were frozen in liquid nitrogen, reserving 1 ml for further splitting into 10 cm dishes for the serum free expression stage. Once confluent, the cells were incubated in serum free medium for a week. By the end of the incubation period, cells were discarded but the supernatants were collected for a second ELISA to determine the clones which were to be taken

forward for the expression stage (in serum-free conditions). The highest six producing clones were frozen in LN_2, while the others were discarded. Representative figures for the first ELISA results and the chosen clone are shown below.

For R95E (lipofectamine, 11 µg DNA), 41 clones were picked and the highest expressing clones were 6, 12, 13, 14, 15, 25, 26, 30, 31, 33, 37, 38 (Figure 3-1).

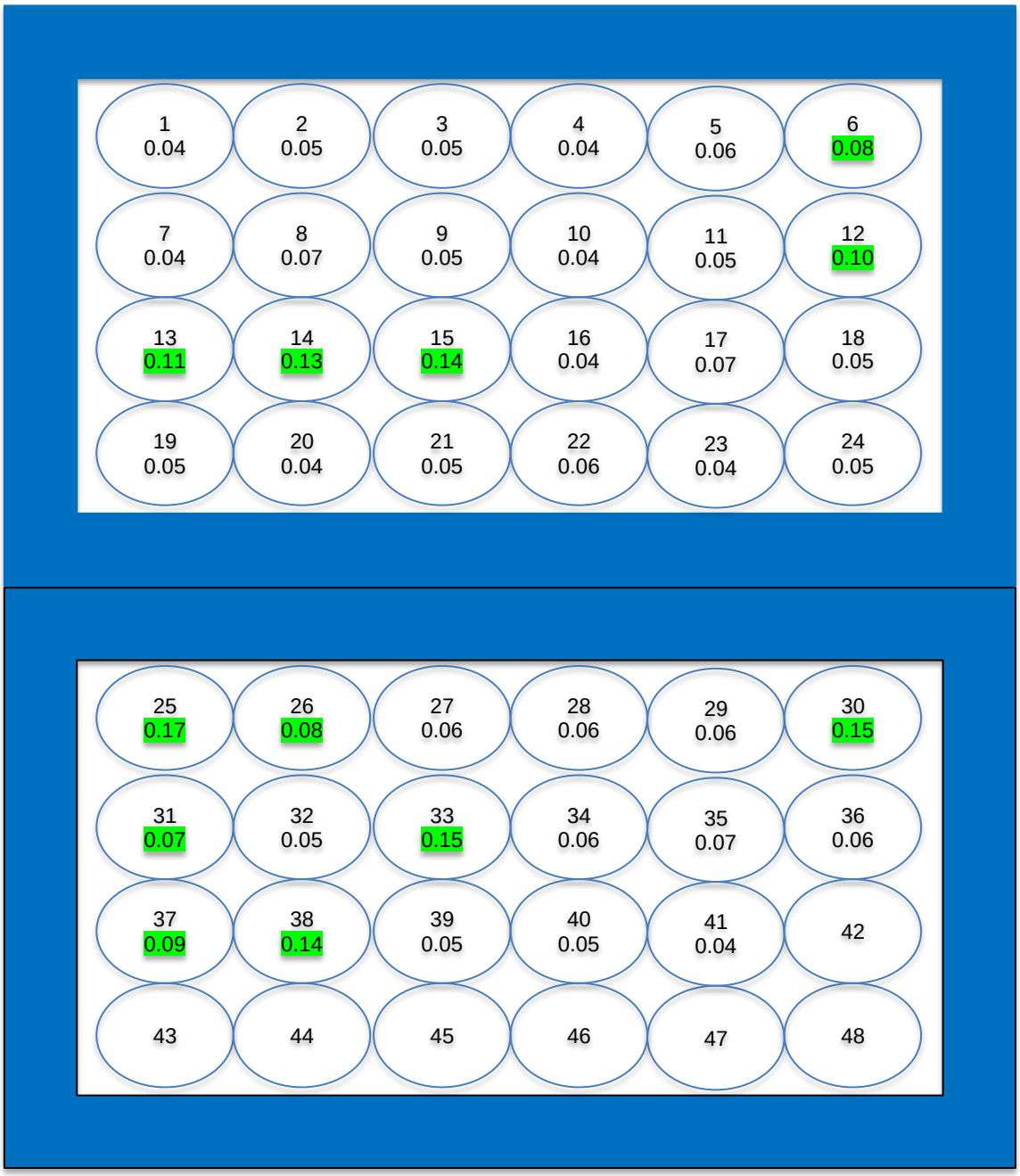


Figure 3-1 Representation of the ELISA plate of R95E clones selection stage. The highest fibrinogen producing (11 µg DNA) clones highlighted in green (6, 12, 13, 14, 15, 25, 26, 30, 31, 33, 37, 38). The numbers represent the ratio of concentration (ng/ml) to confluency at collection (%).

For R95Q (Figure 3-2), plate A (calcium-phosphate, 10 µg DNA), the highest expressing clones were 1, 2, 3, 4, 5, 6, 8, 10, 11, 12, 13, 14, 21, 22, 24. For plate B (calcium-phosphate, 20 µg DNA), the highest expressing clones were 3, 4, 5, 8, 9, 11, 12, 13, 15, 20, 22.

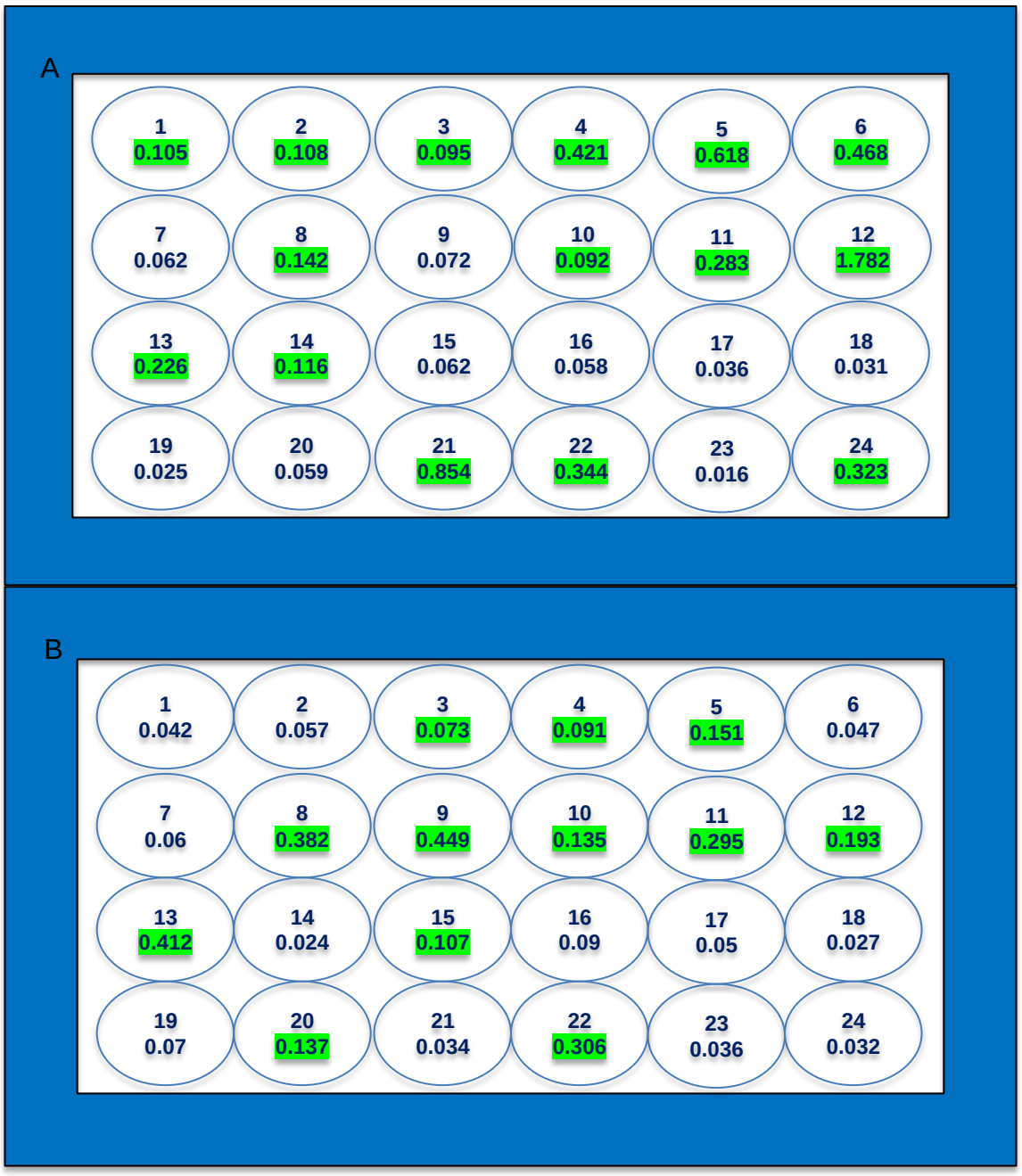


Figure 3-2 Representation of the ELISA plate of R95Q clones selection stage. The highest fibrinogen producing **A** (10 µg DNA) clones highlighted in green (1, 2, 3, 4, 5, 6, 8, 10, 11, 12, 13, 14, 21, 22, 24). The highest fibrinogen producing **B** (20 µg DNA) clones highlighted in green (3, 4, 5, 8, 9, 11, 12, 13, 15, 20, 22). The numbers represent the ratio of concentration (ng/ml) to confluency at collection (%).

For G96V (lipofectamine, 11 µg DNA), 48 clones were picked up and the highest expressing clones were 12, 13, 20, 21, 37, 38, 40, 42, 43, 46, 47, 48 (Figure 3-3).

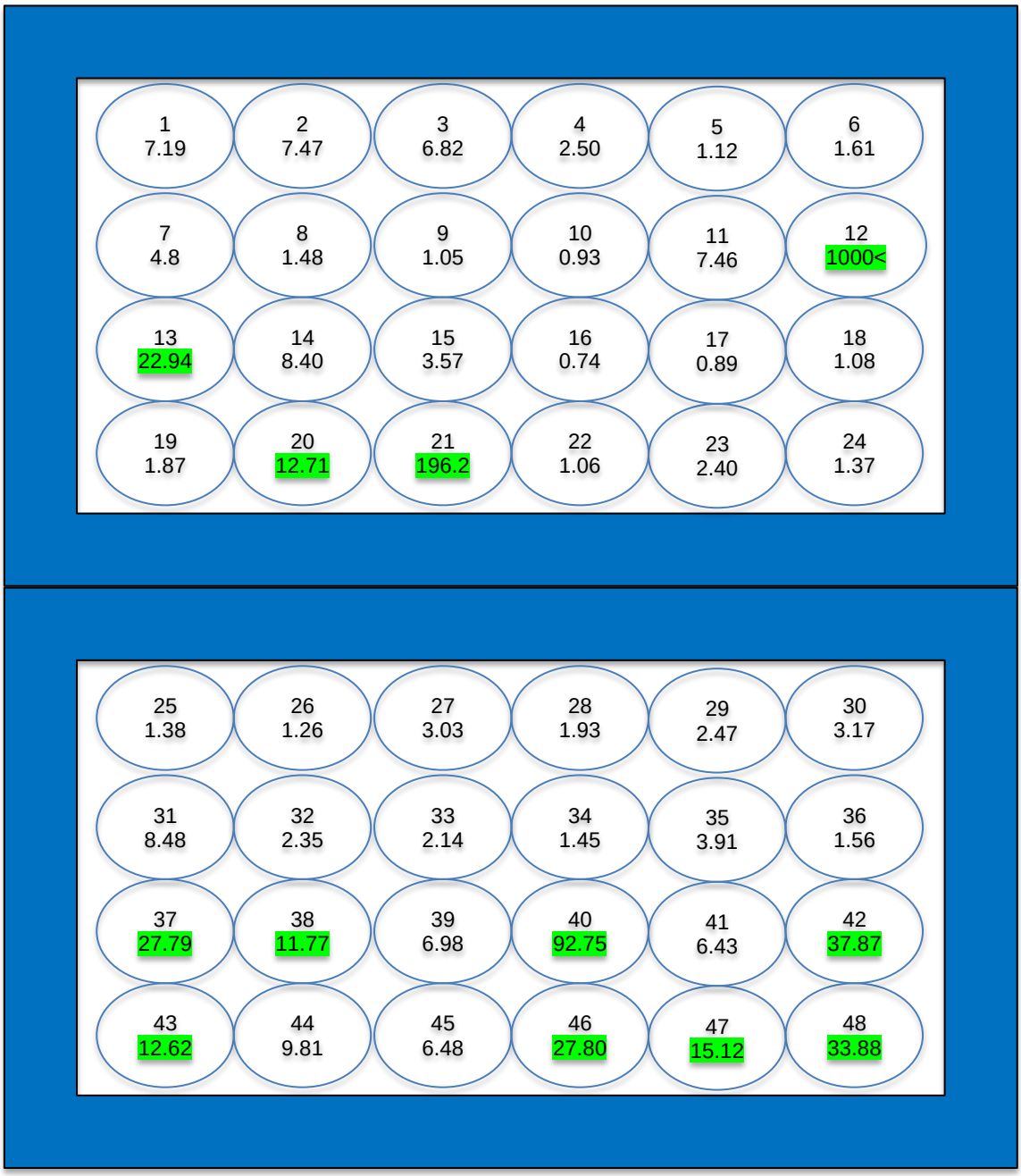


Figure 3-3 Representation of the ELISA plate of G96V clones selection stage. The highest fibrinogen producing (11 µg DNA) clones highlighted in green (12, 13, 20, 21, 37, 38, 40, 42, 43, 46, 47, 48). The numbers represent the ratio of concentration (ng/ml) to confluency at collection (%). Clone number 12 was above the highest level of the absorbance of the standard.

For D97K (lipofectamine, 11 µg DNA), 42 clones were picked up and the highest expressing clones were 1, 3, 4, 5, 7, 18, 24, 27, 28, 29, 37, 42 (Figure 3-4).

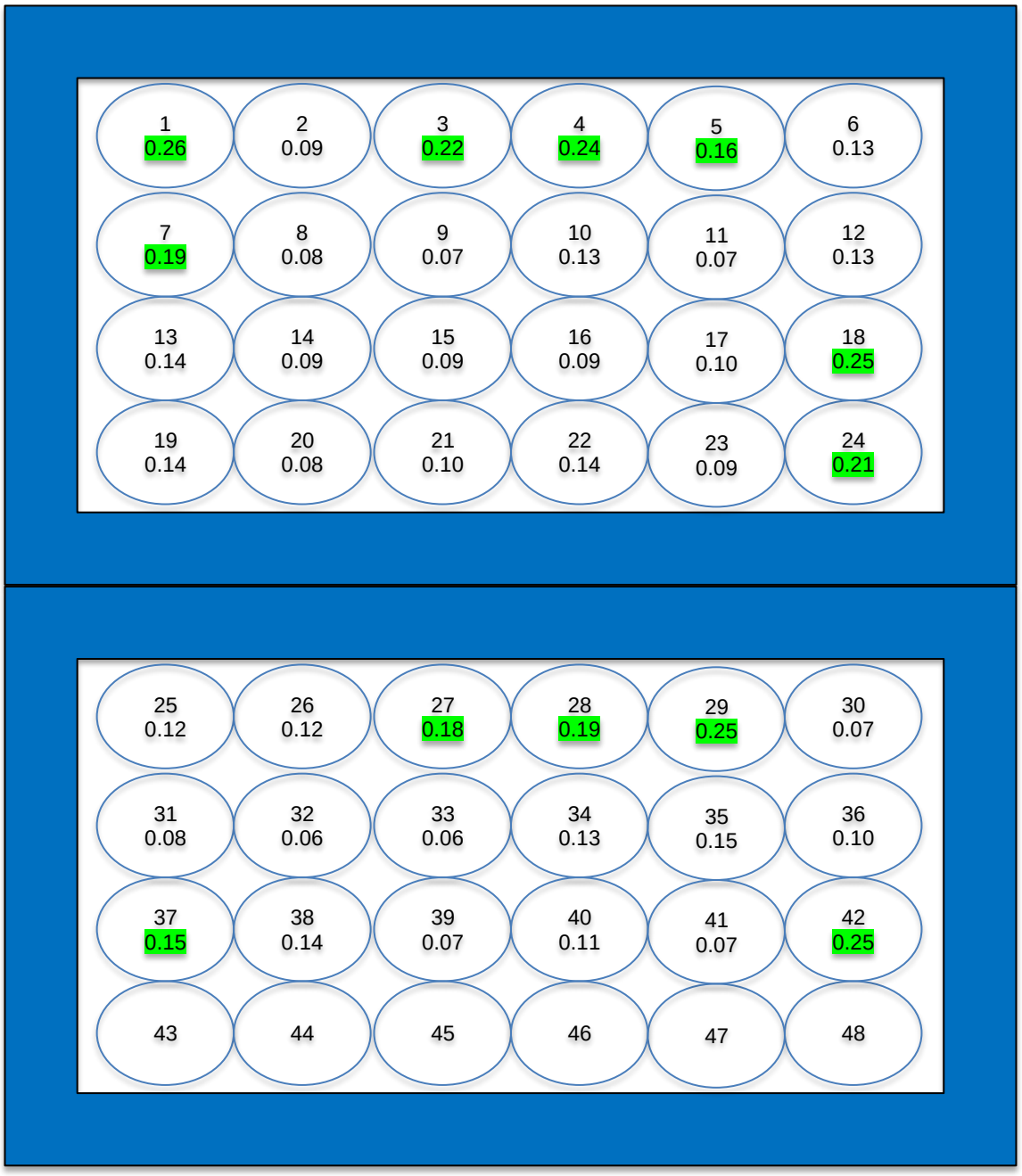


Figure 3-4 Representation of the ELISA plate of D97K clones selection stage. The highest fibrinogen producing (11 µg DNA) clones highlighted in green (1, 3, 4, 5, 7, 18, 24, 27, 28, 29, 37, 42). The numbers represent the ratio of concentration (ng/ml) to confluency at collection (%).

For D97N (Figure 3-5), plate A (calcium-phosphate, 10 µg DNA) the highest expressing clones were 2, 3, 4, 6, 8, 10, 11, 12, 14, 18, 21, 23, 24. Plate B (calcium-phosphate, 20 µg DNA) the highest expressing clones were 1, 2, 6, 9, 10, 12, 13, 15, 16, 20, 21, 24.

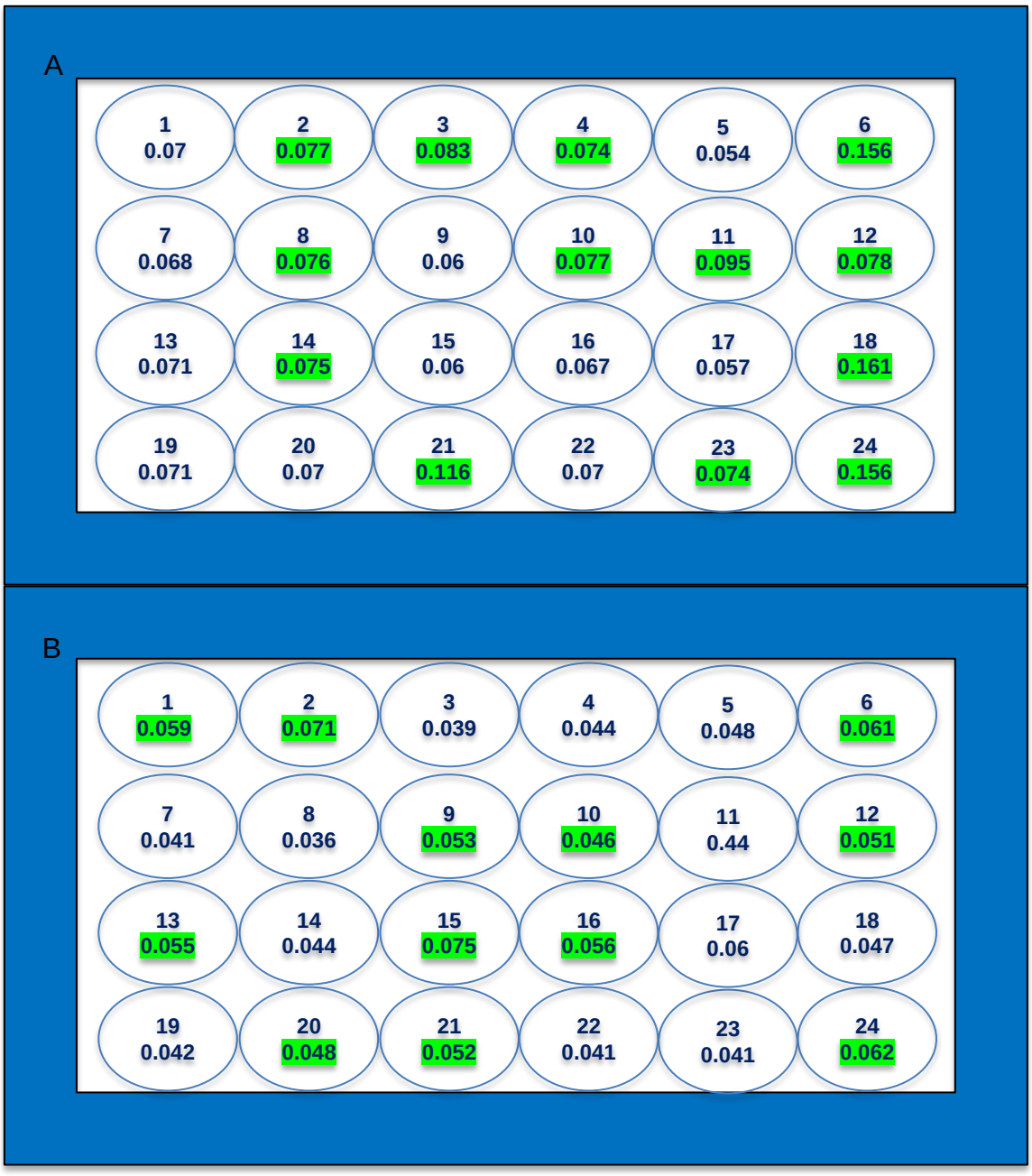


Figure 3-5 Representation of the ELISA plate of D97N clones selection stage. The highest fibrinogen producing **A** (10 µg DNA) clones highlighted in green (2, 3, 4, 6, 8, 10, 11, 12, 14, 18, 21, 23, 24). The highest fibrinogen producing **B** (20 µg DNA) clones highlighted in green (1, 2, 6, 9, 10, 12, 13, 15, 16, 20, 21, 24). The numbers represent the ratio of concentration (ng/ml) to confluency at collection (%).

In the serum free conditions, the highest fibrinogen producing clones by the calcium-phosphate method were A12 for R95Q and A18 for D97N (Table 3-12), in addition to that performed by Miss Ana Filipa Guedes, A16 for F98I. The highest fibrinogen producing clones by Lipofectamine 2000 method were 14 for R95E, 12 for G96V and 37 for D97K (Table 3-13). These clones, which were used for fibrinogen expression in roller bottles, are highlighted in red, while the yellow highlighted clones were frozen in LN_2 as a backup.

Table 3-12 R95Q and D97N highest producing clones in serum free condition are highlighted in yellow. The two clones that proceeded to the expression stage are highlighted in red.

R95Q		D97N	
Clone number	**concentration ng/ml**	**Clone number**	**concentration ng/ml**
A1	123.50	**A2**	6.52
A2	153.59	**A3**	7.49
A3	160.20	**A4**	8.34
A4	97.06	**A6**	10.80
A5	89.96	**A8**	9.55
A6	87.72	**A10**	8.84
A8	44.62	**A11**	7.25
A10	55.36	**A12**	12.63
A11	30.94	**A14**	9.47
A12	266.67	**A18**	16.56
A13	32.39	**A21**	13.94
A14	3.50	**A23**	9.00
A21	27.53	**A24**	10.59
A22	47.51	**B1**	**4.74**
A24	52.76	**B2**	3.48
B3	129.91	**B6**	5.98
B4	82.09	**B9**	5.24
B5	108.43	**B10**	6.45
B8	63.13	**B13**	8.84
B9	56.10	**B15**	10.34
B11	47.45	**B16**	5.17
B12	90.51	**B20**	6.56
B13	84.81	**B21**	9.89
B15	40.87		
B20	53.59		
B22	13.31		

Table 3-13 R95E, G96V and D97K highest producing clones in serum free condition are highlighted in yellow. The two clones that proceeded to the expression stage are highlighted in red.

R95E		G96V		D97K	
Clone number	concentration ng/ml	Clone number	concentration ng/ml	Clone number	concentration ng/ml
6	184.49	12	569.03	1	132.32
12	303.41	13	179.69	3	57.46
13	65.21	20	129.77	4	464.89
14	392.45	21	178.75	5	308.00
15	89.95	37	284.56	7	105.19
25	242.24	38	4.64	18	82.56
26	58.33	40	22.14	24	157.08
30	52.85	42	13.32	27	189.30
31	28.20	43	4.21	28	49.56
33	223.76	46	125.93	29	10.79
37	29.24	47	3.76	37	663.65
38	291.92	48	62.75	42	115.60

3.3.2 Fibrinogen Expression

As with most of the experiments in this project, the expression of the mutants was performed in two different stages, R95Q, D97N and F98I followed by the second set of mutants R95E, G96V and D97K. The expression process itself was composed of two stages, in the first stage the cells and the bottles were prepared to be ready for fibrinogen expression on the roller system in the incubator. This stage included splitting the cells into flasks followed by 10 cm dishes and in the end by seeding them into the bottles. Cells were given adequate time to grow in the bottles until they reached confluency before adding the beads which were aimed at increasing the surface area for the cells to grow. Once the cells had adhered to the beads and confluent again, they were washed with serum free medium and incubated overnight in the same medium. Next, the second stage of ITS medium started by replacing the serum free medium with ITS medium, and the collection consisted of replacing and keeping 100 ml ITS medium per bottle, 3 times a week. ELISA was performed to evaluate the cell ability to produce fibrinogen and 24 collections of the fibrinogen production over eight weeks are shown in Figure 3-6.

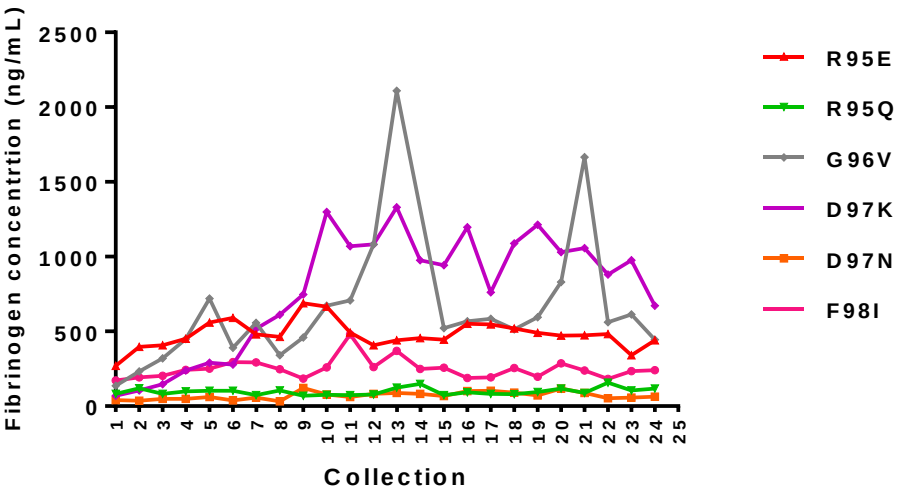


Figure 3-6 Fibrinogen expression over time. The medium samples were collected from the roller bottles three times per week over 8 weeks and ELISA was performed every 2 weeks, and at the end of the collection, to evaluate the production of fibrinogen.

For the first set of mutants (R95Q, D97N, F98I), fibrinogen production was fairly low, this is why the decision was made to use another method for transfection of subsequent mutants. Using Lipofectamine, the expression was enhanced and the amount of fibrinogen produced was increased. However, for R95E and G96V, most of the bottles were terminated early throughout the collection period, ending up with 5 roller bottles of R95E and only 3 bottles of G96V by the end of collection 24. This may be due to the high fibrinogen production, which was not well tolerated by the cells. In the discarded bottles, there were lots of dead cells without any signs of infection. The loss of these bottles had affected the final yield of fibrinogen production.

3.3.3 Fibrinogen Purification

The medium was collected for 8 weeks and precipitated with saturated ammonium Sulphate to precipitate the protein of interest. Ammonium sulphate was added slowly at 4°C to prevent denaturation of fibrinogen by creating an uneven pH around the protein. Foaming was avoided by slowing down the stirring during the addition of ammonium sulphate. The media was centrifuged after an overnight incubation and the pellet was re-suspended in a mixture of protease inhibitors to protect the protein from degradation. The dissolved pellet

was centrifuged again and the supernatant which contained the fibrinogen was stored at -80°C until purification. Fibrinogen was purified using an IF1 antibody (calcium dependent monoclonal antibody against fibrinogen) affinity chromatography using a number of buffers in each step of purification. The first step involves equilibration of the protein in neutral pH 7.4 and low salt in the presence of calcium ion to facilitate its attachment to the column. Next steps are washing of column-bound fibrinogen using high salt and low pH buffers to eliminate any contaminants, in the presence of calcium. The last step is the elution of fibrinogen from the column using an EDTA-containing buffer to chelate the calcium. The protein was stored at -80°C until finishing the purification of all precipitated solutions of each mutant. An example of the purification chromatograms is shown in Figure 3-7 for R95Q, F98I in addition to the wild type, which was kindly provided by Miss Helen McPherson (PhD student). The peaks of the two mutations are considerably lower compared to the wild type.

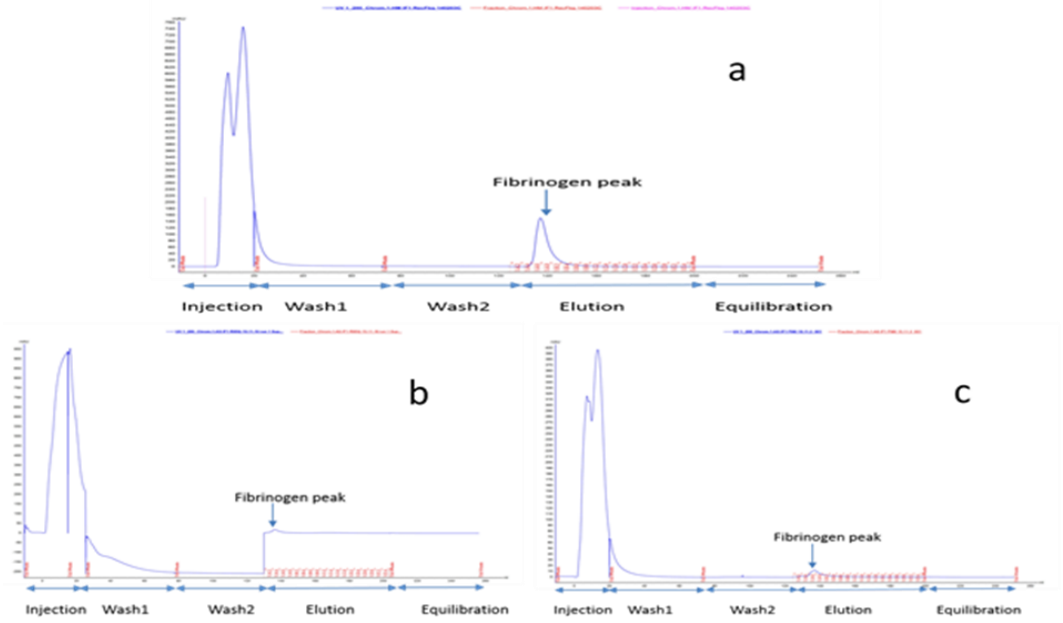


Figure 3-7 An example representation of affinity chromatography purification of (a): WT, (b): R95Q, (c): F98I. The purification steps are injection, wash 1, wash 2, elution, equilibration using an extension coefficient of 1.51. Fibrinogen peak between 120-160 ml for WT, 130-140 ml for R95Q and 120-140 ml for F98I. WT has been kindly provided by Miss Helen McPherson (PhD student).

3.3.4 Concentration and dialysis

The yield was different among the different mutants. The purified fibrinogens for all mutations were concentrated by centrifugation then dialysed for 1 hr, overnight, and 1 hr, by replacing the dialysis buffer each time. The fibrinogen concentration was then measured against the dialysis buffer, using the NanoDrop and further concentrating was performed if required in some mutants to the final concentrations and volumes as shown in Table 3-14.

Table 3-14 Final fibrinogen yield of each mutants and its final volume.

Mutation	Concentration	Volume	Total protein
R95E	1.78 mg/ml	0.6 ml	1.07 mg
R95Q	1.69 mg/ml	1.5 ml	2.53 mg
G96V	0.21 mg/ml	2.5 ml	0.51 mg
D97K	2.60 mg/ml	1.5 ml	3.98 mg
D97N	1.04 mg/ml	1.0 ml	1.04 mg
F98I	2.64 mg/ml	2.0 ml	4.53 mg

3.4 SDS-PAGE

The integrity and the purity of the produced fibrinogen chains for all variants were tested using SDS-PAGE and the results are shown in Figure 3-8. The gel shows that the samples are pure and contain all three fibrinogen chains with no significant degradation or contaminating proteins.

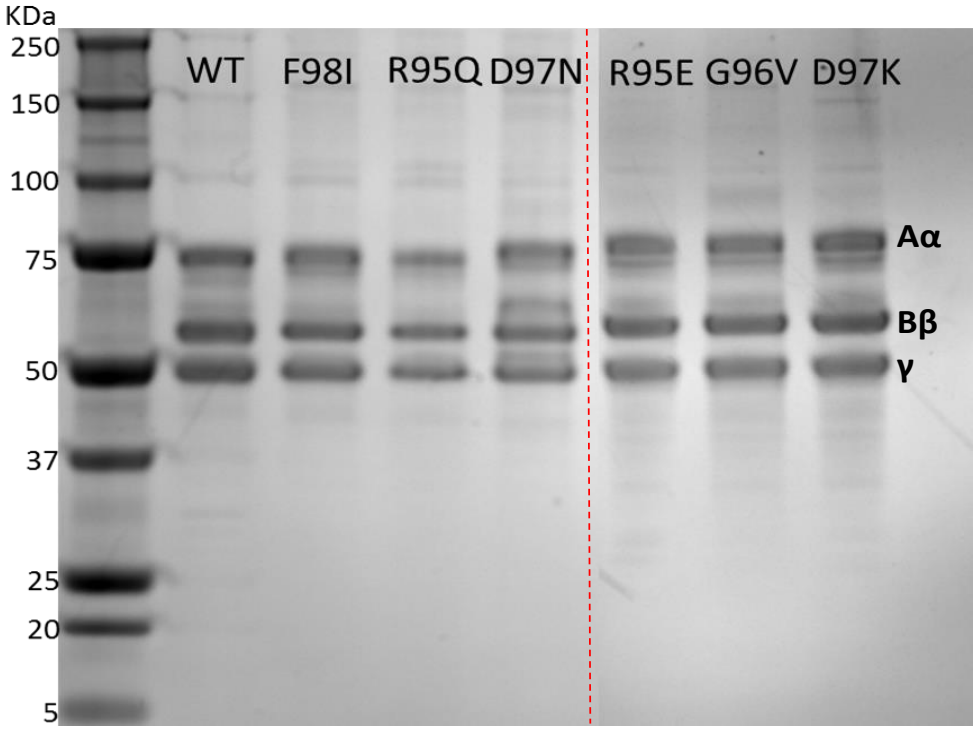


Figure 3-8 SDS page for all mutated fibrinogen against wild type fibrinogen. 2.5 µg of protein were loaded and stained by coomassie blue dye. The gel represents all fibrinogen chains Bβ, γ and the mutated Aα-chain, with clear bands for each polypeptide chain, high purity and integrity and no noticeable protein degradation. The figure is a composite of two gels.

3.5 Discussion

The aim of this project was to test the effects of mutating the RGD sequence, located in the coiled-coil segment of the Aα-chain of fibrinogen, on the interaction of fibrinogen with RBCs.

As indicated above, the design of the proposed mutations relied on changing the charge of the original amino acid to either a neutral or opposite charge as follows

1. **R95E**: Arginine (+ve) to Glutamate (-ve)
2. **R95Q**: Arginine (+ve) to Glutamine (neutral)
3. **D97K**: Aspartate (-ve) to Lysine (+ve)
4. **D97N**: Aspartate (-ve) to Asparagine (neutral)

These changes of amino acids take the size of each amino acid into consideration, so that the replacement new amino acid has approximately the

same size as the original residue. The reason for this strategy was that we could focus on the effect of changing the polar, charge-based interactions only, while excluding or minimising any other factors that may affect the properties of the new mutated fibrinogen.

The next mutation that was produced was G96V, in which Glycine was replaced by Valine which are both hydrophobic. The choice for this mutation was based on a previous report from the literature (Pierschbacher and Ruoslahti, 1984). Pierschbacher and Ruoslahti produced synthetic fibronectin peptides then coupled them to immobilised fibronectin on plastic to study the ability of these peptides to attach to fibroblasts, they found that all the fibronectin synthetic peptides that stimulated this interaction with fibroblast contained the amino acid sequence of Arg-Gly-Asp-Ser or Arg-Gly-Asp-Cys (Pierschbacher and Ruoslahti, 1984). A reduction was found in the attachment-stimulating activity of these peptides with the increase in their size, which they suggested resulted from less accessibility of these peptides to the substrate or lower stability of their conformation (Pierschbacher and Ruoslahti, 1984). The authors tested the attachment ability of these peptides by making different substitutions of the amino acids, one of these substitutions was replacing glycine by valine, which decreased the interaction of these peptides with fibroblasts (Pierschbacher and Ruoslahti, 1984). When these studies were applied to intact fibrinogen and fibrin, no interaction was found between immobilised fibrinogen on plastic surface and fibroblasts (Pierschbacher and Ruoslahti, 1984). Nonetheless, fibrinogen or fibrin polypeptides were transferred to nitrocellulose following their separation on SDS-PAGE, then their attachment to fibroblast was tested; however, the attachment was found to be absent or very weak compared to fibronectin (Pierschbacher and Ruoslahti, 1984). It was suggested that this absence of interaction might be caused by destruction of the binding site on fibrinogen or because this binding site is cryptic. Another suggestion was that the effect of the binding site on fibrinogen might be weakened by its surrounding sequences leading to the loss of its recognition by the fibroblast (Pierschbacher and Ruoslahti, 1984). Comparing the hydrophobic sequences that are surrounding both the RGD sequences (amino and carboxylic) in fibronectin, these RGDs are surrounded by more hydrophilic sequences in fibrinogen (Pierschbacher and Ruoslahti, 1984).

The last fibrinogen mutation produced in this project was F98I, and this is the first time that this naturally occurring mutant has been produced as recombinant fibrinogen. This novel heterozygous mutation was first identified in a woman who had two repeated miscarriages, but she did not present with any history of thrombosis or bleeding (Riedelova-Reicheltova et al., 2014). Laboratory investigations revealed the presence of impaired polymerisation profile and fibrinolysis, in addition to increased platelet aggregation upon activation (Riedelova-Reicheltova et al., 2014). Scanning electron microscopy showed short curly fibrin fibres, which might be a result of decreased interaction between the Aα-98Ile (aliphatic side chain amino acid) with its neighbouring amino acids compared to the Aα-98Phe (bulky aromatic side chain amino acid) that can affect lateral aggregation of the protofibrils (Riedelova-Reicheltova et al., 2014). This mutation is situated just next to the RGD sequence, position 98 in the coiled-coil region, so was of interest to this project.

Producing the mutants was a long process and included some difficulties and complications. The site-directed mutagenesis required a lot of optimisation and manipulation before the mutated colonies were successfully produced, especially for R95Q and D97N. The other mutations were slightly easier to produce because they were started from the last successful conditions of Site-Directed Mutagenesis, although some more manipulations were still required, such as increasing the concentration of polymerase. Sequencing faced some mismatches when aligning the mutated fibrinogen plasmid with the published sequence of the protein. A new updated fibrinogen Aα-chain nucleobases sequence was used in which there was no effect on the amino acid sequences of the original fibrinogen. Transfection was quite a long process and every step depended on the previous one.

In the clone selection stage, sometimes the cells were re-split to lower concentrations because it was not possible to harvest single cell clones if they were not sparse enough. Looking at the highest three clones that were chosen to proceed to the expression stage for the first set of mutants (R95Q, D97N and F98I), all of them were from the transfection with 10 µg of DNA, indicating this concentration is optimal for transfection. However, the Calcium-Phosphate

transfection was replaced by Lipofectamine 2000 transfection for R96E, G96V and D97K to increase transfection efficiency and only one concentration of DNA was used (11 µg). Comparing the two methods, in the former, HEPES saline solution was mixed with a solution that contained $CaCl_2$ and DNA to produce a layer of calcium phosphate and DNA precipitate onto the cells, glycerol shock is used in this method to increase the efficiency of the transfection (Kingston et al., 2003). In the latter method, a positive cationic lysosome surrounded the negative anionic DNA to facilitate its entry inside the cells through the negative charged cell membrane (Dalby et al., 2004). After transfection was completed and single clones were selected, medium was harvested from each clone for ELISA to evaluate fibrinogen production and keep the highest producing clone. Another ELISA was performed for the production in serum-free media of those high producing clones to choose the highest producing clone to be used in serum-free conditions. The results of these two ELISAs showed an increase in the efficiency of the production of fibrinogen when Lipofectamine was used. However, for R95E and G96V, cell death was high during the expression stage for no obvious reason. One explanation may be related to the high fibrinogen production that was not tolerated by the cells. Another possibility is bacterial infection, which can be difficult to diagnose in atypical cases. Random insertion of the vector in the genome could be a third reason. This high production of fibrinogen did not help in the net yield of fibrinogen especially in the case of G96V, followed by R95E, because the cells died and many bottles were prematurely discarded, so the high production could not overcome the low amount of collected media. The loss of some of the protein during the precipitation, purification and concentration steps was also a limiting factor in obtaining high amounts of protein. After purification, the integrity of the produced mutated fibrinogens was tested by SDS-PAGE and showed that the produced fibrinogens contain intact fibrinogen chains Bβ and γ in addition to the mutated Aα-chain. All chains showed high purity and integrity. The produced fibrinogen was later used in the functional analysis of the clot structure, as well as the interaction between fibrinogen and RBCs in relation to the effect of these mutations, detailed in the next Chapters.

Chapter 4 Effect of the Mutations on Clot Structure

4.1 Introduction

The first step after successful production of the fibrinogen mutants (R95E, R95Q, G96V, D97K, D97N) in the Aα-RGD (95-97) sequence, in addition to the novel recombinant production of F98I (Riedelova-Reicheltova et al., 2014), was to study the effect of these mutations on the clot structure and stability. Fibrin fibre thickness and network density are major determinants of clot structure. Clots with thick fibres and loose fibrin conformation lyse faster than compact clots that are made up of thinner fibres (Collet et al., 2000). This most likely due to the fact that lysis is more dependent on the clot three-dimensional configuration rather than the fibre diameter (Standeven et al., 2005, Collet et al., 2000). The difference in lysis rate between coarse clots that are made up of thick fibres and fine clots with thin fibres may be because the binding fronts of tPA are wider in the coarse clot and moved faster when compared with fine clots (Weisel, 2007). Different factors can affect the polymerisation process and subsequently the clot conformation, including pH and ionic strength, calcium and chloride concentrations as well as other proteins in the plasma (Di Stasio et al., 1998, Carr et al., 1986).

Factors that affect clot structure have an impact on its mechanical properties, which are essential for its haemostasis and wound healing functions (Standeven et al., 2005, Weisel, 2005, Di Stasio et al., 1998). Turbidity is a method that is used widely to monitor clot formation as a function of time (Weisel and Nagaswami, 1992). Alteration of fibrinogen and thrombin concentrations both have effects on the rate of polymerisation and hence final clot properties (Hantgan and Hermans, 1979). Increasing thrombin concentrations reduce the time to removal of fibrinopeptides, thereby decreasing the time of protofibril formation, as well as number of protofibrils packed in by lateral aggregation, which is known as the lag phase, hence affecting the clot structure producing clots with less stiffness (Hantgan and Hermans, 1979, Weisel and Nagaswami, 1992, Domingues et al., 2016). In clots made of purified fibrinogen, high thrombin concentrations also decrease the final turbidity, which reflects the formation of thinner fibres because turbidity is directly related to the average size of fibre bundles or even a single fibre, which is known as maximum absorbance (OD) (Carr and Hermans, 1978, Weisel and Nagaswami, 1992, Undas et al., 2012).

There is no turbidity increase during the initial lag phase of half-staggered, double stranded protofibril formation, but turbidity increases rapidly as protofibril lateral aggregation takes place (Wolberg, 2007, Carr and Hermans, 1978).

Alterations in fibrinogen concentrations also affect the polymerisation profile as well as clot structure. Higher fibrinogen levels shorten the lag phase, while increasing the fibre size and overall turbidity (Standeven et al., 2005). Calcium was found to affect clot structure by altering fibre thickness since it enhances lateral aggregation of protofibrils (Carr et al., 1986). It was suggested that binding of calcium ion to fragment D protects this fragment from being attacked by plasmin and induces certain plasmin resistant conformations of fibrinogen. These conformations are favourable for fibrinogen function in gelation and polymerisation, which may be stabilised by the effect of calcium ions (Haverkate and Timan, 1977, Okada and Blomback, 1983). The degree of crosslinking by FXIII may affect the structural changes of the clot at increasing calcium concentration and also increases in the rigidity of the gel, while in the absence of calcium no crosslinking can take place (Okada and Blomback, 1983). Although the release of both fibrinopeptides is only slightly affected by calcium concentrations, the presence of calcium decreases the activation required for gelation taking in consideration the shortening in the clotting time. Short polymers and compact clots are present with short clotting time while long polymers and porous structures are present with long clotting time (Okada and Blomback, 1983).

This chapter will assess the secondary structures of the new proteins to confirm if the mutations affect the fibrinogen structure and function. The effect of the mutations on clot structure and function were studied using some of the well-known techniques in this field. The ability of these new mutated fibrinogens to clot was tested by a clotability method followed by turbidity and lysis studies in which the effects of the mutations on clotting time, fibre thickness, and rate of lysis were evaluated. Clot fibre density was assessed by confocal microscopy.

4.2 Results

4.2.1 Effect of the Mutations on Secondary Structure of the protein by Circular Dichroism Spectra

Circular dichroism (CD) spectra were performed on the mutants in two different batches due to the availability of proteins at that time. CD spectra were used to study the effect of the mutations on the secondary structure of the protein. The results of each set of mutants were presented against the relevant WT and blank. CD spectra curves in Figure 4-1 show a slight decrease in α-helical content for the neutrally charged Q95 and N97 mutants, indicating that the charged residues might be important for the correct folding of the protein. However, this slight change in the secondary structure is considered minimal and largely does not affect protein functions.

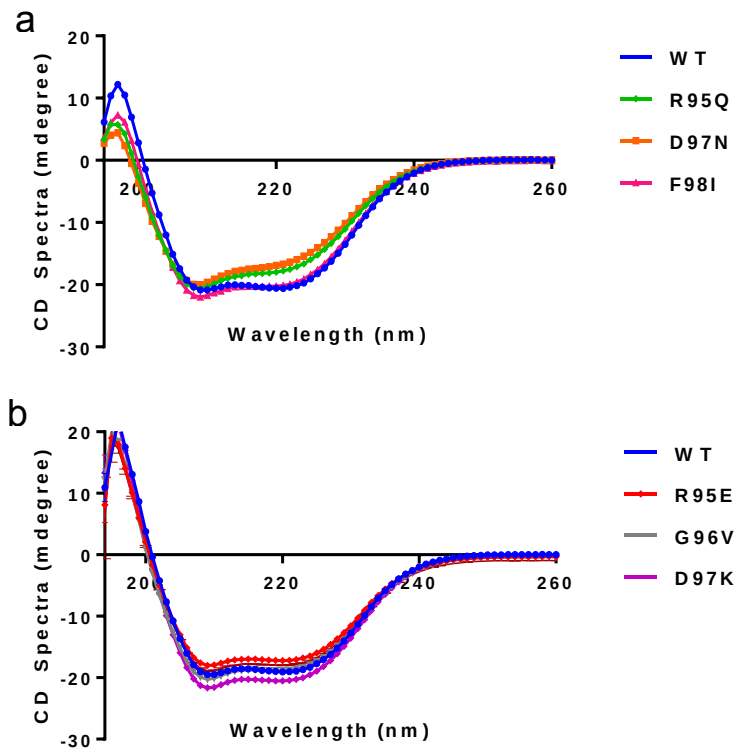


Figure 4-1 Effect of the mutations on the integrity of the proteins using CD spectra. Total of 0.2 mg/ml of fibrinogen in final volume of 200 µl TBS. Experiments were performed (n=1). **a)** First set of mutatnt (R95Q, D97N, F98I) compared to WT. **b)** Second set of mutants (R95E, G96V, F98I) compared to WT.

A closer view of the α-helical content itself is shown in Table 4-1 which showed the slight decrease of R95Q and D97N as well as a slight increase of D97K. The overall structural analysis show that the secondary structure components of the mutants were almost the same as the WT, apart from the slight changes mentioned above.

Table 4-1 Effect of the mutations on the composition of the secondary structure of the proteins using CD spectra. Total of 0.2 mg/ml of fibrinogen in final volume of 200 µl TBS. n=1

195-260 nm	WT %	R95E %	R95Q %	G96V %	D97K %	D97N %	F98I %
Helix	31.3	29.7	27.9	31.8	33.9	26.8	29.6
Antiparallel	10.6	11.6	13.9	10.6	9.4	15	12.4
Parallel	8.8	9.3	9.4	8.7	8.1	9.7	9
β-turn	17.2	17.6	18.1	17.2	16.8	18.4	17.7
Random Coil	31.8	33.1	31.8	30.9	29.6	32.3	31.0

4.2.2 Effect of Mutations on Clotability

Fibrinogen clotability assay was performed in order to test the ability of the recombinant proteins to form a clot. The way in which the experiment was performed relied on the volume of the reaction constituents without consideration of the differences in concentrations between mutants because this way was more suitable for the low amount of available proteins. However, when the experiment was repeated for the purpose of protocol optimisation by a member of the group taking into account the concentration of the protein rather than relying on its volume, there was no significant difference from the data that was obtained by adjusting the concentrations. The concentration of the proteins was measured before adding the activation mix then in the supernatant after clot formation. The clotability is expressed as percentage of the amount of protein incorporated into the clot over the original protein concentration (Figure 4-2).

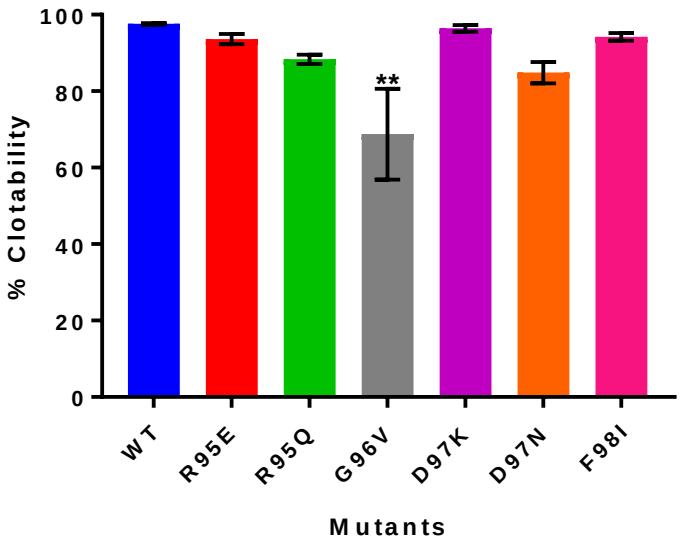


Figure 4-2 Effect of the mutations on the fibrinogen clotability. Total of 20 µl fibrinogen, 30 µl TBS, 20 µl activation mix (5 mM $CaCl_2$, 0.1 U/mL thrombin), incubated at 37°C water bath for 2 hrs. Concentration of fibrinogen was measured before and after the addition of the activation mix then the percent clotability was calculated. Significance compared to WT **P<0.01. Error bars represent Standard Error of mean (SEM) (n=3). One-way ANOVA multiple comparisons in GraphPad prism, Dunnett's post hoc test were used.

As shown in Figure 4-2, R95E, D97K and F98I had similar clotability to WT, followed by R95Q and D97N having a slight decrease but this did not reach a statistical significance. G96V had significantly lower clotability of around 68% compared to the WT. The higher similarity in clotability between WT fibrinogen and the oppositely charged mutants (negative E95 and positive K97) may suggest that the charge of the side chain in these RGD residues provides fibrinogen with more stability. Hence, when the RGD was mutated by charged residues, clotability was more similar to the WT rather than when the sequence was mutated by neutral residues. A charged residue at this point in the polypeptide chain may be important for correct folding of the protein domain.

4.2.3 Effect of the Mutations on Clot Formation and Fibre Thickness by Turbidity assay

Fibrinogen R95Q, D97N, F98I

Before the production of all mutants was completed, turbidity was first performed for the first set of mutants (R95Q, D97N, F98I) in a 384-well plate. The experiment was performed using 0.5 mg/ml of fibrinogen and 0.1 U/ml of thrombin. Looking at the polymerisation profile in Figure 4-3a, both R95Q and D97N had clearly lower maximum absorbency than WT whereas F98I was only slightly lower. The maximum OD profile in Figure 4-3b, which refers to fibre diameter or thickness (Metassan et al., 2009) showed significantly thinner fibres for R95Q and D97N compared with WT and to a lesser extent, F98I showed thinner fibres than WT. R95Q had a clearly and significantly longer lag phase compared to the WT in agreement with its slow clotting tendency that appeared on the polymerisation profile. On the other hand, there was no statistically significant differences between the lag phase of D97N and F98I compared to the WT as shown in Figure 4-3c.

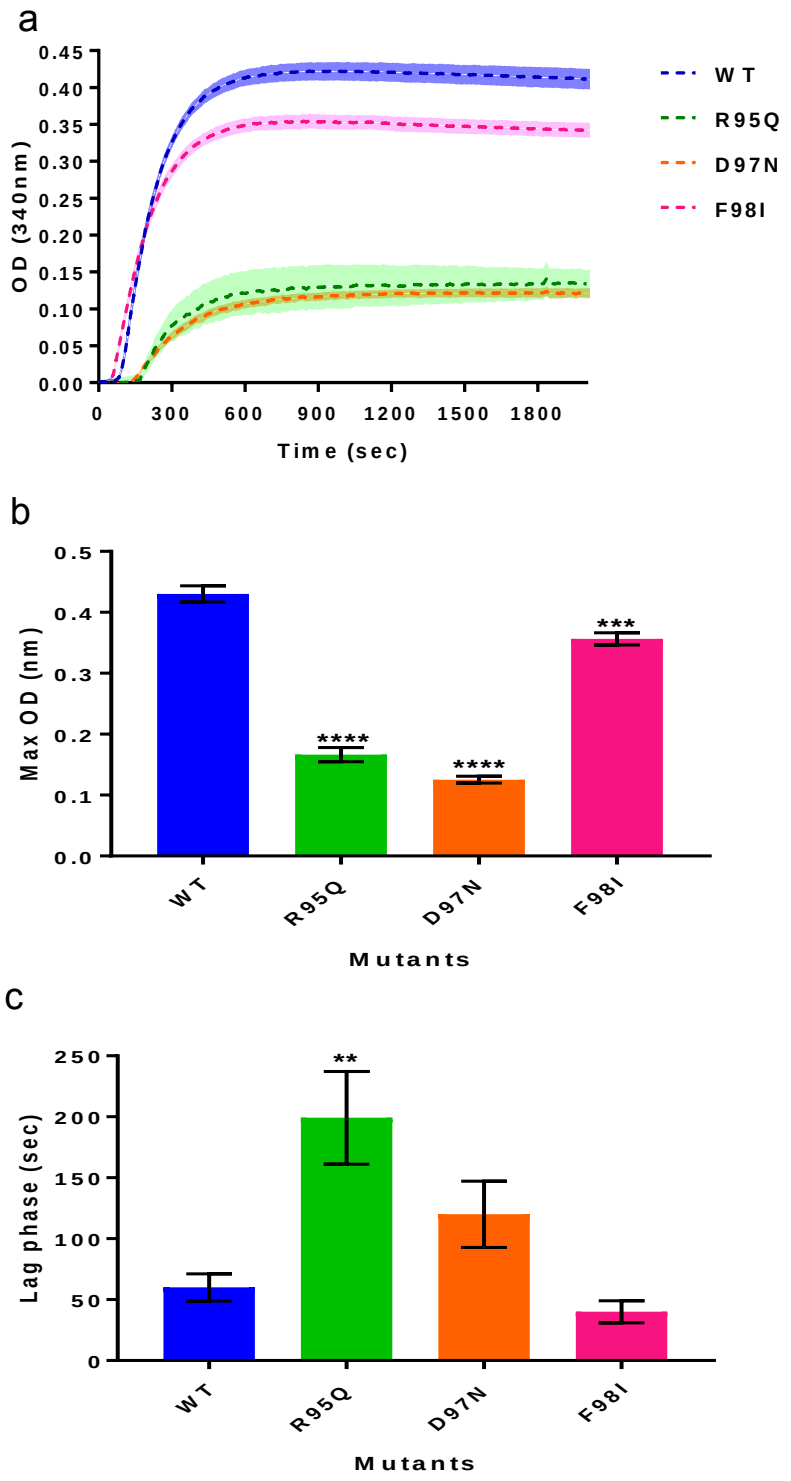


Figure 4-3 Turbidity analysis for WT, R95Q, D97N, F98I in 384-well plate. Total of 0.5mg/ml fibrinogen, 5mM $CaCl_2$, 0.1 U/mL thrombin. **a)** Absorbency measurements were made every 12 sec for 2 hrs at 340 nm. **b)** Maximum OD in the turbidity analysis for fibre thickness assessment. **c)** Lag Phase representation of clotting time. Significance compared to WT **P<0.01, ***P<0.001, ****P<0.0001. Error bars represent Standard Error of mean of the intra assay variations (SEM) (n=3). One-way ANOVA multiple comparisons in GraphPad prism, Dunnett's post hoc test were used.

Using Lower Concentrations of Fibrinogen

Because of the lower availability of the second set of mutants (R95E, G96V and D97K), a decision was made to use lower concentrations of fibrinogen in the turbidity experiment. Before proceeding with the turbidity experiments using the lower fibrinogen concentrations in the 384-well plate, a couple of test experiments were performed to confirm the concentrations of thrombin and fibrinogen that could be used to produce turbidity curves with sufficient changes in maximum absorbency, appropriate lag phase in the order of a few hundred seconds and acceptable polymerisation rates. Normal WT fibrinogen (0.5 mg/ml fibrinogen + 0.1 U/ml thrombin) was compared with two different combinations of WT fibrinogen and thrombin which were 0.2 mg/ml fibrinogen + 0.1 U/ml thrombin and 0.2 mg/ml fibrinogen + 0.04 U/ml thrombin.

Average results of the two test experiments are shown in Figure 4-4. As might be expected, the turbidity curves (Figure 4-4a) are overall lower due to the lower fibrinogen concentration for both the tested concentrations of thrombin; however, using the original amount of thrombin (0.1 U/ml) showed better profile with a similar appearance to the WT. The thickness of fibres represented by the maximum OD (Figure 4-4b) was almost the same for both tested concentrations showing significantly thinner fibres compared to the normal WT condition, but the lag phase (Figure 4-4c) was significantly longer with 0.04 U/ml thrombin concentration compared to WT condition. Considering these effects of decreasing the fibrinogen concentration, a decision was made to use the combination of (0.2 mg/ml fibrinogen + 0.1 U/ml thrombin) in the turbidity of the second set of mutants.

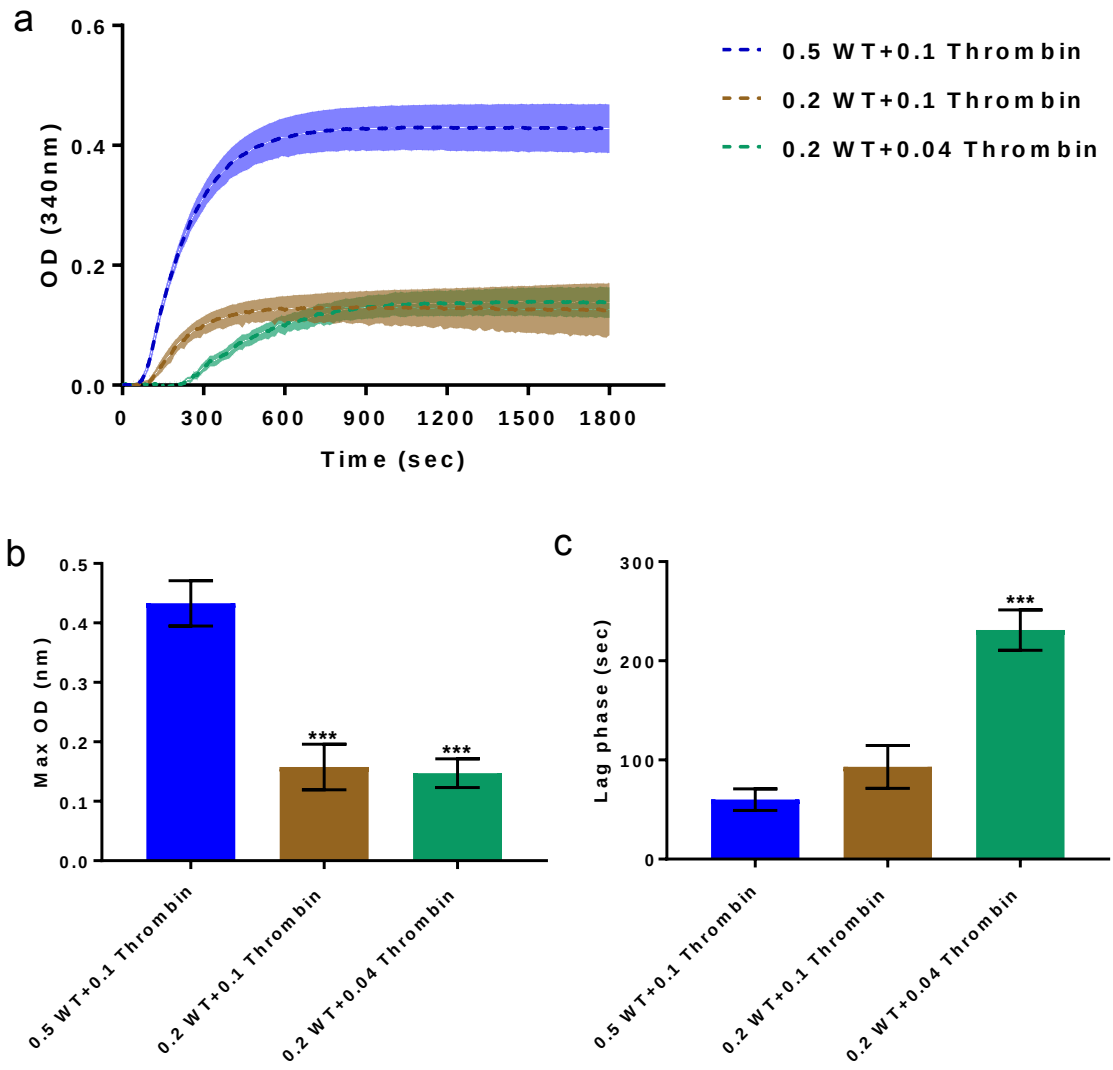


Figure 4-4 Turbidity analysis test for lower fibrinogen concentration in 384-well plate. Original WT (0.5 mg/ml fibrinogen, 5 mM $CaCl_2$, 0.1 U/mL thrombin) compared to two different combinations (0.2 mg/ml WT, 5 mM $CaCl_2$, 0.1 U/mL thrombin) and (0.2 mg/ml WT, 5 mM $CaCl_2$, 0.04 U/mL thrombin). **a)** Absorbency measurements were made every 12 sec for 2 hrs at 340 nm. **b)** Maximum OD in the turbidity analysis for fibre thickness assessment. **c)** Lag Phase representation of clotting time. Significance compared to WT ***P<0.001. Error bars represent Standard Error of mean of the intra assay variations (SEM) (n=2). One-way ANOVA multiple comparisons in GraphPad prism, Dunnett's post hoc test were used.

R95E, G96V, D97K

Polymerisation profiles (Figure 4-5) illustrated lower maximum absorbency for all the mutants compared to WT, which was more pronounced for R95E and G96V, as shown in Figure 4-5a. The maximum OD profile in Figure 4-5b showed significantly thinner fibres for both R95E and G96V compared to WT. D97K also had thinner fibres but less significantly than the other two mutants. The lag phase profile in Figure 4-5c showed a significantly longer time to lateral aggregation for R95E compared to the WT. G96V came next with a slight increase of the lag phase, whilst D97K had a slightly shorter lag phase than WT, but these effects of the latter two mutants did not reach statistical significance.

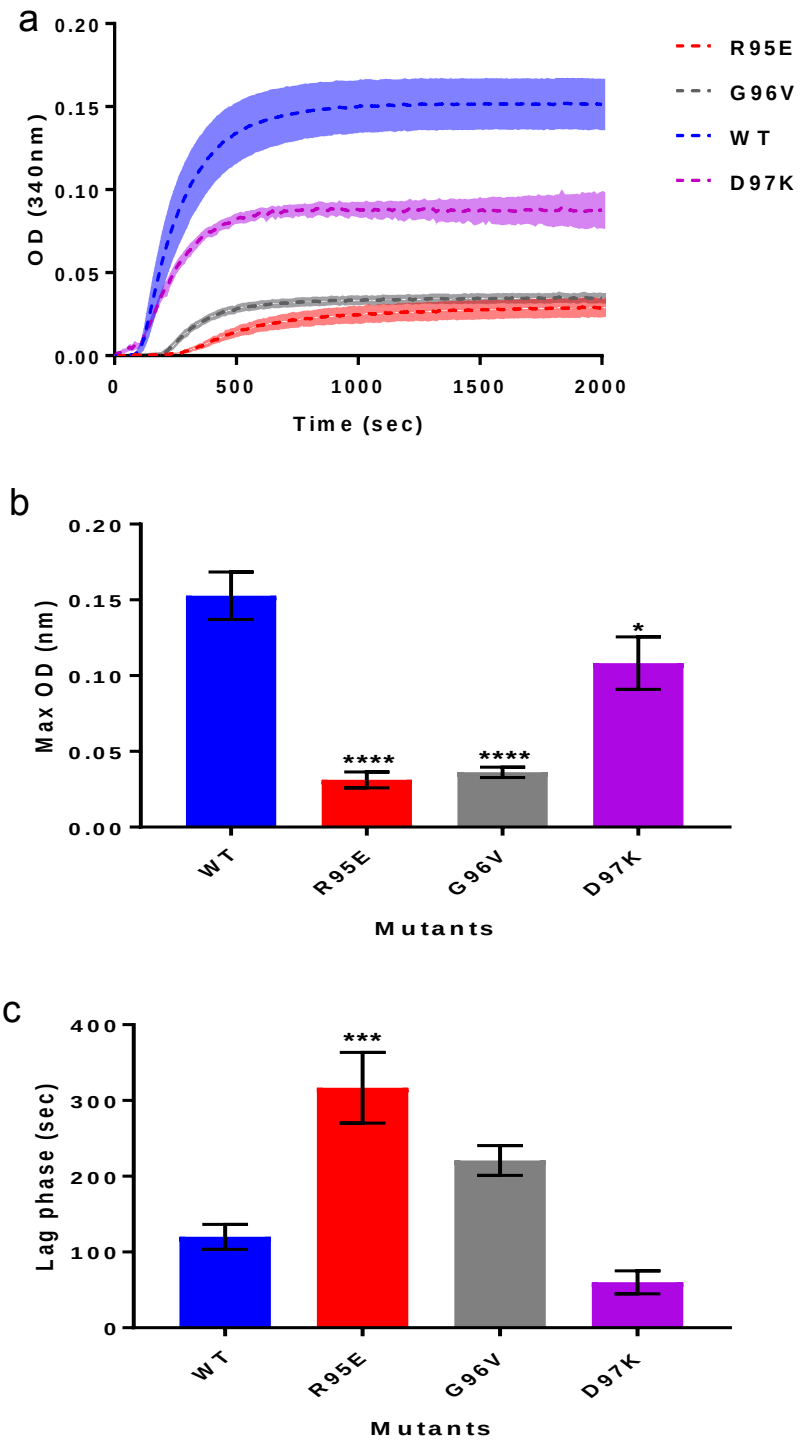


Figure 4-5 Turbidity analysis for WT, R95E, G96V, D97K in 384-well plate. Total of 0.2 mg/ml fibrinogen, 5 mM $CaCl_2$, 0.1 U/mL thrombin. **a)** Absorbency measurements were made every 12 sec for 2 hrs at 340 nm. **b)** Maximum OD in the turbidity analysis for fibre thickness assessment. **c)** Lag Phase representation of clotting time. Significance compared to WT *P<0.05, ***P<0.001**,** ****P<0.0001, Error bars represent Standard Error of mean (SEM) (n=3). One-way ANOVA multiple comparisons in GraphPad prism, Dunnett's post hoc test were used.

The results of both sets of mutants were normalised to their relevant WT in order to be comparable in one graph. Maximum OD representing fibre thickness in Figure 4-6a was similar for R95E, R95Q, G96V, D97K and D97N showing significant thinner fibres that that of WT. However, this effect failed to reach statistical significance for F98I. In case of lag phase Figure 4-6b, normalised values for all mutants showed consistent results with their original ones being a significantly slower for R95Q and R95E. However, there were no statistically significant differences for the lag phase of G96V, D97K, D97N and F98I compared to the WT.

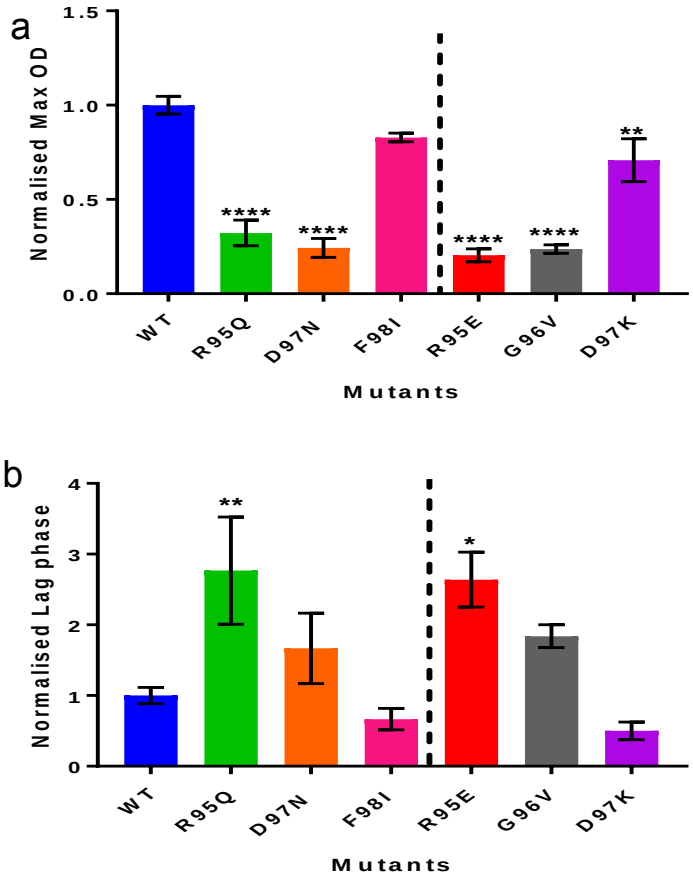


Figure 4-6 Normalised values of the turbidity analysis to compare the effect of all mutants to the WT. Total of 0.5 mg/ml fibrinogen to the left of dotted line or 0.2 mg/ml fibrinogen to the right of dotted line, 5 mM $CaCl_2$, 0.1 U/mL thrombin. **a)** % of the lag phase representing clotting time. **b)** % of the Max OD in the turbidity analysis for fibre thickness assessment. Significance compared to WT *P<0.05, **P<0.01, ****P<0.0001. Error bars represent Standard Error of mean (SEM) (n=3). One-way ANOVA multiple comparisons in GraphPad prism, Dunnett's post hoc test were used.

4.2.4 Effect of the Mutations on Clot Lysis

In this experiment, G96V was not used because there was not enough protein left, and D97N was analysed only once due to the limited amount of protein available. Lysis profiles Figure 4-7 a and b were divided into two graphs according to ease of presentation and to avoid overlap between the mutants. The lysis was assessed by calculating the time required for half of the clot to lyse for each mutant, as shown in Figure 4-7c. When comparing all the mutants to the WT, it appears that the oppositely charged mutants (negative E95 and positive K97) had a slightly elongated time to half-lysis that failed to reach statistical significance, whereas the other mutants had a slightly shorter half-lysis time that did not reach statistical significance. R95E required the longest time to clot and the longest time to lyse, but D97K required a slightly shorter time to clot and longer time to lyse, compared to WT. R95Q and D97N required longer time to clot and shorter time to lyse than WT. F98I clots lysed slightly faster than WT. However, there were no statistically significant differences between the lysis time of the mutants and the WT.

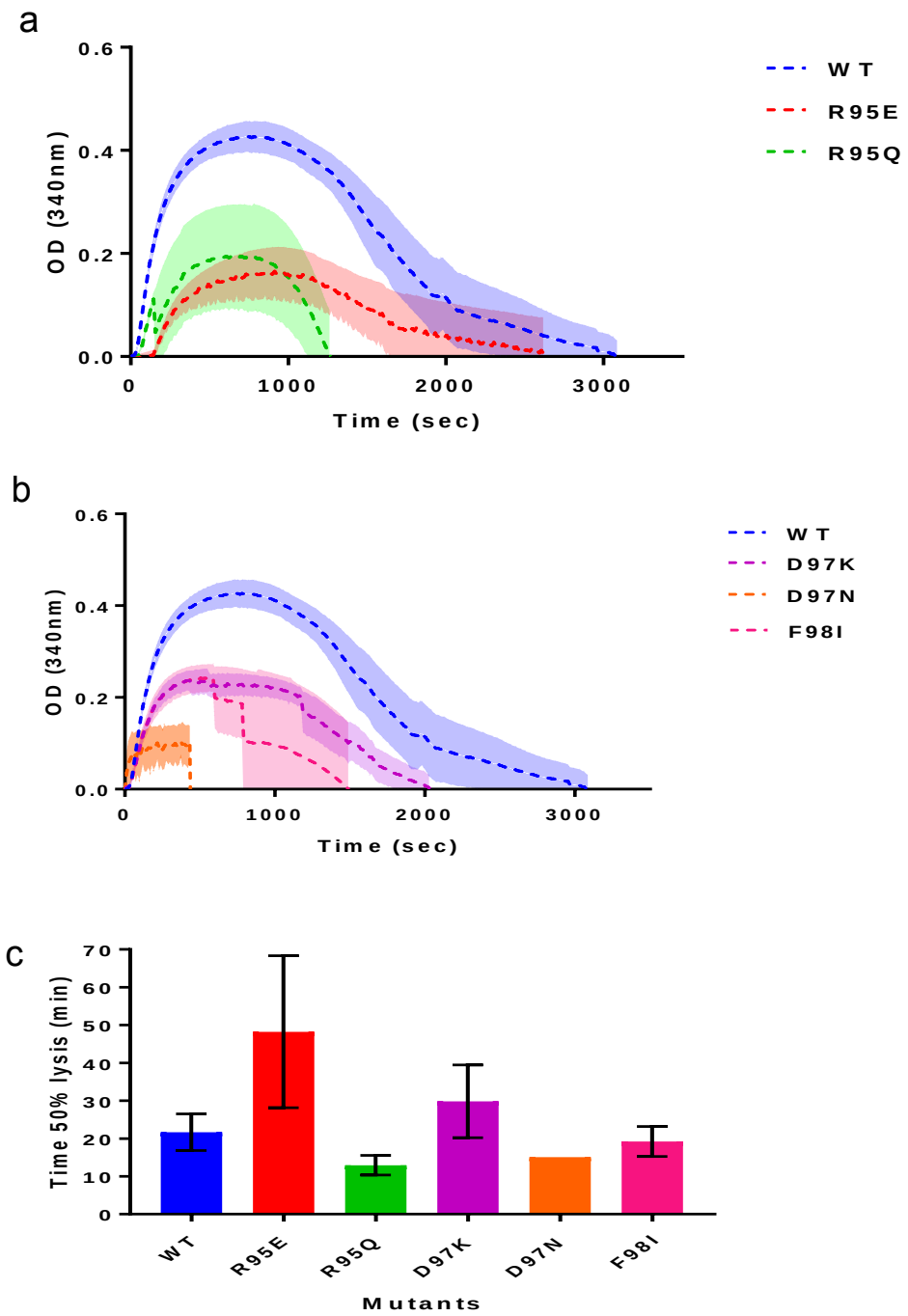


Figure 4-7 Turbidity and lysis analysis for WT and all mutants in 384-well plate. Total of 0.5 mg/ml fibrinogen, 5 mM $CaCl_2$, 0.1 U/mL thrombin, 100 pM tPA, 0.24 µM plasminogen. Absorbency measurements were made every 12 sec for 2 hrs, 37°C at 340 nm. **a)** Lysis curve of R95E and R95Q compared to WT. **b)** Lysis curve of D97K, D97N and F98I compared to WT. **c)** Time to 50 % lysis of all mutants compared to WT. Error bars represent (SEM). n=3, D97N (n=1). The lysis profiles are presented in two separate curves to avoid overlapping between mutants and for clarity of presentation. One-way ANOVA multiple comparisons in GraphPad prism, Dunnett's post hoc test were used.

4.2.5 Effect of the Mutations on Clot Structure using Laser Scanning Confocal Microscopy

The effects of the mutations on fully hydrated clot three-dimensional structure were analysed by confocal microscopy. Clots were formed by incubating fluorescently-labelled fibrinogen of each variant with thrombin and $CaCl_2$ overnight, and then imaged using the laser scanning confocal microscope. Representative images of clots from each mutant and the WT are shown in Figure 4-8. The images showed that all mutants had shorter fibres, which were more branched than the WT. Fibre counts are shown in Figure 4-9. R95Q showed significantly higher whilst D97N almost identical fibre count compared to WT, although the fibres looked shorter. R95Q fibres were highly branched and formed quite large branching points, giving the clot a heterogeneous appearance with larger pores. D97N fibres were less defined, clumped in a lot of branching points, with smaller pores showing a heterogeneous appearance. R95E formed significantly less dense clots compared to the WT, with slightly shorter and less branched fibres, forming a more porous clot. D97K and F98I had a significantly higher fibre count than WT, with shorter and more branched fibres. D97K formed larger pores, whilst F98I showed smaller pores, compared to WT. The general appearance of all mutants is in agreement with the turbidity data which indicated thinner fibres for all mutants. G96V was not used in this experiment due its concentration and amount being too low. Comparing with the turbidity curve, the higher the fibre number of D97K and F98I, the faster the clotting and the opposite is true for the other mutants.

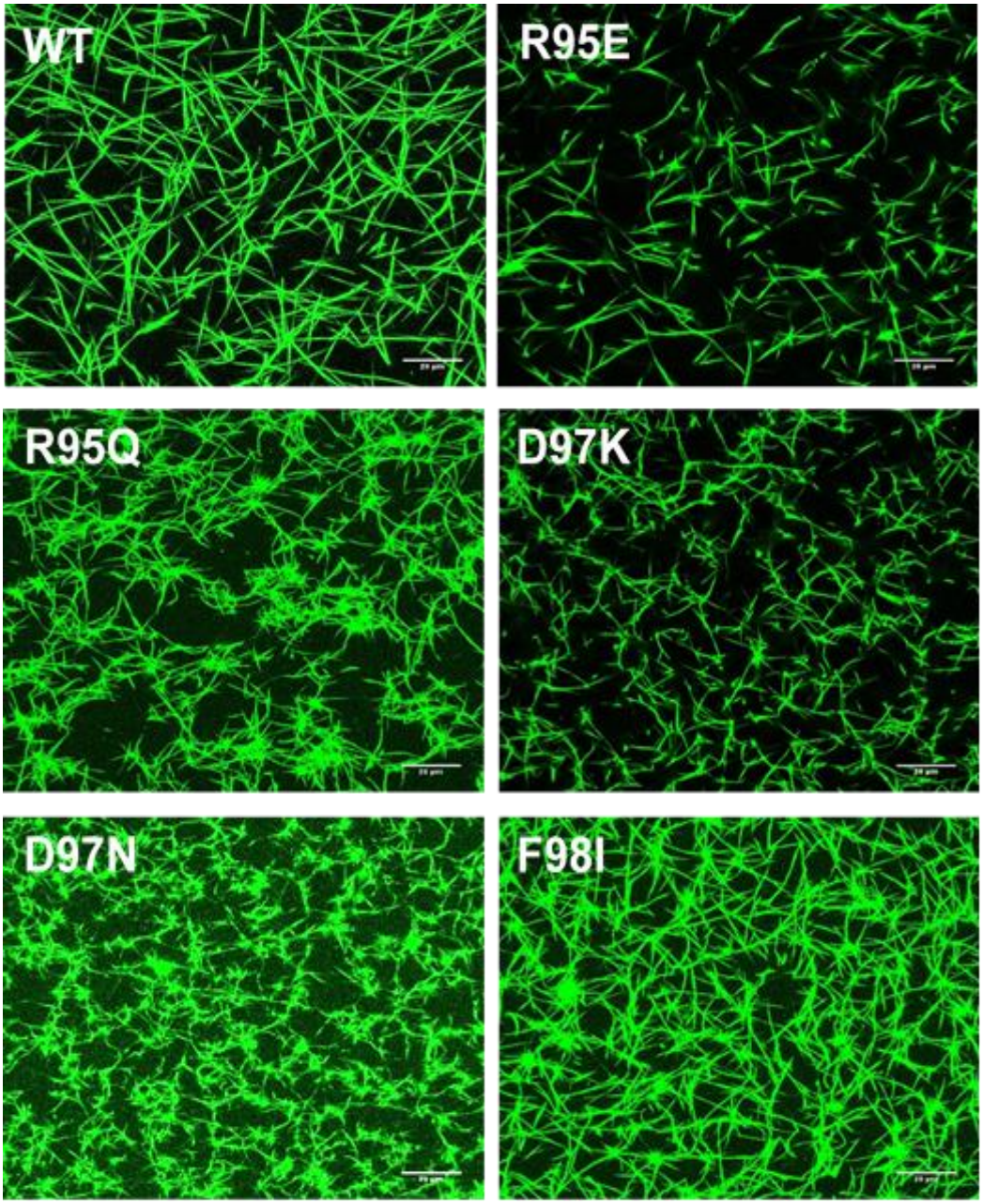


Figure 4-8 Representative images of the mutated fibrinogen clots taken by laser scanning confocal microscopy. Each clot is made by 0.5 mg/mL unlabelled fibrinogen, with 25 µg/mL Alexa^{488}-fibrinogen, 5 mM $CaCl_2$, 0.1 U/mL thrombin, final volume 40 µl. n=3. Scale bar= 20 µm.

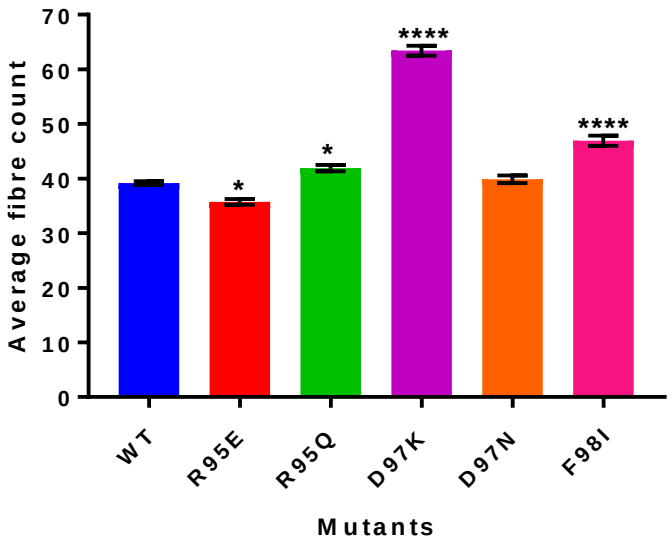


Figure 4-9 Average fibres count of mutated fibrinogen clots taken by laser scanning confocal microscopy. Each clot is made by 0.5 mg/mL unlabelled fibrinogen, with 25 µg/mL Alexa^{488}-fibrinogen, 5 mM $CaCl_2$, 0.1 U/mL thrombin, final volume 40 µl. Error bars represent Standard Error of Mean (SEM). n=3. Significance compared to WT, *P<0.05, ****P<0.0001. One-way ANOVA multiple comparisons in GraphPad prism, Dunnett's post hoc test were used.

4.3 Discussion

The structure of clots can indicate their tendency to increase the risk of cardiovascular diseases, e.g. clots formed with compact structures of thin fibres and small pores that are resistant to fibrinolysis (Hooper et al., 2012). Assessment of fibrin involvement in thrombotic risk can be achieved by the direct analysis of clot structure and fibrinolysis (Hooper et al., 2012), and targeting fibrin clot properties was suggested for finding novel therapies for thrombotic and cardiac diseases (Sumaya et al., 2018).

The results of this chapter indicate the structural and functional effects of the mutations in fibrinogen including its ability to clot, its polymerisation profile, as well as the clot ability to lyse, and its structure. Although no clear changes in secondary structure of the proteins, which may affect fibrinogen function, were observed, all mutants showed changes in their turbidity and lysis profiles which

varied in their extent. Clear changes in the clot structure were also observed by confocal microscopy. A summary of the results is shown in Table 4-2.

Table 4-2 Summary of the results of the effect of the mutations on clot structure and properties, compared to the WT. Arrows represent the increase or decrease in each particular parameter. One arrow represents a statistically non-significant effects, two arrows represent statistically significant effects. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

	R95E	R95Q	G96V	D97K	D97N	F98I
Clotability	93.7%	88.4%	68.8% **	96.4%	84.9%	94.2%
Max OD	↓↓ ***	↓↓ ****	↓↓ ****	↓↓ *	↓↓ ****	↓↓ ***
Lag phase	↑↑ ****	↑↑ **	↑	↓	↑	↓
Time to ½ lysis	↑	↓	-	↑	↓	↓
Clot structure	Shorter and slightly less branched	Shorter and highly branched	-	Shorter and highly branched	Much shorter and highly branched	Shorter and highly branched
Fibre density	↓↓ *	↑↑ *	-	↑↑ ****	≈	↑↑ ****
α-helix count	≈	↓	≈	↑	↓	≈

The analysis of CD spectra showed slight changes in the amount of α-helical content of the secondary structures of R95Q and D97N, compared to WT. This indicates that the loss of charge in the RGD site may affect the structure of the protein although these changes were minimal. For all other mutants R95E, G96V, D97K and F98I, there were no changes in the fibrinogen secondary structures caused by the mutations.

Compared to the WT which had 97.6% clotability, all mutants showed similar clotting tendency except for G96V. This may in part be due to its very low concentration compared to the other mutants. While the method is dependent on the volume of the solutions and not on protein concentration, very low concentrations may increase the variability and noise of the assay. Mutating the centre of the RGD may have more effect on altering the clotting tendency of the fibrinogen molecule maybe because the mutation disturbs the core of the region, and consequently the coiled coil of the molecule, which provides an essential backbone to the longitudinal shape of the fibrinogen and fibrin protein. Otherwise, alteration in the tertiary structure may occur since no changes in the secondary structure were observed by the CD spectra for this mutant.

Turbidity profiles showed clearly lower maximum absorbency for R95E, R95Q, G96V, D97N and F98I and to a lesser extent for D97K compared to the WT. Looking at the maximum OD curves, fibrin fibres made by all mutants were significantly thinner than the WT. Looking at the lag phase profiles, the rate of formation of fibrin fibres varied between the mutants; being significantly delayed for R95E and R95Q compared to the WT, followed by G96V and D97N. For D97K and F98I, there were no statistically significant differences in rates of formation of the fibres compared to the WT. These results were consistent with the normalised results. These changes reflect the importance of the RGD sequence in producing normal clots and restoring the normal function of fibrinogen. Turbidity curves indicating effects of mutating the Aα-chain RGD sequence clearly showed alterations in structure of clots that were formed by the new mutated fibrinogens. It was observed that mutating this site of fibrinogen affected the fibre thickness producing thinner fibres. The effect of the mutations may occur by inhibiting the protofibril lateral aggregation process, which results in thinner fibres. This may be explained by the delay in the lag phase, reflecting a delay in the formation of the fibrin fibres and their growth to the extent in which lateral aggregation starts to occur (Weisel and Nagaswami, 1992, Weisel, 2007).

Turbidity and lysis profiles showed that the effects of mutations on fibrinolysis did not reach statistical significance. Neutral charged mutants; Q95 and N97 showed a very small decrease in lysis time, followed by F98I, whereas the charged

mutants; negative R95E and positive D97K showed a slightly slower half-lysis time compared to the WT, but these changes did not reach statistical significance. Comparing the opposite results of clotability and lysis that were obtained by the neutral charge changes in the residues Q95 and N97 to the charged residues of negative E95 and positive K97 may reflect the importance of the presence of charge in fibrinogen molecule in order to provide the molecule with more stability. The more normally clotable charged mutants required slightly more time to lyse in comparison to the slight decrease in clotability that was observed for the neutral mutants.

Another way to assess clot structure is the use of laser scanning confocal microscopy to study the three-dimensional structure of fully hydrated fibrin network (Blomback et al., 1989). Analysing the confocal images, mutations in the Aα-RGD sequence influenced fibre thickness, in agreement with the turbidity results with all recombinant fibrinogens (R05E, R95Q, G96V, D97k, D97N, and F98I) showing thinner fibres compared to the WT. Clots were described as coarse and fine, depending on the degree of lateral aggregation of the protofibrils assuming that the coarse clots have more branching point forming a network of bundle fibres (Muller et al., 1984). However, branching junctions were found in the fine clots which may be formed as a result of twisting of two or more protofibrils around each other, this action could be the determinant of the clot strength (Muller et al., 1984). According to this assumption all clots formed by the produced Aα-RGD mutants were fine clots because they all had thinner fibres and as mentioned above this may be due to inhibition of the lateral aggregation (Weisel and Nagaswami, 1992, Weisel, 2007). Contrary to Muller's *et al* suggestions, R95E and D97K had slightly less branching points than the WT and the other mutants but they had high clotability and required slightly more time to lyse. The slight increase in clot stability of R95E and D97K may be due to the opposite change in charge of the residues at positions 95 and 97. The highly altered clot structures for R95Q and D97N may explain their low maximum absorbency in turbidity and is consistent with the thinner fibres which were measured by turbidity. These two mutants may have much impaired protofibril lateral aggregation compared with the other mutants. F98I had significantly more fibres which were shorter, more branched and less porous than the WT which

agreed with the slightly lower maximum absorbency, hence thinner fibres, measured by turbidity. These changes which were observed by confocal images in this project, agreed with the structural appearance by SEM of the natural form of this mutant in which the fibres were short and curly (Riedelova-Reicheltova et al., 2014). In the study by Riedelova-Reicheltova *et al*, regarding the naturally occurring form of F98I, it was found that this mutation had lower interaction with its neighbouring amino acids compared to the WT using molecular modelling. The authors suggested that this alteration may affect the protofibril lateral aggregation to form the short and curly fibres (Riedelova-Reicheltova et al., 2014). According to Riedelova-Reicheltova *et al*, interaction of the new aliphatic side chain of isoleucine instead of the bulky aromatic side chain of phenyl alanine with its aromatic surrounding amino acids may be another cause of the presence of thinner fibres (Riedelova-Reicheltova et al., 2014). Due to its limited optical resolution, inability to measure the thickness by confocal microscopy tends to limit the comparison between the turbidity and the confocal images to visual assessment rather than calculated analysis (Blomback et al., 1989).

The advantages of studying clot structure by spectrophotometry methods were addressed by Wolberg *et al.*, in that fibrin fibre structure and clot lysis can be evaluated using plate-reading spectrophotometers that only require small sample volumes, and are easy to use for multiple sample analysis (Wolberg et al., 2002). Compared to the end-point, single sample spectrophotometers, these plate-reading spectrophotometers are found to be susceptible to a degree of errors owing to uncaptured scattered light. These errors can be negligible in case of turbidity because they do not affect the ability to detect changes in clot structure (Wolberg et al., 2002).

Assessment of clot structure may aid the diagnosis of some diseases which are caused by altered clot structure and function such as cryptogenic stroke (Undas et al., 2009a). Another example was the case of the woman who had the multiple miscarriages which were caused by the naturally occurring F98I fibrinogen variant, and who had no obvious thrombotic symptoms. The laboratory assessment however showed alterations in clot structure which may explain the

patient’s symptoms, and are consistent with our results (Riedelova-Reicheltova et al., 2014).

In summary, the mutations demonstrated effects on fibrin clot structure represented by the changes in polymerisation profiles, lysis rate, fibres thickness, fibre branching, and clot heterogeneity. However, the mutations did not significantly affect the clotability (apart from G96V) of the proteins or their secondary structures.

Chapter 5 Plate-Based Fibrinogen-RBC Binding Studies

5.1 Introduction

RGD is a sequence which is known to be the recognition site on fibrinogen and some other proteins for the $\alpha_{IIb}\beta_3$ integrin on platelets (Xiao et al., 2004, Oyama et al., 2017). These peptides are present in two different sites on the Aα-chain of fibrinogen, residues 95-97 and 572-574 (Hook et al., 2017).

This project's hypothesis was that altering the RGD sequence would reduce the interaction of fibrinogen with the RBCs. This reduction is depending on the assumption that the site of interaction of the RBC with fibrinogen is β-integrin dependent and might share some structural similarities with the platelets integrin (Carvalho et al., 2010). The study by Carvalho *et al.* has indicated that Eptifibatide, (an $\alpha_{IIb}\beta_3$ inhibitor) leads to decrease, but not full elimination of binding between fibrinogen and RBCs, when tested by AFM (Carvalho et al., 2010). This was in a comparison study between RBCs and platelets in the presence of calcium ion. In the case of platelets, Eptifibatide blocked the binding with fibrinogen completely. These findings indicated that the binding site of fibrinogen on RBCs is not $\alpha_{IIb}\beta_3$ but a related integrin (Carvalho et al., 2010).

Red blood cells are the most abundant blood cells in the body, and subsequently they are major regulators of blood rheology and flow (De Oliveira et al., 2012). RBCs can be used in studying the mechanism of cell adhesion, cellular interaction and involvement in thrombosis and cardiovascular disease (Lavalle et al., 1996, Chien and Jan, 1973). On the other hand, because RBCs are anucleated cells, they are prone to *in vitro* loss of function and viability, by accumulation of waste, nutrient depletion, in addition to morphological and other biochemical changes. In the early 1970s, their role in cell to cell interactions, which may be involved in RBC aggregation, was investigated. A study by Chien and Jan suggested that RBC aggregation was not the result from a balance between attractive and repulsive electrostatic interactions on the RBC surface, but due to interactions between the RBC surface and macro molecules that are present in the suspension (Chien and Jan, 1973). Another early study suggested a possible relationship between RBC and fibrin(ogen). The authors suggested the existence of a possible proteolytic activity in the RBC wall that could alter fibrin structure (Murray and Rearick, 1974).

Later studies suggested some sort of interaction between fibrinogen and RBCs, based on the fact that fibrinogen induces RBC hyper-aggregation (De Oliveira et al., 2012). In another study, it was found that Monafram (a platelet $\alpha_{IIB}\beta_3$ inhibitor) was capable of slowing down RBC aggregation, but not the strength of this aggregation, which was regardless of the presence or absence of platelets (Gafarova et al., 2012). The authors hypothesised the existence of a specific receptor on the RBC that interacts with fibrinogen and is able to speed up the aggregation in normal conditions, and that this receptor is different from the platelet receptor for fibrinogen. In some of their experiments they found that binding of fibrinogen to the erythrocyte unknown receptor resulted in abnormal increase in the strength of the aggregation of the cells (Gafarova et al., 2012). In the presence of fibrinogen, the authors found that Monafram can decrease the strength of this aggregation. Although the effect of Monafram on RBC aggregation was independent of the platelet count, they found that the presence of platelets had some effect on the strength of RBC aggregation, however the mechanism of this effect remained undetermined (Gafarova et al., 2012). These data are in agreement with the possibility of a fibrinogen receptor on the RBC membrane that is related to (and also different from) the platelet $\alpha_{IIB}\beta_3$ integrin receptor for fibrinogen (Carvalho et al., 2010).

Due to the fact that blocking the receptor on the RBC membrane decreased the interaction with fibrinogen, and assuming that the fibrinogen $A\alpha R_{95}G_{96}D_{97}$ sequence is responsible for the interaction with RBC, new fibrinogen variants with mutated $A\alpha R_{95}G_{96}D_{97}F_{98}$ sequence should show altered ability to bind to RBCs. This chapter examines this interaction through the development of binding assays in static conditions that are plate-based.

5.2 Results

5.2.1 Effect of the Mutations on Interaction between Red Blood Cells and Fibrinogen by Static Plate-Binding Assay

In order to study the presence of any kind of binding between fibrinogen and RBCs, and the effect of the mutations on this binding, several interaction experiments have been developed. Effect of mutations (R95E, R95Q, G96V, D97K, D97N and F98I) on the binding of fibrinogen with RBCs was tested in static condition using a maxisorp plate to develop a plate-binding assay. Before using the mutants, several steps of optimisation processes were performed. The optimisation can be divided into three stages; choosing a way to measure the binding, coating with fibrinogen to measure the released haemoglobin from the RBCs, and finally coating with RBCs to measure bound fibrinogen to the RBCs by fibrinogen ELISA.

Mode of Measuring the Binding

Three modes of measuring the binding between fibrinogen and RBCs were tested, using 5% v/v DiD dye, auto-fluorescence (Casella et al., 2004) and haemoglobin release. Initially, the plate was coated with 80 µg/ml of fibrinogen, blocked with 1% w/v BSA, before different RBCs were added in different Haematocrit (Ht) levels of 5, 10, 25, 50, and 100%. All plates were washed and wells were filled with PBS for fluorescence reading, and water to haemolyse the cells for haemoglobin reading. The haemoglobin readings were taken on a range of wavelengths (650-700 nm) to select a wavelength that works best with the newly developed technique. The results of the three types of experiments suggested that the use of released haemoglobin, as indicator for the binding between fibrinogen and RBCs, was the best option. For DiD-labelled RBCs, the results are presented in Figure 5-1. The signals were too high to be detected and the readings showed “overflow” at 650 nm (data not shown). For the other wavelengths except 660 nm, there was no detection of fluorescence on the DiD-labelled RBCs (Figure 5-1). At 660 nm, there was a slight change in DiD-labelled RBCs signal, reflecting a decrease in binding with increasing haematocrit, which

looked unconvincing. The auto-fluorescence readings were similar among all concentrations of the RBCs and among the different wavelength tested (650-700 nm, data not shown), suggesting that this method was not the correct way to assess the binding between fibrinogen and RBCs. Relying on these data, both auto-fluorescence and DiD dye were excluded as indicators of the binding between fibrinogen and RBCs.

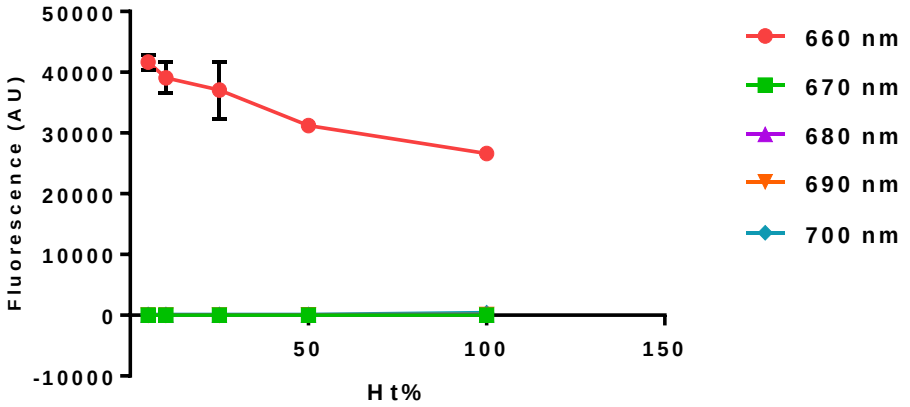


Figure 5-1 Plate-Binding assay of fibrinogen interaction with RBCs. Plates were coated with 80 µg/ml fibrinogen and DiD-labelled RBCs test concentrations were 5, 10, 25, 50, 100 % Ht. Signals at 660 nm showed a decrease in the interaction between fibrinogen and RBCs as the haematocrit of RBCs increase. Other wavelengths showed no interaction between fibrinogen and RBCs, subsequently, they are overlaid by 670 nm.

The third method for measuring the binding of fibrinogen to RBCs was the release of haemoglobin by haemolysed RBCs. The 96-well plate was scanned for different wavelengths (400-557 nm) to confirm the optimum wavelength for haemoglobin measurements. Figure 5-2 shows 400-414 nm provided the highest level of detection.

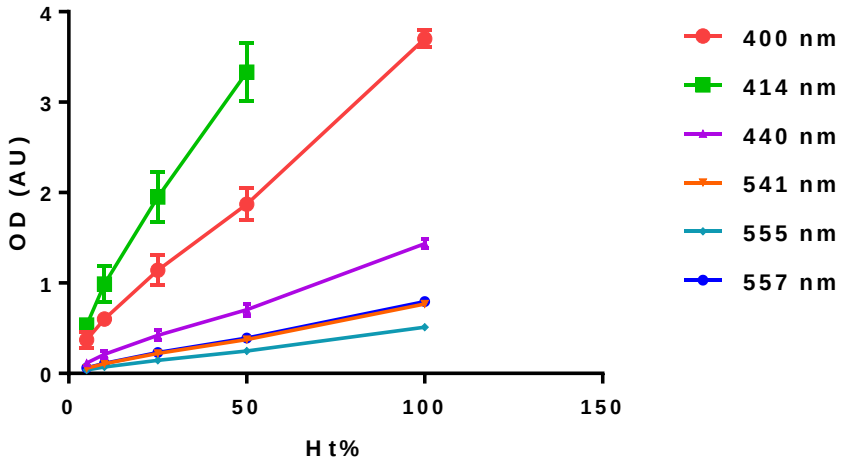


Figure 5-2 Plate-binding assay of fibrinogen interaction with RBCs to test the optimum absorbance wavelength of released haemoglobin. Wavelength of 400-414 nm showed the highest level of detection for haemoglobin released from fibrinogen-bound RBCs. Plate was coated with 80 µg/ml fibrinogen and RBCs test concentrations were 5, 10, 25, 50, 100% Ht.

Fibrinogen Coating

In this stage, the experiment was performed as described in the methods section 2.10 but with minor adjustments. The plate was coated with different concentrations of fibrinogen (20-80 µg/ml) then different concentrations of RBCs (5-50 % Ht) were added to the plate. Lower concentrations of RBCs (1, 2.5 % Ht) were also tested, as well as lower concentrations of fibrinogen (1, 2.5, 5, 10 µg/ml), but no binding was detectable (data not shown). The data of those experiments suggested the optimum value of 50% Ht for RBCs and 20 µg/ml of fibrinogen, as shown in Figure 5-3. Following coating with fibrinogen, the plate was initially blocked with BSA, but this was subsequently excluded as a precautious measure to ensure that the binding signal did not result from the attachment of RBCs to BSA.

It was noticed that the blood would wash off during the washing steps and gather around the edges of the wells. This loss of RBCs was the cause of repeating the experiment several times to try to find out a way to overcome this issue. When RBCs were washed off the plate this was affecting the values between the

duplicates and causing high intra-assay variation. Several precautions were undertaken over the different optimisation experiments to try to overcome the washing off RBCs. These adjustments to the method ranged from decreasing the number of washes after each addition of samples, changing the mode of washing using pipetting instead of discarding the contents by inversion, to draining the plate contents on paper tissues; this did not work especially well. The difference between the absorbance of the duplicates, which was represented on Figure 5-3 by large error bars was still an issue that resulted from the washing off of RBCs. Accumulation of RBCs around the edges may cause an increase in the haemoglobin absorbance signal reflecting an artefactual increase in RBCs content rather than the binding between fibrinogen and RBCs.

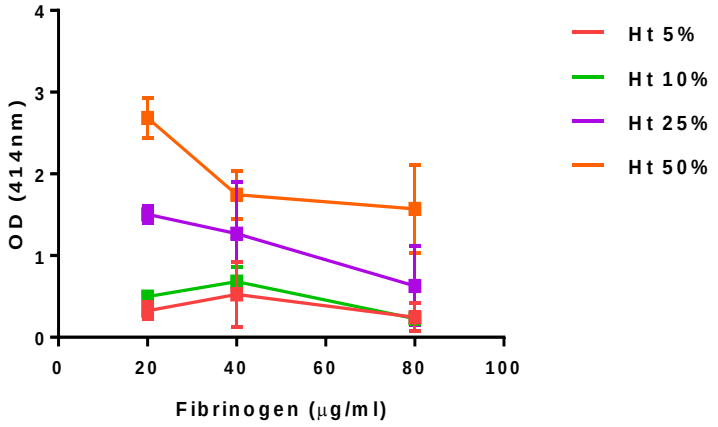


Figure 5-3 Representative graph of the optimisation steps of plate-binding assay between fibrinogen and RBCs. Plates were coated with 20, 40, 80 µg/ml fibrinogen, RBCs test concentrations were 5, 10, 25, 50 % Ht. 20 µg/ml fibrinogen and 50% Ht was the best combination.

Red Blood Cells Coating

The next stage for optimising the plate-binding assay method was changing the coating of the plate to RBCs instead of fibrinogen. Surprisingly the RBCs attached well to the plate and did not wash off after the addition of fibrinogen. A new design of the protocol was introduced to accommodate the change in the experimental conditions as described in methods section 2.10. In this case, the plate was coated with RBCs then fibrinogen was added to the coated RBCs. The

binding of fibrinogen to RBCs consisted of haemolysing the RBCs with water and measuring the amount of fibrinogen in the lysate by fibrinogen ELISA.

In this method, the same combinations of RBCs 5, 10, 15, 20, 25 % Ht were tested with 5, 10, 20, 40, 80 µg/ml fibrinogen (Figure 5-4). The results of this set-up showed that 5% Ht was the optimum concentration of RBCs for coating, whilst 20 µg/ml of fibrinogen showed strong binding between fibrinogen and RBCs, comparable with higher concentrations of fibrinogen (40 and 80 µg/ml). On this basis 20 µg/ml of fibrinogen was chosen to proceed with the mutants considering the low amount of protein which was available.

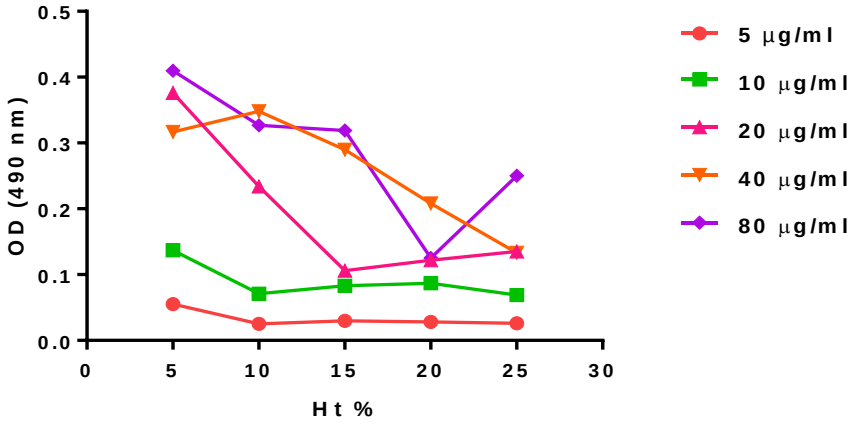


Figure 5-4 ELISA-based plate-binding assay between fibrinogen and RBCS. Plates were coated with RBCs (5, 10, 15, 20, 25 % Ht), fibrinogen test concentrations were 5, 10, 20, 40, 80 µg/ml. ELISA on the lysate was used to detect the fibrinogen that bound to RBCs. 5% Ht and 20 µg/ml fibrinogen showed the best combination.

Confirming the Source of Fibrinogen

In order to exclude that the data produced in the previous section were due to fibrinogen binding to the plate, two different conditions were used. One row of the 96-well plate was coated normally with 5% Ht RBCs then washed. When fibrinogen was added to the plate, it was added to the coated RBCs row, in addition to an extra row where no RBCs were present. A series of fibrinogen concentrations were used including very low concentrations; (0.625, 1.25, 2.5, 5, 10, 20 µg/ml), as shown in Figure 5-5. Fibrinogen was incubated, washed and

then 100 µl/well of water were added to the plate to haemolyse the RBCs and to transfer the contents to the new plate, which was coated with fibrinogen antibodies for ELISA. The results in the figure showed binding between fibrinogen and RBCs but no binding of fibrinogen to the wells of the second plate. This reflected that only RBC-bound fibrinogen was transferred to the new plate for ELISA and there was no increase in the binding signal of the fibrinogen when RBCs were not present. This confirms that the results of the fibrinogen-RBCs plate binding assay reflected the RBC-bound fibrinogen and not the added fibrinogen.

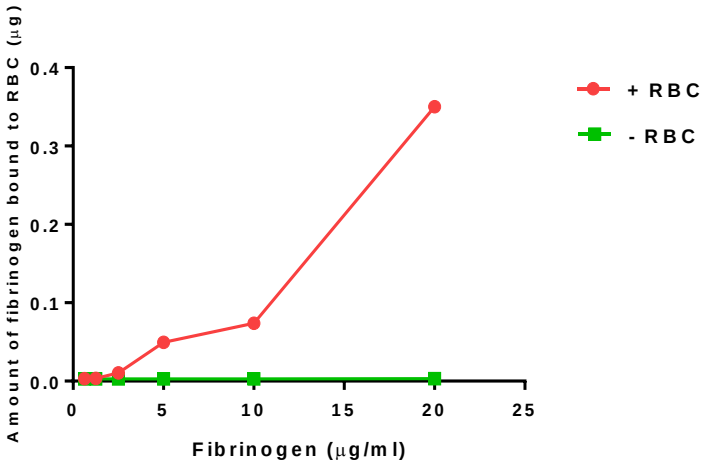


Figure 5-5 Comparison between RBC-free fibrinogen (green) and RBC-bound fibrinogen (red) by fibrinogen ELISA of plate-binding assay. Fibrinogen 0.625, 1.25, 2.5, 5, 10, 20 µg/ml were added to the plate with and without 5% Ht RBCs. RBC-bound fibrinogen has shown increase of detected signal of fibrinogen whereas there was no change in the RBC-free fibrinogen signal.

Amount of Fibrinogen in Blanks

In order to exclude that the data produced in the previous section were not due to pre-existing fibrinogen on the surface of RBCs, fibrinogen ELISA was performed on lysates from serial RBC haematocrits, without addition of fibrinogen (Figure 5-6). There was an little detectable amount of fibrinogen detected on RBCs (~0.004 µg/ml), indicating that the washed RBCs do not have an amount of fibrinogen on their surface that affects the reading of fibrinogen concentrations following addition of our recombinant fibrinogens.

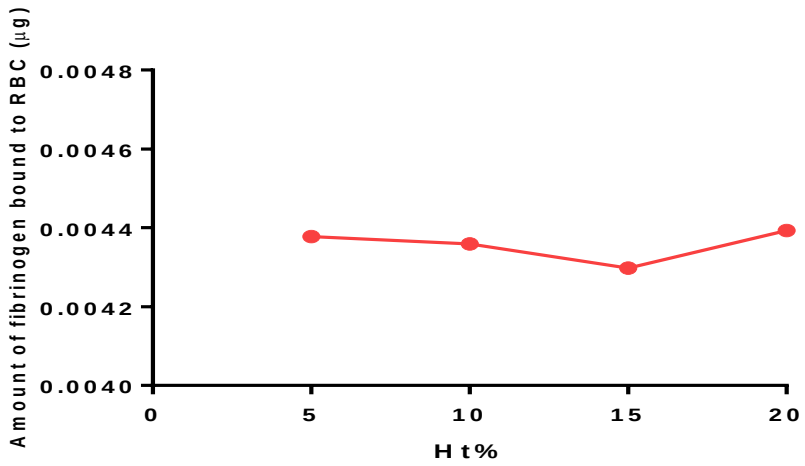


Figure 5-6 Detection of fibrinogen amounts on serial concentrations of RBC without added fibrinogen. Plates were coated with RBCs (5, 10, 15, 20 % Ht). Fibrinogen in the lysate was detected by ELISA, and showed no change in the amount of RBC-bound fibrinogen, confirming that washed RBCs have minute amount of fibrinogen on their surfaces.

Effect of Mutations on Fibrinogen-RBC Binding

The next step was to start using the fibrinogen variants (R95E, R95Q, G96V, D97K, D97N, F98I, and WT) to test if they have any effect on the binding of exogenous fibrinogen to RBCs. The experiment was undertaken in two different sets because fibrinogen variants were produced in different batches as described in methods section 2.10. Plates were coated with RBCs (5% Ht) before 20 µg/ml of fibrinogen were added to test the effect of the mutations on the fibrinogen binding to the RBCs. The RBCs were then haemolysed, and the lysate was transferred to a plate pre-coated with fibrinogen antibodies in order to quantify the amount of fibrinogen present in the lysate by ELISA. The results of these experiments are shown in Figure 5-7.

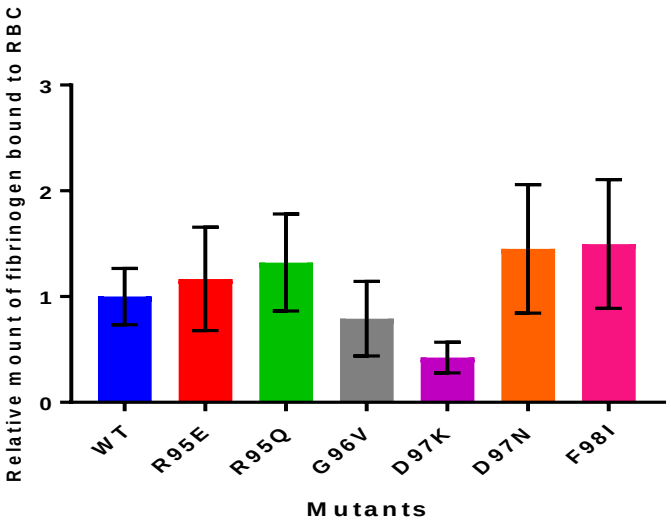


Figure 5-7 RBC-Fibrinogen-plate binding assay for the mutants. Plates were coated with RBCs (5 % Ht), and 20 µg/ml recombinant fibrinogen were added. There were no significant differences in the amount of binding between the mutants and the RBCs compared to WT. Error bars represent Standard Error of Mean (SEM). (n=4). . One-way ANOVA multiple comparisons in GraphPad prism, Dunnett's post hoc test were used.

There were no statistically significant differences in the amount of fibrinogen bound to RBCs compared to the WT showing a slight increase for R95E, R95Q, D97N and F98I and a slight decrease for G96V, with a more pronounced, but still not significant decrease for D97K, compared to the WT. Altogether, these data suggested that the mutations did not significantly alter the interaction between fibrinogen and RBCs and there was no significant difference in the binding compared to the WT.

Binding of Fibrinogen Mutants to RBCs from Fibrinogen-Knockout Mice

To confirm any effects of the mutations on the binding of fibrinogen to RBCs were not due to a pre-existing fibrinogen on the surface of the RBCs, blood from fibrinogen-KO mice was used in this experiment so only the influence of recombinant fibrinogen was present. The blood was washed and the RBCs were isolated using the same method as human blood (methods section 2.10). As for the previous section, there was no significant effect of the mutations on the binding of fibrinogen to the RBCs from fibrinogen-KO mice, compared to WT (Figure 5-8). All mutants showed similar binding to that of WT, except a slight,

but not significant, increase for F98I, that agreed with the results obtained using human blood. D97K binding was still lower, but to a much lesser extent, which could be considered similar to WT.

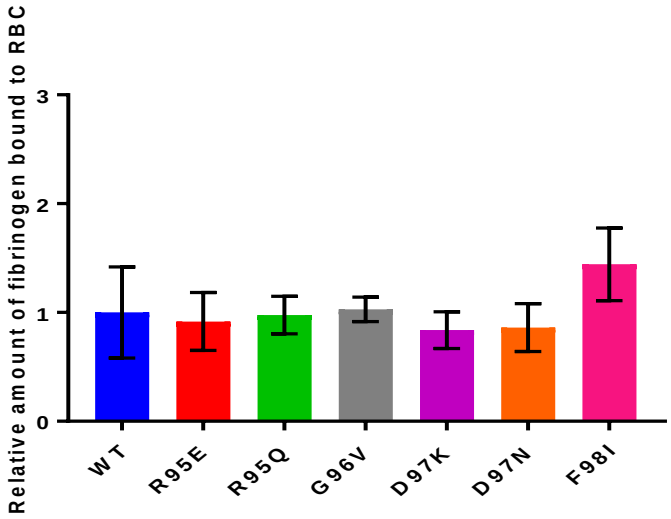


Figure 5-8 Plate-binding assay for mutant fibrinogens and RBCs from fibrinogen Knockout mice. Plates were coated with RBCs (5 % Ht), and 20 µg/ml recombinant fibrinogen were added. The results showed statistically non-significant changes between the binding of mutants and that of WT. Error bars represent Standard Error of Mean (SEM). (n=3). One-way ANOVA multiple comparisons in GraphPad prism, Dunnett's post hoc test were used.

5.2.2 Effect of the Mutations on Interactions between Fibrinogen and RBCs in Solution.

Another method to test the interaction between fibrinogen and RBCs in solution was established. This method was adapted from the plate-binding assay, where now the interaction between fibrinogen and RBCs was performed in solution, before the binding was measured using a plate-based assay. Another difference between the two methods was that the evaluation of the binding of fibrinogen to RBCs occurred through analysis of the released haemoglobin.

The first step consisted of combining fibrinogen and RBCs in solution as described in methods section 2.11. The mixture was then transferred onto a plate pre-coated with fibrinogen binding antibodies. Following binding of fibrinogen (and the bound RBCs) to the plate, and washing steps, the amount of

haemoglobin released due to osmotic shock after addition of water was measured as an indication of binding.

The results of this method are shown in Figure 5-9. The differences in the binding of fibrinogen to RBCs between R95E, R95Q and D97N mutants and the WT did not reach statistical significance being slightly increased, in agreement with the plate-binding assay results. D97K showed a slightly higher but statistically non-significant binding of fibrinogen and RBCs compared to that of WT, in contradiction with the plate-binding assay experiments. However, G96V and F98I showed similar binding to that of WT, unlike the data obtained in the plate-binding assay. However, these changes were minimal, as for the previous method and can therefore be considered as similar to WT.

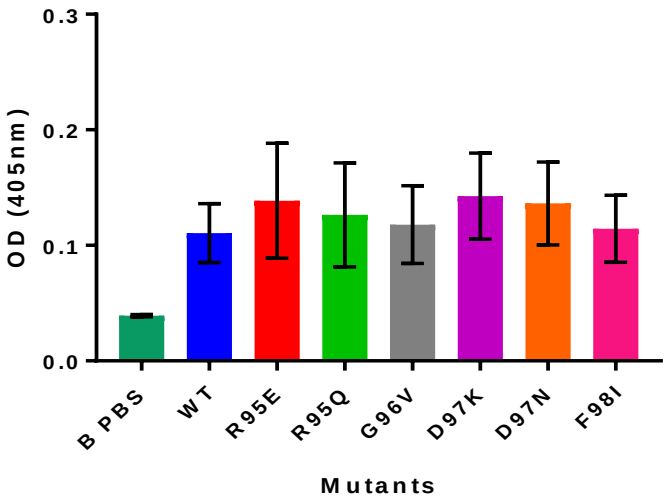


Figure 5-9 Effect of mutations on fibrinogen-RBC binding by plate-based solution-interaction assay. Total of, 20 µg/ml fibrinogen were mixed with RBCs (5% Ht). No statistically significant differences in the binding of mutants to the RBCs compared to WT were observed. **B PBS**: blank of only PBS. Error bars represent Standard Error of Mean (SEM). n=3. One-way ANOVA multiple comparisons in GraphPad prism, Dunnett's post hoc test were used.

5.3 Discussion

This chapter describes several experiments to test the effect(s) of the produced mutants (R95E, R95Q, G96V, D97K, D97N and F98I) on the binding of fibrinogen to the RBC, in comparison to WT. This binding was hypothesised to be through

the RGD site, which is in the coiled-coil residues 95-97 of the Aα-chain of fibrinogen (Carvalho et al., 2018), and which was mutated in the recombinant fibrinogens studied. The amount of fibrinogen bound to the RBCs showed small and non-significant changes for all mutants. A slight but statistically non-significant increase of the binding between fibrinogen and RBCs was observed for D97N and F98I and to a lesser extent for R95E and R95Q, whilst there was a non-significant decrease of the binding for G96V and D97K compared to WT.

Plate-binding assay was the first choice because the aim was first to assess the effect of the mutations on interaction between the RBCs and fibrinogen, and this technique has been used previously to study the interaction of fibrinogen with different components such as FXIII and platelets (Achyuthan et al., 1994, Leung and Nachman, 1982). Achyuthan *et al.* found some advantages in using microtiter plate binding assay over other methods. When studying the interaction of FXIII and FXIIIa with different forms of fibrinogen, this method only requires very low amounts of sample and can be applied for a large number of samples (Achyuthan et al., 1994). They also found that this method showed high specificity and sensitivity in addition to its quick application. They were able to identify fibrin binding site with FXIII purified from human plasma and recombinant FXIII produced in bacteria (Achyuthan et al., 1994). This study developed binding assays based on a previous study by Leung and Nachman, who labelled proteins including fibrinogen with radioactive iodine to study their interaction with platelet glycoprotein Thrombospondin, which was adsorbed on the plate. The radio-active complex was then analysed by SDS-PAGE (Leung and Nachman, 1982). Similarly, in this chapter, fibrinogen was added to the wells coated with RBCs then quantified by ELISA. This is a colorimetric method relying on the direct proportional increase of the colour in relation to the amount of fibrinogen. Another example for using the plate-binding assay is the colorimetric method that was used by Bellavite *et al.*, who suggested that the use of multi-well microplate is of benefit particularly to assess different reaction conditions. In this case the authors used the assay to evaluate platelet adhesion by determining the activity of platelet Acid Phosphatase enzyme, which was proven to be a good indicator for platelet mass and number (Bellavite et al., 1994). In our case, changes in colour were an indication of the amount of fibrinogen present in the reaction.

A large number of optimisation steps were undertaken before the method was established to measure the interaction of fibrinogen with RBCs. The final design was to coat the plate with RBCs, and then in a separate step the fibrinogen that bound to the coated RBCs was measured by fibrinogen ELISA. At the start of the experimental development when the plate was coated with fibrinogen, RBCs wash off was highly variable and limited the usability of the method. This wash off of the RBCs from the plate may be caused by their relative weak attachment to the plate.

The results of the plate-binding assay reflect that the mutations may enhance the RGD sequence to express more affinity and interactions rather than blocking it. It is possible that the mutations induced conformational changes that exposed the RGD sequence for more interaction. These data, although minimal, were opposite to what was hypothesised at the start of this project. The hypothesis for these mutants in the RGD site (95-97) was that they would block the binding between fibrinogen and RBCs, assuming that this sequence is the binding site on fibrinogen. Because the effect of the differently charged mutant (negative E95 to the neutral Q95) on the binding between fibrinogen and RBCs were almost similar to the WT, this may exclude any effect of charge on the binding between fibrinogen and RBCs in static conditions using plate-binding assays. Binding of positive K97 was lower than that of neutral N97, but the D97K was variable in its effect among the different techniques so this difference between these two differently charged mutants in the same site may actually be negligible. These two mutations on D97 were opposite in the fibrinogen-RBCs plate-binding assay when human blood was used, but not when fibrinogen-KO mice blood was used, or in the solution Binding assay.

All mutants were tested for their effect on the binding between fibrinogen and RBCs by plate binding assay again using murine fibrinogen-knockout blood. This was to confirm that fibrinogen amount which was detected by ELISA was due to the added mutated fibrinogen and not related to any pre-existing fibrinogen already bound to and present on the RBCs from the blood and released by haemolysis. This was confirmed by measuring the fibrinogen concentrations from lysates of different RBCs haematocrit. In this case, only very low amount of

fibrinogen was detected, with no difference among the different haematocrits. If the fibrinogen, which was detected by ELISA resulted from RBCs, it would increase as the haematocrit increases. Adding mutated fibrinogen to the fibrinogen KO mice showed no significant difference in the binding of fibrinogen to the RBCs compared to the WT, confirming that the plate-binding assay method excluded the RGD (95-97) site from being the prime binding site of fibrinogen and RBCs. A small, but statistically non-significant, increase in the F98I was observed in the experiments using blood from fibrinogen-knockout mice, which agreed with the prothrombotic tendency associated with the naturally occurring F98I mutation found in a patient with dysfibrinogenaemia and multiple miscarriages in pregnancy (Riedelova-Reicheltova et al., 2014). This mutant was found to play a role in increasing thrombosis by altering clot structure, as described in the previous chapter section 4.3.

Another method of binding was established in this project, which was aimed at examining the binding interaction in solution rather than in static conditions. The idea for this method was to establish another method that may be considered more physiologically relevant. In this method, released haemoglobin after RBCs haemolysis was measured to evaluate the binding between fibrinogen and RBCs. The results of this method were consistent with the previous method in showing no significant effect of the mutants on the binding of fibrinogen to RBCs, compared to the WT. D97K and G96V had different effects to that produced by plate binding assay, reflecting the fluctuation of the effect of these two mutants between the different methods. The structural conformation of these mutants may be weaker than the other mutants making them more affected by the state of the binding either in liquid or static state.

The results based on this assay suggested that RBCs do not bind fibrinogen through this RGD site but the binding will require further testing using different assay.

In conclusion, the two methods for quantification of the binding between fibrinogen and the RBCs showed that the mutations in the Aα-95-97 RGD did not significantly affect the binding. This may mean that this RGD sequence is not the

primary site on fibrinogen to bind RBCs. To fully confirm these findings, the more sensitive flow cytometry technique was used next.

Chapter 6 Solution-Based Fibrinogen-RBC Binding Studies

6.1 Introduction

Flow cytometry may be used to analyse millions of blood cells in the samples even with no requirement for pre-experimental sample preparation (Seliger et al., 2012). Flow cytometry is able to identify a single cell type and isolate it in a group of cells such as platelets or RBCs in the whole blood or in mixed sample of cells (Lazarus et al., 1995). Platelets are difficult to be handled in the laboratory because they are easily activated during blood sampling and *in vitro*, but flow cytometry has the ability to overcome this problem by isolating the specific groups when gating on size, allowing for the identification of the required cells specifically (Lazarus et al., 1995). This can be achieved by using two-parameter analysis such as in case of platelets; the size which is represented by forward light scatter (FSC) combined with fluorochrome-labelled platelet antibodies (Lazarus et al., 1995).

In this chapter, the identification of RBCs was performed using specific labelled-antibodies against RBCs rather than relying solely on size gating to exclude smaller cells such as platelets. The use of these antibodies was a way to detect the binding of the labelled fibrinogen to the labelled RBCs, to ensure that the signal was specific to RBCs and not to other cells.

A new method for evaluating the binding interactions between fibrinogen and an unknown site on RBCs, and the effect of the produced mutations in the Aα-95-98 RGDF sequence on this binding, was established and optimised. The effect of the mutations on the fibrinogen binding to the RBCs in solution was performed first by using the haematocrit as the indicator for the amount of RBCs for the reaction. This was changed later because the signal from the RBCs was low indicating that more antibodies were required. A titration of the optimum amount of antibodies required for labelling the RBCs was performed to improve the signal. The principle of this experiment was to detect the binding of fluorescently labelled fibrinogen with fluorescently labelled RBCs.

6.2 Results

My initial plans were to optimise a number of conditions before analysing the binding of the mutated fibrinogens to RBCs (data not shown). Recombinant fibrinogens were used first in the actual experiment in combination with a specific RBC haematocrit (Ht). To label RBCs, CD235a^{PE} antibodies were added to the compact RBCs in a ratio of 1:11 v/v then diluted to the required amount for the experiment which was 0.01- 5% Ht. The low signals obtained using this method suggested that the amount of antibodies used was not enough to produce a clear signal of the binding between fibrinogen and RBCs. Subsequently, a titration experiment was performed to evaluate the required amount of antibodies in relation to a specific cell count of RBCs. Next, the combination of RBC-CD235a^{PE} was tested in relation to AlexaFluor^{488}-labelled fibrinogens.

1.2.1 Initial optimisation work

A few experiments were performed using a wide range of conditions with a combination of different concentrations of RBCs and fibrinogen. Regarding fibrinogen, the choice of the required concentration of AlexaFluor^{488}-labelled fibrinogen was limited due to the low availability of recombinant fibrinogens. Two different concentrations were tested; the concentration which was used for laser scanning confocal microscopy (5 µg/ml), in addition to a lower concentration (1 µg/ml). The choice of 5 µg/ml concentration was made because the signal was too low with the lower concentration. Commercial fibrinogen was used in the optimisation steps and the controls.

For the RBCs, a large range of concentrations was used and more attempts were required to decide the best RBC haematocrit to be used in the experiment. Preparations of the reaction mixtures started from 50% Ht down to 0.01% Ht. 50% Ht was prepared in the first experiment together with 5 % Ht, but was not used on the flow cytometer, because the 5% Ht gave signals that were too high. On the other hand, 0.01% Ht showed too low signals by the flow cytometer. In the middle of the range, 1% Ht was used and gave strong signals.

The flow cytometer optimisation experiments were performed in a way similar as described in the methods section 2.12, but with slight changes as shown in

Figure 6-1. The CD235a^{PE} antibodies were added to the RBCs in a ratio of 1:11 v/v and then incubated at 4˚C in the cold room for 20 min according to the manufacturer's instructions. The RBCs were then combined with AlexaFluor^{488}-labelled fibrinogen in final concentrations ranging between 0.01-5% Ht and 5 µg/ml fibrinogen in a final volume of 400 µl. The following steps were similar to the methods section; 30 min incubation on a roller at room temperature, wash, centrifugation, re-suspension followed by flow cytometer analysis.

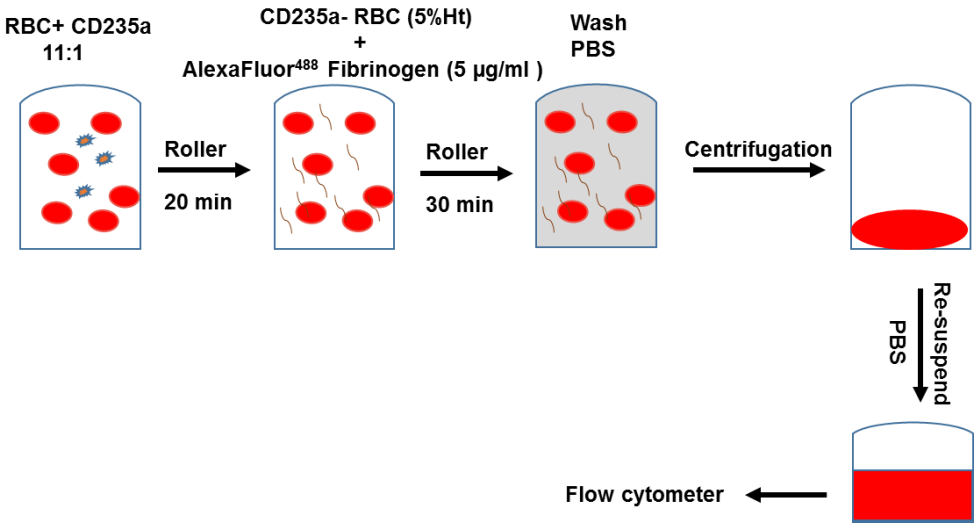


Figure 6-1 Schematic representation of the binding between fibrinogen and RBCs in solution using flow cytometer. Antibodies CD235a^{PE} were added to RBCs in 1:11 ratio v\v and incubated for 20 min. 5 µg/ml of AlexaFluor^{488}-labelled fibrinogen were combined with 0.01-5% Ht RBCs final concentrations in final volume of 400 µl then incubated for 30 min, washed, centrifuged, re-suspended then taken to the flow cytometer.

Two different buffers were tested in the optimisation process: PBS and HEPES (150 mM NaCl, 0.1 % D-glucose. 2.7 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, pH 7.45) (de Witt et al., 2014). No difference on the RBC binding to fibrinogen was observed with the HEPES buffer, so PBS was used for all subsequent experiments. Another factor was tested for its effect on the CD235a^{PE} antibodies was the temperature. Incubation in the cold room or at room temperature did not have any effect on the binding of CD235a^{PE} antibodies to RBCs, so room temperature incubation was chosen for all subsequent experiments.

The binding between fibrinogen and RBCs was assessed using two gating strategies. The first involved gating to exclude the debris only, relying on including all cells because the RBCs were labelled. However, this was further divided into two types of inclusion criteria; one including only single positive cells; the other including all cells even if they were in clumps. The second gating strategy involved gating based on size, where small cells such as platelets were excluded to ensure the presence of RBCs only. This type of gating was not taken into consideration in later experiments because the CD235a^{PE} antibodies are specific for the RBC and ensure that only labelled RBCs are evaluated.

6.2.1 Evaluation of RBC Concentration

After confirming 5 µg/ml as the best concentration of AlexaFluor^{488}-labelled fibrinogen to be used in the experiment, more work was done to confirm the optimal concentration of the RBCs that would show the best binding with the chosen amount of labelled fibrinogen and be appropriate to be processed in the flow cytometer at the same time. Two lower concentrations (0.1% and 0.01% Ht) of RBCs were used, either labelled or unlabelled, with controls described in Figure 6-2. In this experiment, the temperature was tested as stated above to confirm that the temperature for the RBC-labelling step did not affect the binding between fibrinogen and RBCs. An additional tube of CD235a^{PE}-labelled RBCs was prepared and incubated at room temperature on the roller instead of the cold room. The results are shown in Figure 6-3 (all cells gating strategy) and Figure 6-4 (size gating strategy).

For both gating strategies, the results showed no difference between the cold room and the room temperature incubation of the RBCs with the labelling antibodies, subsequently, the choice was made to incubate at room temperature on a roller to ensure a better mixing of the cells and antibodies. The signals with 0.01% Ht were lower than that of 0.1% Ht, so the decision was to confirm the choice of 0.1% Ht.

The results confirmed that interactions occur between the AlexaFluor^{488}-labelled fibrinogen and the CD235a^{PE}-labelled RBCs, by flow cytometry, showing no signal for the controls as expected. The aim of using flow cytometry in this thesis chapter was to study the binding between RBCs and fibrinogen in solution, this

binding was proposed to occur via an integrin receptor on the RBC membrane. Excluding clumps may exclude parts of the RBC membrane that carry the integrin on its surface and bind fibrinogen, subsequently affecting the total binding level. To ensure that all possible expected binding between the RBCs and the integrin was included, an all-cells inclusion criterion was chosen in the final method when the mutants were used in the experiment. Gating with size was excluded as well since the CD235a^{PE} antibodies are specific for the RBCs which mean that no contamination with other cells should be present.

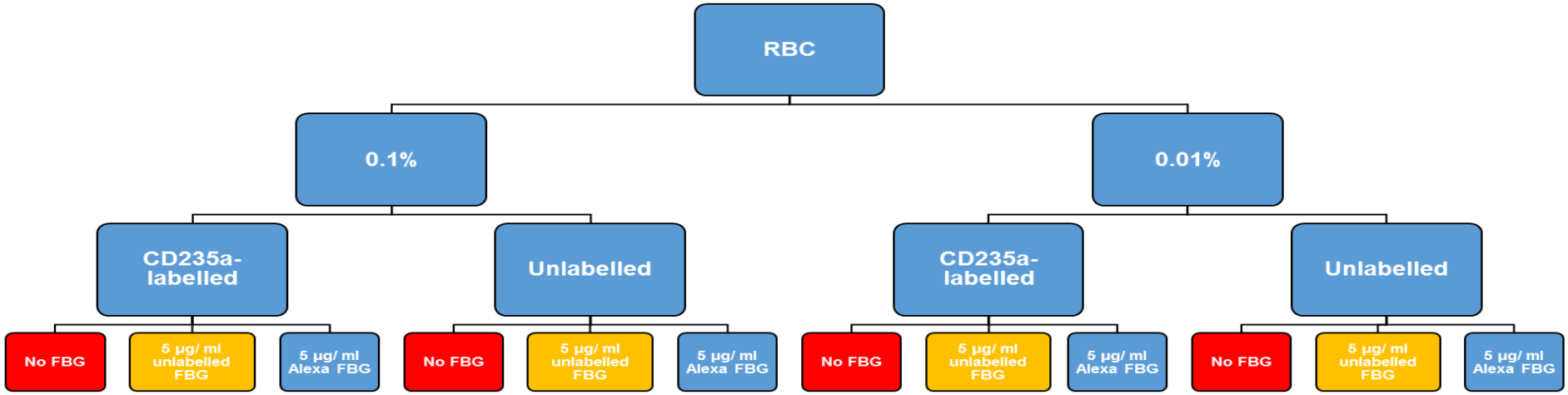


Figure 6-2 Schematic representation to the reaction conditions of the final optimisation experiment for flow cytometry analysis of fibrinogen-RBCs binding. Red boxes represent the control RBCs without adding fibrinogen. The yellow boxes represent the controls of commercial unlabelled fibrinogen. The small blue boxes represent the reaction conditions of commercial AlexaFluor^{488}-labelled fibrinogen. In all cases either unlabelled or CD235a^{PE} labelled RBCs were used. **FBG:** fibrinogen.

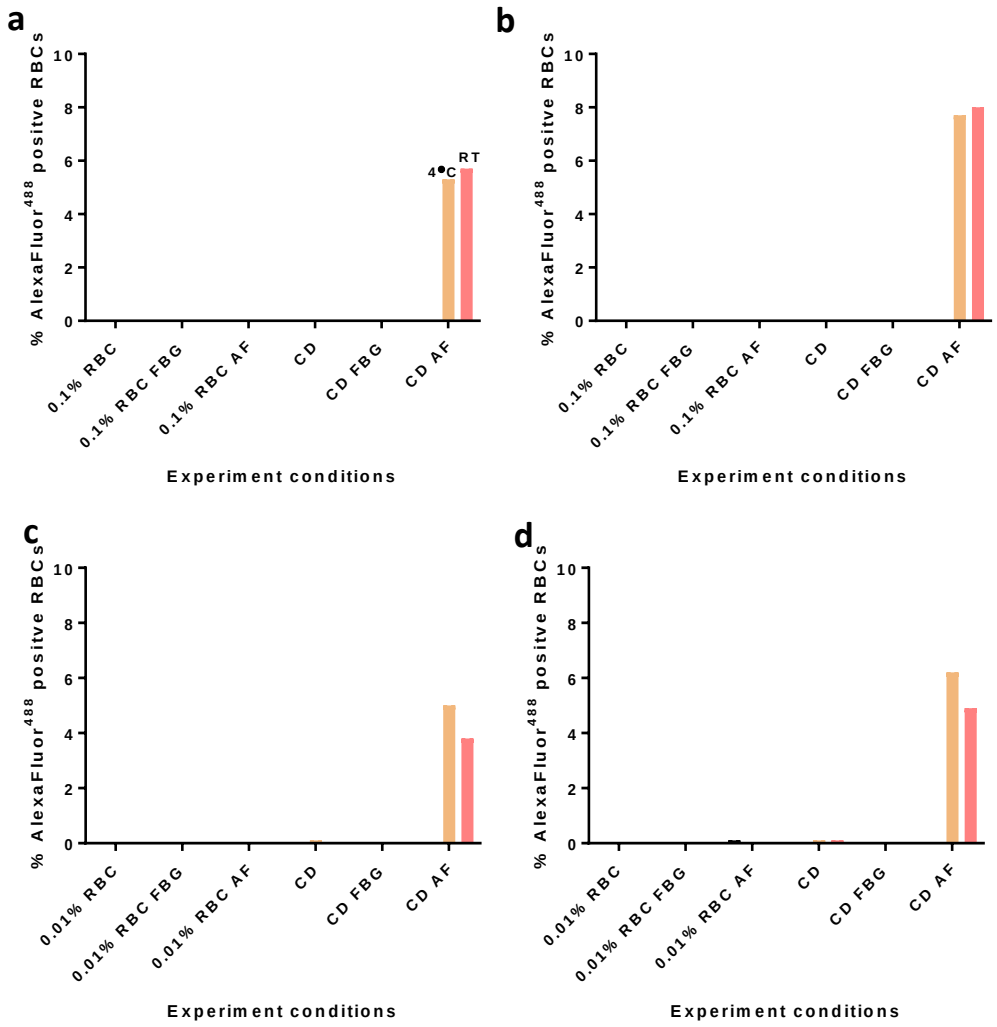


Figure 6-3 Optimisation reaction of binding interaction between fibrinogen and RBC, no gating was used. Beige and pink bars represent cold room (4°C) and room temperature (RT) incubation respectively. **a)** % of single CD235a^{PE}-labelled RBCs (0.1% Ht) showing binding with AlexaFluor^{488}-labelled fibrinogen. **b)** % of all CD235a^{PE}-labelled RBCs (0.1% Ht), including clumps, showing binding with AlexaFluor^{488}-labelled fibrinogen. **c)** % of single CD235a^{PE}-labelled RBCs (0.01% Ht) showing binding with AlexaFluor^{488}-labelled fibrinogen. **d)** % of all CD235a^{PE}-labelled RBC (0.01% Ht), including clumps, showing binding with AlexaFluor^{488}-labelled fibrinogen. **RBC:** unlabelled RBCs. **RBC FBG:** unlabelled RBCs + unlabelled fibrinogen. **RBC AF:** unlabelled RBCs + AlexaFluor^{488}-labelled fibrinogen. **CD:** CD235a^{PE}-labelled RBCs. **CD FBG:** CD235a^{PE}-labelled RBCs + unlabelled fibrinogen. **CD AF:** CD235a^{PE}-labelled RBCs + AlexaFluor^{488}-labelled fibrinogen. Unlabelled and AlexFluor^{488}-labelled fibrinogens were used at 5 µg/ml. The figure shows that there was no binding in the controls compared to the reaction conditions.

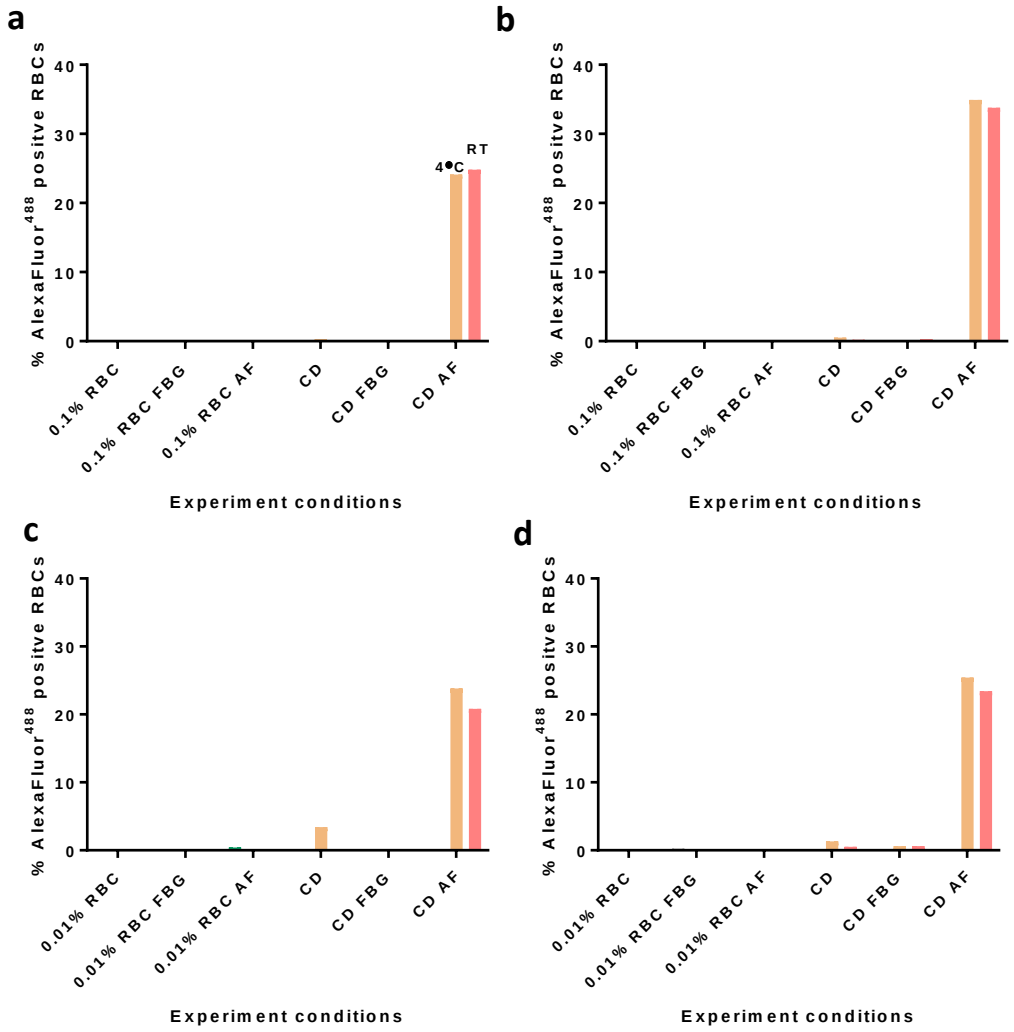


Figure 6-4 Optimisation reaction of binding interaction between fibrinogen and RBC, using gating on size of the cells. Beige and pink bars represent cold room (4°C) and room temperature (RT) incubation respectively. **a)** % of single CD235a^{PE}-labelled RBCs (0.1% Ht) showing binding with AlexaFluor^{488}-labelled fibrinogen. **b)** % of all CD235a^{PE}-labelled RBCs (0.1% Ht), including clumps, showing binding with AlexaFluor^{488}-labelled fibrinogen. **c)** % of single CD235a^{PE}-labelled RBCs (0.01% Ht) showing binding with AlexaFluor^{488}-labelled fibrinogen. **d)** % of all CD235a^{PE}-labelled RBC (0.01% Ht), including clumps, showing binding with AlexaFluor^{488}-labelled fibrinogen. **RBC:** unlabelled RBCs. **RBC FBG:** unlabelled RBCs + unlabelled fibrinogen. **RBC AF:** unlabelled RBCs + AlexaFluor^{488}-labelled fibrinogen. **CD:** CD235a^{PE}-labelled RBCs. **CD FBG:** CD235a^{PE}-labelled RBCs + unlabelled fibrinogen. **CD AF:** CD235a^{PE}-labelled RBCs + AlexaFluor^{488}-labelled fibrinogen. Unlabelled and AlexFluor^{488}-labelled fibrinogens were used at 5 µg/ml. The figure shows that there was no binding in the controls compared to the reaction conditions.

6.2.2 Effect of the mutations on fibrinogen binding to RBCs in solution

All mutants (R95E, R95Q, G96V, D97K, D97N and F98I) in addition to the WT were tested for their effect on the binding between fibrinogen and RBCs. The experiment was repeated three times using the conditions that were selected above and shown in Figure 6-5. The controls were 0.1% Ht unlabelled RBCs, 0.1% Ht unlabelled RBCs + 5 µg/ml commercial AlexaFluor^{488}-labelled fibrinogen, 0.1% Ht CD235a^{PE}-labelled RBCs, 0.1% Ht CD235a^{PE}-labelled RBCs + 5 µg/ml commercial AlexaFluor^{488}-labelled fibrinogen. The tests were 0.1% Ht CD235a^{PE}-labelled RBCs + 5 µg/ml AlexaFluor^{488}-labelled recombinant fibrinogen. The experiment was performed as described in the methods section 2.12 and shown in Figure 6-1, except that the amount of RBCs used was dependent on the haematocrit (0.1 % Ht CD235a^{PE}-labelled RBCs) instead of on RBC count as described in the methods.

A representation of the scatter-plot generated from the flow cytometry data is shown in Figure 6-6a, for the WT. The plot is composed of four quarters which were chosen based on the location of each negative control tested separately:

1. **Quarter 1 (Q1):** AlexaFluor^{488}-labelled fibrinogen positive cell
2. **Quarter 2 (Q2):** Double positives (AlexaFluor^{488}-labelled fibrinogen and CD-235a^{PE} RBCs)
3. **Quarter 3 (Q3):** Double negatives.
4. **Quarter 4 (Q4):** CD-235a^{PE} positive RBCs.

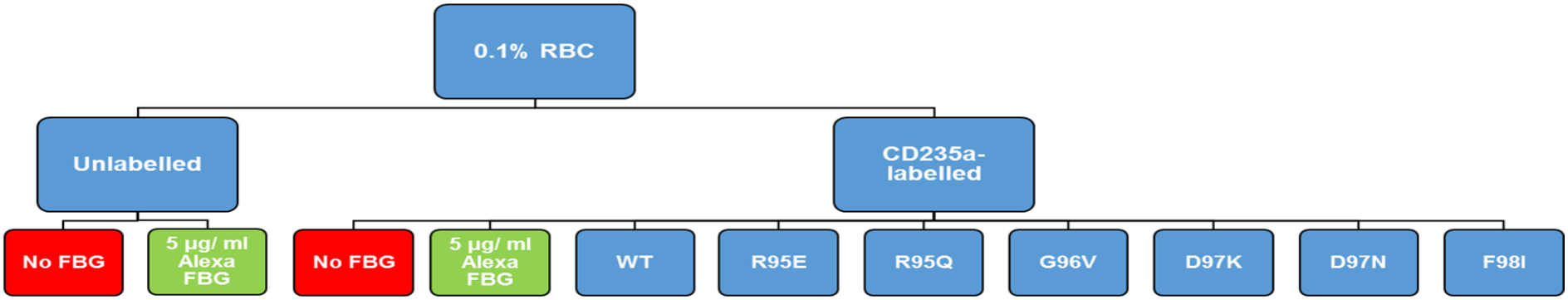


Figure 6-5 Schematic representation of the reaction conditions for flow cytometry analysis of fibrinogen-RBCs binding. Red boxes represent the controls RBCs without adding fibrinogen. The green boxes represent the controls of commercial AlexaFluor^{488}-labelled fibrinogen. The small blue boxes represent the different AlexaFluor^{488}-labelled variants of fibrinogen (5 µg/ml) FBG: fibrinogen.

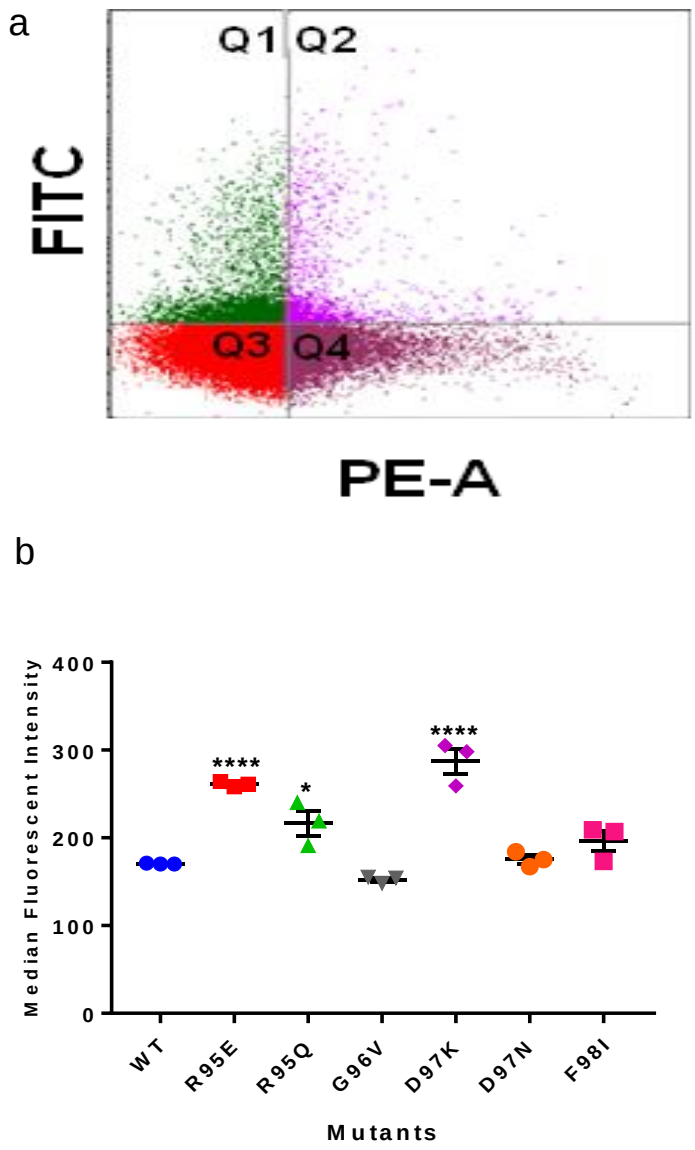


Figure 6-6 Effect of different mutations on the binding between fibrinogen and RBCs. a) Representative scatter-plot graph for fibrinogen WT. **Q1:** AlexaFluor^{488}-fibrinogen positive cells, **Q2:** Double positive cells, **Q3:** Double negatives cells, **Q4:** CD235a^{PE} positive cells (n=1). **b)** 5 µg/ml of AlexaFluor^{488}-labelled variants in solution with 0.1% Ht (MFI: Mean Fluoresence Intensity). R95E, R95Q and D97K showed significant increase binding to RBCs, compared to the WT. The other mutants showed no change in binding. Error bars represent Standard Error of Mean (SEM). n=3: *P<0.05, ****P<0.0001. One-way ANOVA multiple comparisons in GraphPad prism, Dunnett's post hoc test were used.

The cells on the scatter-plot in Figure 6-6a were accumulating towards the centre of the plot showing no distinct single cell for each population so it was not possible to distinguish individual cells from aggregates. This may be because the amount of CD-235a^{PE} antibodies was too low compared to the cell count

which means that not every positively bound RBC was shown. Although the results shown in Figure 6-6b represent significant increases of binding to RBCs for fibrinogens R95E, R95Q and D97K, the experiment was thought to require more optimisation, and the physiological relevance of such increase remains unclear. G96V, D97N and F98I binding were similar to WT. Based on these data, a validation experiment, including a titration of the amount of the CD-235a^{PE} antibodies was required before proceeding with the experiments on the mutated fibrinogens again.

6.2.3 Validation of the Flow Cytometry Results by Titration of the Antibodies

In order to validate the method further, a titration of two steps was performed. The first step was to choose the right amount of CD235a^{PE} labelling antibodies relative to the number of cells, whereas the second step was to choose the optimum amount of CD235a^{PE}-labelled RBCs relative to the concentration of fibrinogen that had been used (5µg/ml). Due to the limitation of the available fibrinogen, its concentration had to be kept constant.

Optimal RBC Number / Antibody Ratio Determination

The first step was to find out the optimum ratio of RBCs count to the amount of CD235a^{PE} labelling antibodies. Three different cell counts were used (10^6, 10^5, and 10^4 RBC/ml) in combination with 1:11 v/v (manufacturer's instructions) of CD235a^{PE} antibodies. Because the final volume of the fibrinogen and RBCs was 400 µl, 40 µl of the antibodies were added to each tube of the different RBC count giving a final reaction volume of 440 µl. The cells were incubated on a roller at room temperature for 20 min, washed and re-suspended as above then taken to the flow cytometer. The cell number with the highest signal was selected to proceed to the second step. The results are shown in Figure 6-7, together with a control of 1% Ht unlabelled RBCs. RBCs at 10^6 and 10^5 cell/ml were too concentrated and had to be diluted by adding PBS; subsequently these two concentrations were excluded. One difficulty arose when processing the cells for the experiment, which involved a centrifugation step after the final wash; the

RBCs agglutinated and the pellet required more mixing to re-suspend. This agglutination may be because of the high attachment tendency of the RBCs to different surfaces, which was observed in its ability to stick to the plates in the binding assay experiment. The presence of fibrinogen on the surface of the bound RBCs may also induce RBC hyper-aggregation (de Almeida et al., 2009).

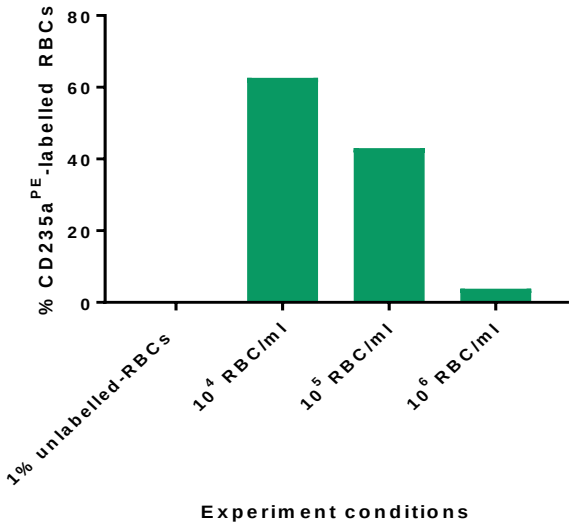


Figure 6-7 Titration of the number of RBCs to the amount of CD-235a^{PE} antibodies. Three different cell counts to the same amount of antibodies (1:11 v:v) were tested. 1% unlabelled RBCs were used as a negative control.

Effect of Adding Fibrinogen to the Ratio

The second step was to find the best RBC count that gives the highest binding signal when incubated with 5 µg/ml fibrinogen. This step relied on the previous step that 10^4 RBC/ml:40 µl antibodies was the best ratio to be used in the fibrinogen-RBC binding method. 10^3 RBC/ml and 10^2 RBC/ml were also used in addition to the 10^4 RBC/ml. To keep the ratio between the RBCs and antibodies constant, the amount of antibodies that was added to each tube was as follow:

1. 10^2 RBC/ml + 0.4 µl CD235a^{PE} antibodies
2. 10^3 RBC/ml + 4 µl CD235a^{PE} antibodies
3. 10^4 RBC/ml + 40 µl CD235a^{PE} antibodies

At this stage fibrinogen was added after washing the cells, and incubated at room temperature on roller for 20 min. Cells were then washed, re-suspended and sent to the flow cytometer for analysis.

Figure 6-8 shows the result of the best RBC count to provide optimal binding signal with fibrinogen. Again, 10^4 RBC/ml showed the highest binding signal to fibrinogen. However, the figure shows a degree of interaction for both 10^3 and 10^2 RBC/ml, but both conditions were excluded because after the washing and centrifugation steps, the pellet was very small to the degree that it was not clearly visible. The difficulty of re-suspending the pellet after the final wash for the 10^4 RBC/ml condition was observed again and raised the concern of possibly affecting the results. Consequently, before proceeding with the experiment using the mutants and to confirm if these observations were reproducible, this titration experiment was repeated again. A new condition was introduced in the repeated titration to try to overcome the agglutination issue of the RBCs which involved adding a lower amount of the antibodies.

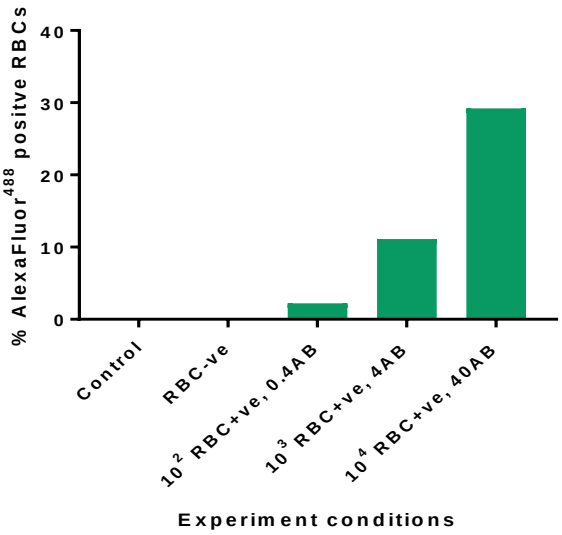


Figure 6-8 Titration of RBCs concentration to a set amount of fibrinogen binding. Control: 1% unlabelled RBCs. **RBC-ve:** 1% unlabelled RBC + AlexaFluor^{488}-fibrinogen. **10^2 RBC+ve, 0.4AB:** 10^2 RBC/ml + 0.4 µl CD235a^{PE} + AlexaFluor^{488}-fibrinogen. **10^3 RBC+ve, 4AB:** 10^3 RBC + 4 µl CD235a^{PE} + AlexaFluor^{488}-fibrinogen. **10^4 RBC+ve, 40AB:** 10^4 RBC/ml + 40 µl CD235a^{PE} + AlexaFluor^{488}-fibrinogen. AlexFluor^{488}-labelled fibrinogen was used at 5 µg/ml.

Final validation experiment to confirm RBC/ antibodies ratio

In this last optimisation experiment and for the same concentration of RBCs (10^4 RBC/ml), three different volumes of the CD235a^{PE} antibodies were used (40, 30, 20 µl). Again 10^4 RBC/ml + 40 µl CD235a^{PE} antibodies had the highest binding signal with fibrinogen (Figure 6-9), but agglutination of the cells was still a problem. Although it showed less binding signal compared to the amount of antibodies, 20 µl antibodies was chosen to minimise the effect of agglutination on the fibrinogen-RBC binding.

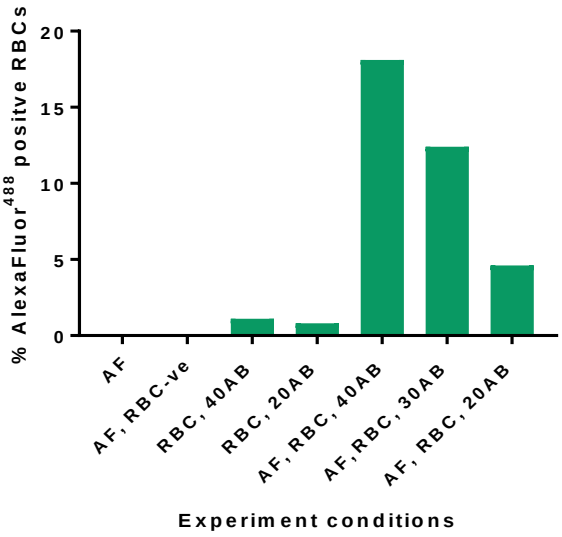


Figure 6-9 Titration of the amount of antibodies to a set concentration of RBCs. AF: AlexaFluor^{488}-fibrinogen only. **AF, RBC-ve:** Alexa AlexaFluor^{488}-fibrinogen + 1% unlabelled RBCs. **RBC, 40AB:** RBCs + 40 µl CD235a^{PE}. **RBC, 20AB:** RBCs + 20 µl CD235a^{PE}. **AF, RBC, 40AB:** RBC + 40 µl CD235a^{PE} + AlexaFluor^{488}-fibrinogen. **AF, RBC, 30AB:** RBCs + 30 µl CD235a^{PE} + AlexaFluor^{488}-fibrinogen. **AF, RBC, 20AB:** RBCs + 20 µl CD235a^{PE} + AlexaFluor^{488}-fibrinogen. Amount of 10^4 RBC/ml and 5 µg/ml AlexFluor^{488}-labelled fibrinogen were used, unless specified.

6.2.4 RBC-Fibrinogen Binding Using the New Method

Next, the mutants were used for the final assessment of the effects of the mutations on the binding between fibrinogen and RBCs, in solution using the flow cytometry following the protocol stated in material and methods section 2.12. Briefly, 10^4 RBC/ml + 20 µl CD235a^{PE} were combined with 5 µg/ml AlexaFluor^{488}-labelled recombinant fibrinogen in a final volume of 420 µl, incubated for 30 min at room temperature, washed and re-suspended in PBS then analysed by flow cytometry. The results are shown in Figure 6-10 as MFI.

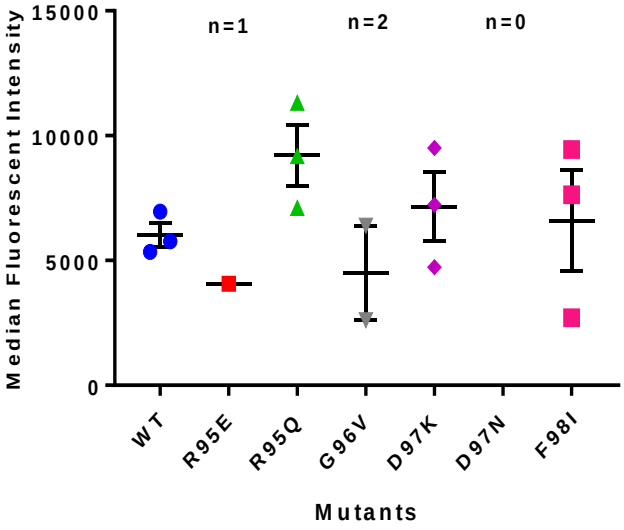


Figure 6-10 Effect of different mutants on the binding between fibrinogen and RBCs. Total of 5 µg/ml of Alexa Fluor^{488}-labelled variants in solution with 10^4 RBC/ml + 20 µl of CD235a^{PE}. All mutants showed statistically non-significant changes in the binding to the RBCs, compared to the WT. Error bars represent Standard Error of Mean (SEM). n=3 (except R95E n=1, G96V n=2, D97N n=0). One-way ANOVA multiple comparisons in GraphPad prism, Dunnett's post hoc test were used.

As shown in Figure 6-10, R95E was analysed only once whereas G96V was analysed twice, due to the limited amounts of labelled proteins available for these two mutants. The pattern of the effects of the mutants is consistent with the results that obtained by plate-binding assay and solution assay, where the differences in the binding between fibrinogen and RBCs were statistically non-significant showing slight increase for R95Q, D97K (except plate-binding assay), D97N and F98I, whilst G96V showed a slight decrease compared to the WT.

Although R95E showed a statistically non-significant differences in binding signal being a slightly higher than the WT in other methods, here it showed a slightly lower MFI than the WT, but this would need repeating as there was only one measurement done for this mutant. When compared to the WT, the slight changes in binding between fibrinogen mutants and RBCs failed to reach statistical significance. Figure 6-11 (histogram) and Figure 6-12 (scatter-plot) show representative data from one experiment for each mutant. The results of these two figures do not strictly reflect the actual results since they are from one experiment only.

Figure 6-11 shows histograms of WT compared to each mutant separately, these figures are representative and they do not reflect the actual results because they were chosen from the experiment that include the single R95E sample analysis. The histogram shows a clear separation between the negative RBCs population (top left) that represent no binding of fibrinogen, and the positively fibrinogen-bound RBCs (bottom right). In this case, the negative population was higher than the positive one. In Figure 6-12, Q1 and Q4 are the AlexaFluor^{488}-fibrinogen and CD235a^{PE} positive quadrants respectively. Q3 is the double negative quadrant whereas Q2 is the double positive quadrant from which the MFI was calculated reflecting the degree of binding between fibrinogen and RBCs. Although the double positive population in Q2 looks very low, it shows double positive cells that are distributed as a single cell population. Although it is shown in the figures that the positive signal of binding between the RBCs and each of the mutants was very low compared to the negative population, the presence of this signal was good evidence indicating the presence of binding between the RBC and fibrinogen, which was successfully detected by the flow cytometry method. However, the mutations had no significant effect on the binding of fibrinogen to RBCs, compared to WT, when tested in solution.

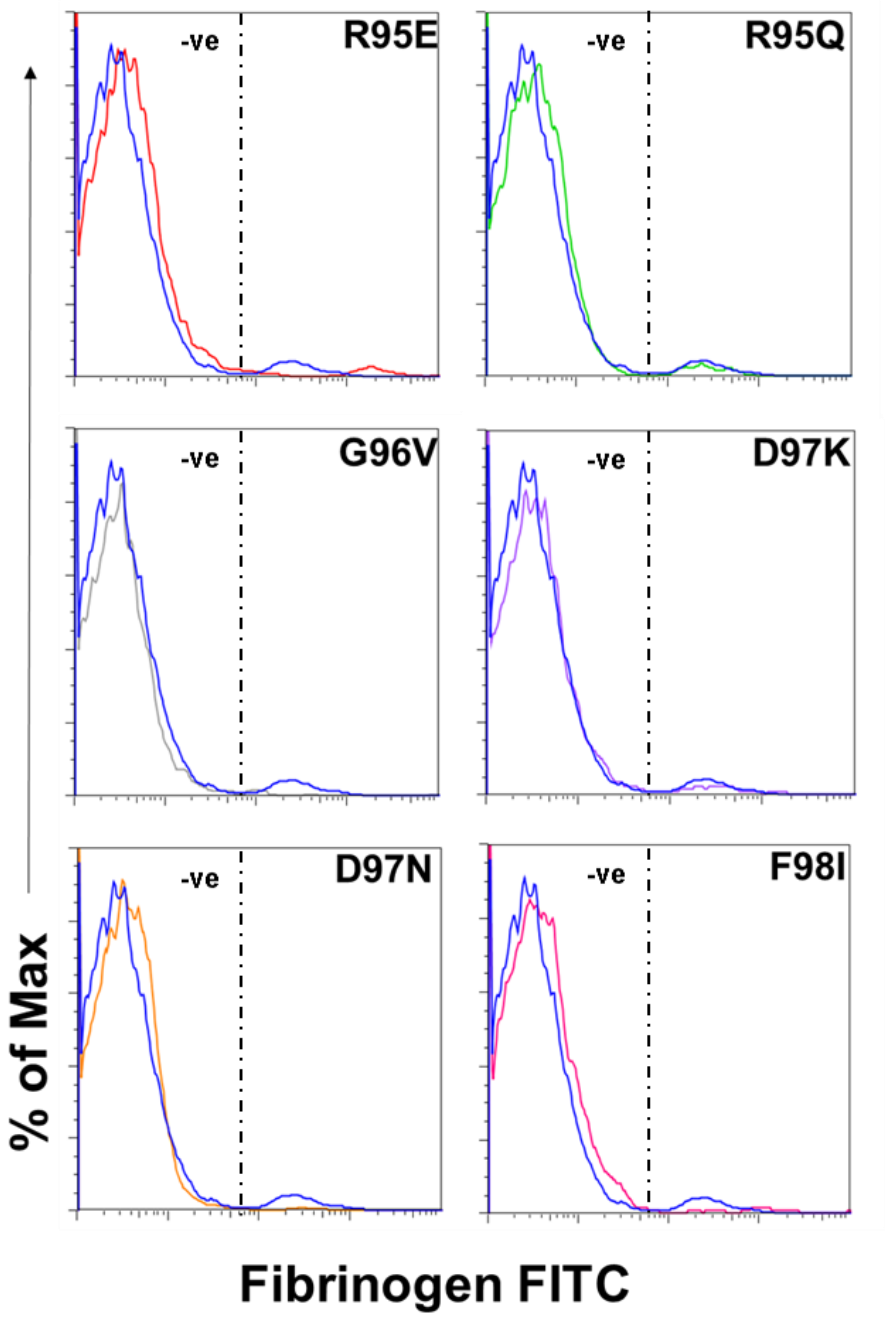


Figure 6-11 Representative histogram indicating the increase of binding of fibrinogen. Blue lines represent the recombinant WT. Coloured lines represent the mutants. The left area represents the negative population of RBCs whereas the right area represents the RBCs that have bond to fibrinogen. Histogram gated on RBC showing fibrinogen binding and calculated by the FlowJo7 software (n=1)

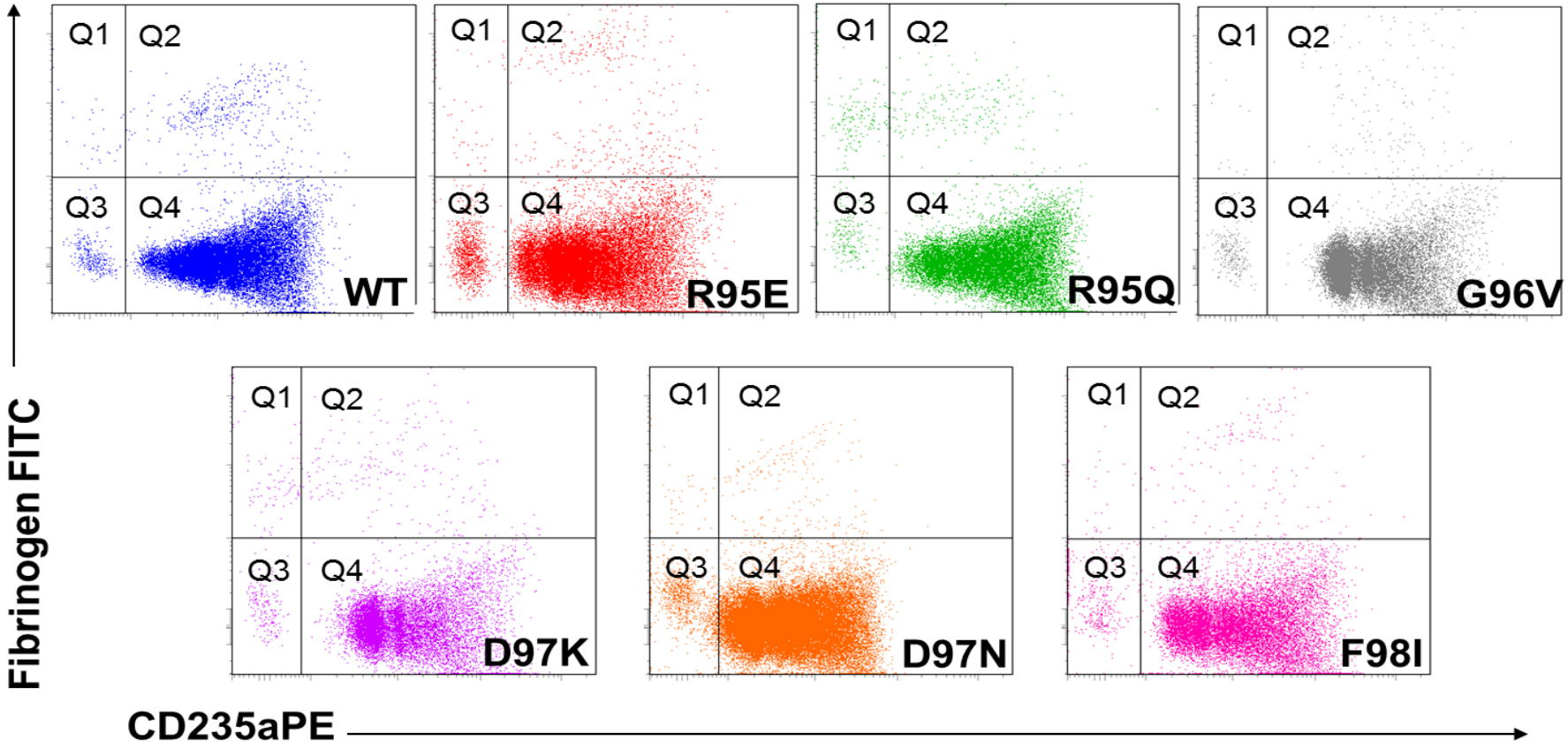


Figure 6-12 Representative scatter-plots produced by the flow cytometer. Side scatter represents AlexaFluor^{488}-fibrinogen intensity (in the FITC channel) and forward scatter represents CD235a^{PE} intensity. Q1: AlexaFluor^{488}-fibrinogen positive cells, **Q2:** Double positive cells, **Q3:** Double negatives cells, **Q4:** CD235a^{PE} positive cells (n=1).

6.3 Discussion

Analysis of fibrinogen binding to the RBCs in solution using flow cytometry has shown that compared to the WT, there were no statistically significant differences between the mutants and the WT showing a slight increase in the binding of R95Q, followed by D97K and to a lesser extent for F98I, whilst G96V showed a slight decrease in binding together with R95E (n=1). All these effects failed to reach statistical significance. The data correlated largely with the results of the fibrinogen-RBC plate-binding assay and solution binding assay. The effects of the mutations were fluctuating between each other, but in general they were just marginally changed compared to the WT.

In this study, flow cytometry was explored to find a robust and well-established solution-based method to study the binding between fibrinogen and RBCs. Lominadze and Dean (2002) previously measured the interaction between RBCs and fibrinogen using labelled fibrinogen as has been done in this thesis, but they evaluated the binding using fluorescence measurements by spectrophotometer and quantified the binding sites using phase-contrast microscopy (Lominadze and Dean, 2002). Specific binding between RBCs and fibrinogen was found by their study, which they suggested to be integrin dependent and they were able to inhibit this binding using an RGDS peptide that is an integrin antagonist (Lominadze and Dean, 2002).

Agglutination of the RBCs as a result of the presence of the CD235a^{PE} antibodies was a slight issue, which was the reason for using less CD235a^{PE} antibodies to ensure that all cells were taking into account. The agglutination may be caused by the binding of RBCs to fibrinogen, which may induce erythrocyte hyper-aggregation in addition to the high tendency of the RBCs to attach to surrounding surfaces, which was also observed in other methods of this thesis or by centrifugation as in the case of flow cytometry (de Almeida et al., 2009). Some studies which used flow cytometry for studying the interaction between fibrinogen and platelets, such as Kasahara *et. al*. (1987), ensured that no aggregation of platelets took place in order to measure the interaction of individual platelets, so they avoided centrifugation of platelets and used low density platelets (2 x 10^8

cells/ml) suspension (Kasahara et al., 1987). These authors demonstrated that by not centrifuging the platelets, non-bound fibrinogen would be present in the sample; however, they confirmed that its effect was negligible. So they were able to measure the fluorescence intensity of individual platelets with sufficiently weak signal from non-bound fibrinogen (Kasahara et al., 1987).

Two more studies investigated platelets and fibrinogen interactions using flow cytometry for its ability to detect the binding of a single cell to fibrinogen. (Warkentin et al., 1990, Abrams et al., 1990). Warkentin *et al.* avoided using centrifugation in their protocol, because it can cause damage to the platelets' membrane. They found that this damage is minor and should not affect fibrinogen binding response to the agonist activated platelets *in vitro*, however, this could cause damage to the platelets, which were activated *in vivo* (Warkentin et al., 1990). These authors diluted whole blood and measured the binding between fibrinogen and platelets using FITC-conjugated polyclonal anti-fibrinogen antibodies. They found that this was a sensitive method for measuring fibrinogen, but that the binding could be non-specific, because the antibody cannot discriminate between free and platelet-bound fibrinogen (Warkentin et al., 1990). On the other hand, Abrams *et al*., chose to use monoclonal antibodies that were fluorescein-labelled against platelet-specific glycoprotein GPIb antibodies, so there was no need for washing or centrifugation, because labelled-platelets are easily distinguished from other cells when used in flow cytometry (Abrams et al., 1990). Although the CD235a^{PE} antibodies are specific for RBCs, all experiments were performed on washed RBCs rather than whole blood to be consistent with the other experiments presented in this thesis. In this project, as mentioned above, a final wash was performed after the incubation of fibrinogen with labelled RBCs to eliminate non-bound fibrinogen. This final wash was established in the initial method relying on haematocrit. In this method, there was no effect of the antibodies on agglutination, because the amount of antibodies was low. On the other hand, to overcome the agglutination limitation in the later method, the amount of CD235a^{PE} antibodies was decreased to a minimum that still can show effect on the binding.

The use of a monoclonal antibody to provide required specificity was the reason for using CD235a^{PE} in this thesis. Monoclonal antibodies were used previously in flow cytometry analysis to determine the number of RBC membrane-derived vesicles in thalassaemia patients in comparison to normal individuals (Pattanapanyasat et al., 2004). In whole blood, vesicles of RBC membrane were identified depending on their size using the FSC and side light scattered (SSC). They used two colour immunostaining of specific FITC/PE conjugated monoclonal antibodies to glycophorin A and CD41a to further distinguish the RBC vesicles from platelets and their vesicles (Pattanapanyasat et al., 2004). The authors found that this method has advantages of using small volumes; it is quick, reproducible and easy to perform (Pattanapanyasat et al., 2004).

The effect of the mutations in the RGD sequence of the amino-terminal half of fibrinogen Aα-chain was suggested to non-significantly enhance the binding between fibrinogen and RBCs instead of inhibiting them, especially for R95Q, D97N, F98I followed by D97K compared to the WT. The statistically non-significant effect of G96V showed decrease in binding to RBCs whilst R95E binding was studied only once and was non-significantly lower than the WT. The enhancing effects of the mutations in the RGD can be a result of conformational changes in the tertiary structure that might expose the sequence for the interaction with fibrinogen, or because the initial binding between fibrinogen and RBCs might induce conformational changes in the secondary structure that again expose the RGD for more binding (Riedelova-Reicheltova et al., 2014). In the first flow cytometer experiments there were some significant increases in the mutants with oppositely charged amino acids (negative E95 and positive K97) and to a lower extent of significance for the neutral Q95. However, the other mutants showed no changes. When the method was altered to increase the amount of available CD235a^{PE}-labelled RBCs for interaction with fibrinogen, the significance of the results was lost, but they were more reproducible. The cells were presented in distinct populations rather than the clumps that were found in the first method. The optimisation experiment was performed using commercial AlexaFluor^{488}-labelled fibrinogen and not the recombinant WT, and an observation which needs more investigation was that the effect by commercial fibrinogen differs somehow from the WT. When the experiment was optimised

with commercial fibrinogen, the binding signal was slightly stronger than when recombinant fibrinogen was used. The effect was still consistent, but not as clear as when commercial fibrinogen was used. This difference between the two types of fibrinogen may be due to the nature of each of them: commercial fibrinogen is purified from human plasma, and provided in a lyophilised form which is then reconstituted in water, whilst recombinant fibrinogen is expressed in cultured cells then purified. However, WT was prepared by the same process as the mutant fibrinogens, making it a better control. The difference between commercial and recombinant fibrinogen was observed in later stages of the project. Therefore, optimisation experiments after this observation were done using the WT in the following experiments.

The non-significant response and the inability to inhibit the binding between RBCs and fibrinogen by the different mutants may reflect that the binding of RBCs to fibrinogen is largely independent from the fibrinogen RGD sequence, in agreement with the other plate-based experiments of RBC-fibrinogen interactions.

In conclusion, there was no effect of the RGD mutations on the binding of fibrinogen to RBCs in solution. There was no clear inhibition for this binding, but a statistically non-significant enhancement was observed for some mutants.

Chapter 7 Study of the Integrin Responsible for Fibrinogen Interaction with Platelets and RBCs

7.1 Introduction

Platelets are an important cell type in the body. Physiologically they play a key role in haemostasis by balancing bleeding and thrombosis, but at the same time, they play a key role in thrombotic diseases, such as myocardial infarction, stroke and cerebral infarction (Remijn et al., 2002, Savage et al., 1998). Platelet activation starts with agonist-receptor binding and ends with platelet aggregations, secretion and shape change (Shattil et al., 1985). This activation is required for their binding to fibrinogen, and there are >100,000 copies of $\alpha_{IIb}\beta_3$ on the surface of activated platelet (Shattil et al., 1985). Platelets are activated in response to physiological agonist such as ADP, thrombin, and collagen binding to their respective receptors on the platelet membrane. This interaction between platelets and agonists induces shape changes and activates the $\alpha_{IIb}\beta_3$ integrin which is a glycoprotein heterodimer complex acting as a receptor for fibrinogen (Shattil et al., 1987, Shattil et al., 1989, Frojmovic et al., 1994). Binding of fibrinogen to this receptor can be used to assess platelet activation, or as in this project, adhesion since fibrinogen is an important cofactor for aggregation as well as adhesion of the platelet (Shattil et al., 1989). The $\alpha_{IIb}\beta_3$ integrin binds fibrinogen through a recognition sequence on fibrinogen composed of three amino acids; Arg-Gly-Asp (RGD) (Farrell et al., 1992). The interaction between this RGD sequence on fibrinogen γ-chain and the integrin is well-defined (Farrell et al., 1992). However, the role of the RGD sequence on the Aα-95-97 of fibrinogen, which is the focus of this PhD thesis, remains unclear.

In platelets, actin cytoskeleton is shown to be part of the regulation of the integrin $\alpha_{IIb}\beta_3$ function, since impairment of actin polymerisation inhibits agonist-induced binding of the integrin to fibrinogen (Bennett et al., 1999). It is suggested that the affinity of the integrin $\alpha_{IIb}\beta_3$ to soluble ligands such as fibrinogen is increased by releasing the cytoskeletal constraints on the integrin. This release occurs by increasing actin filaments turnover which is induced by inside-out signalling in platelets (Bennett et al., 1999). Inside-out signalling is the process of binding platelet agonists to the $\alpha_{IIb}\beta_3$ integrin caused by its activation; the integrin is converted from its low affinity form on platelet surface to high affinity form capable of binding to its ligands (Coller and Shattil, 2008, Oberprieler et al.,

2007). Releasing the cytoskeletal constraints was found to increase cell adhesion avidity by increasing the chances of random meetings between the integrin and its ligand (Bennett et al., 1999).

Platelet plasma membrane and lipid bilayer are coated by a membrane skeleton that is a lattice-like structure. This skeleton membrane acts as a signal transduction regulator due to the nature of its structure; it is interconnected with intracellular cytoskeletal elements as well as extracellular glycoproteins (Fox et al., 1993). Platelets normally circulate as flat oval discs, however, when platelets are activated by agonists such as thrombin or ADP, they transform to compact spheres with emerging spikes called filopodia, lamellipodia and veils (Hartwig, 1992). The reorganisation of actin-myosin cytoskeleton leads to changes in the platelet shape, and to the formation of the actin filaments in filopodia (thin finger-like structures) and lamellipodia (thin sheet-like protrusions), both of which are required for platelet spreading (Mattila and Lappalainen, 2008, Roberts et al., 2009). At the site of vascular injury, adhesion of platelets is vital to stop bleeding (Roberts et al., 2009). Spreading of platelets on immobilised fibrinogen was evaluated using fluorescently-labelled phalloidin staining. Phallotoxins are a group of cyclic peptides that bind specifically to actin. Fluorescent phallotoxins have several advantages including that they are small in size, they have long-term stability, and they have stabilising effects against depolarisation of actin, which improves the structural preservation in the preparation (Faulstich et al., 1988).

This chapter describes the interaction between fibrinogen and $\alpha_{IIb}\beta_3$ platelet integrin using platelet spreading assay and the different fibrinogen Aα-chain $R_{95}GDF_{98}$ mutations (R95E, R95Q, G96V, D97K, D97N and F98I). This was to validate the assay evaluating whether this RGDF sequence in the Aα-chain binds platelet $\alpha_{IIb}\beta_3$ integrin (Carvalho et al., 2010), in analogy to studying the role of the fibrinogen mutants in RBC binding.

In order to study the interaction of the RBC integrins with the fibrinogen Aα-chain $R_{95}GDF_{98}$ sequence, antibodies, previously used to identify the potential site of interaction (anti-$\alpha_V\beta_3$), were also used. Another antibody against the α_V integrin was used as well to confirm the specificity of the binding site (Guedes et al.,

2016), in addition to the normal isotype IgG. Study of the binding site will be performed using antibodies against some integrins thought to be involved in the binding of fibrinogen to RBCs. In addition, eptifibatide (an inhibitor of $\alpha_{IIb}\beta_3$ integrin on platelets) will also be used to assess whether the $\alpha_{IIb}\beta_3$ integrin is involved in RBC-fibrinogen binding, like it is for platelet-fibrinogen interactions.

7.2 Results

7.2.1 Studies of the Integrin-Bound Fibrinogen on the Platelets Surface

The effect of the mutations in the RGD sequence on the interaction between fibrinogen and platelet $\alpha_{IIb}\beta_3$ integrin was tested by visualising the spreading of platelets on immobilised fibrinogen. Platelet spreading assay was performed as per the protocol from the Prof Khalid Naseem Laboratory (Roberts et al., 2009) (section 2.13.2) using 100 µg/ml of recombinant fibrinogen and 5 x 10^6 cells/ml. The interaction was assessed using AlexaFluor^{488}-conjugated phalloidin. Before staining, the cells were fixed to ensure that their structures were preserved. The cells were then permeabilised to allow the phalloidin to enter the cells, and washed with PBS to remove any debris before fixation (Wang et al., 1982, Roberts et al., 2009).

A negative control consisted of 5 mg/ml denatured fatty acid free Bovine Serum Albumin (BSA) was coated onto the cover slip. The images in Figure 7-1 show that platelets did not adhere in the control experiment (BSA coating) because their morphology was different from the shape of the spreading platelets on fibrinogen. The control platelets looked irregular in shapes and they did not show the spreading projections of lamellipodia. The spreading of platelets was clear and the cells looked larger than in the control (BSA) experiment. For R95E, the platelet count looked similar to the WT, with a slightly bigger size. For D97K, there were statistically non-significant less platelets, with similar size to the WT. The similarity of the results of both oppositely charged mutants (negative E95 and Positive K97) with the WT reflected again that altering the type of the charge did not affect the ability of fibrinogen to bind the platelets. In coverslips which were coated with R95Q and D97N fibrinogens, platelet count was decreased,

but this did not reach statistical significance, but their size appeared larger than the WT, but again they failed to reach statistical significance. Platelets on coverslips coated with F98I fibrinogen looked slightly larger in size, but almost similar in number to the WT fibrinogen. For all mutants, the platelets showed lamellipodia as well as small, highly stained, retracted cells, but to different extents. Coverslips with WT and oppositely charged mutants (R95E and D97K) seemed to have more of the small, highly stained nodular cells than R95Q, D97N and F98I coverslips, where lamellipodia were observed. G96V fibrinogen was no longer available, so could not be included in this experiment. D97N was studied only once for the same reason of limited amount available.

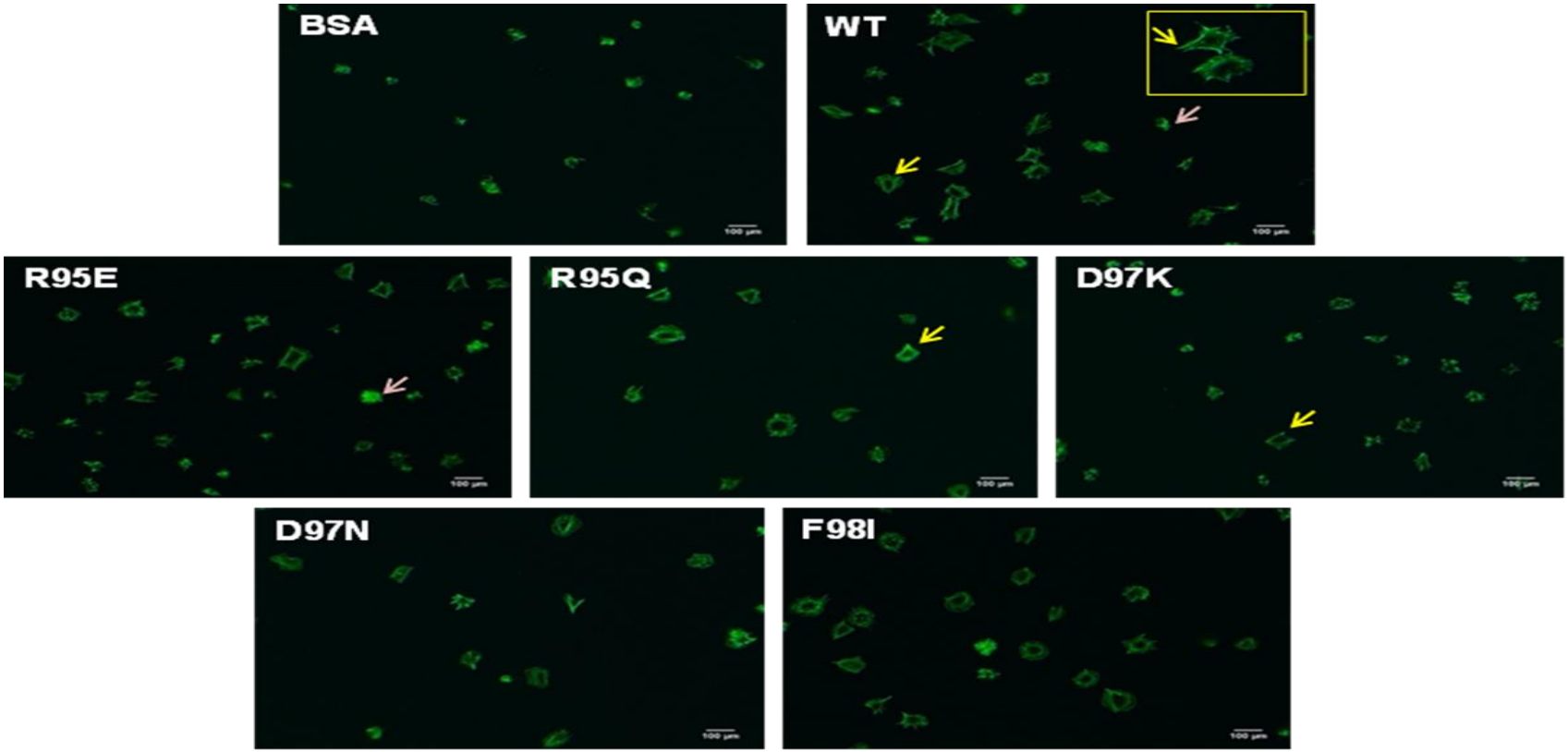


Figure 7-1 Comparison of platelets spreading on coverslips coated with mutant fibrinogens. Total of 100 µg/ml fibrinogen, 5 x 10^6 platelet/ml, 1 unit/coverslip of AlexaFluor^{488}-phalloidin, and BSA control were used. Images were taken by 63x lens and processed using FIJI software, applying automatic threshold. Scale bar = 100 µm. n=3 (except D97N, n=1). Platelets are coloured in green. Yellow arrows point to spread platelets with lamellipodia, whilst pink arrows point to retracted platelets. Yellow box is a magnifying part to show the lamellipodia.

The images were analysed using FIJI by applying automatic threshold and the analysis of spreading results are showed in Figure 7-2

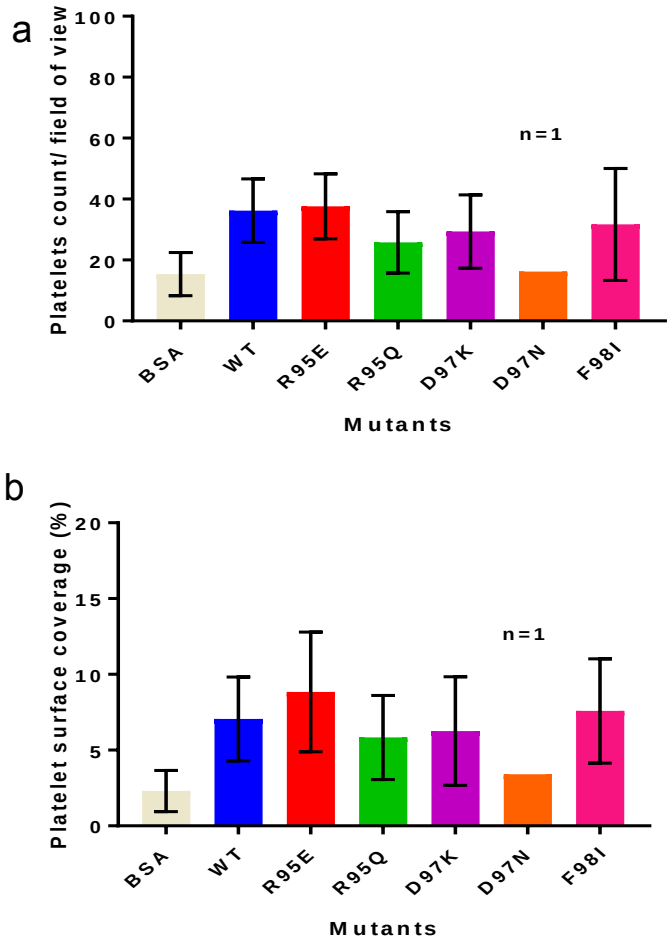


Figure 7-2 Quantitative comparison of the effect of different mutants on platelets spreading on fibrinogen-coated coverslips. Total of 100 µg/ml fibrinogen, 5 x 10^6 platelet/ml, 1 unit/coverslip AlexaFluor^{488}-phalloidin. **a)** Platelet count. **b)** % coverslip covered with platelet. Images were taken by 63x lens, field of view (1790.52X1341.60 micron), and processed using FIJI, applying automatic threshold. Error bars represent Standard Error of Mean (SEM). n=3 (except D97N, n=1). One-way ANOVA multiple comparisons in GraphPad prism, Dunnett's post hoc test were used.

Platelet counts in Figure 7-2a showed that platelet adhesion for all mutants was almost similar to WT, except a slight decrease in the R95Q followed by D97K compared to the WT. Nonetheless, D97N appeared the most obvious decreased mutant compared to the WT, but this was tested only once because there was not enough protein of this mutant. The effect of the mutations on the percentage of area covered by platelets was in agreement with the platelet counts

Figure 7-2b, and showed that R95E slightly increased the spreading, whilst R95Q induced a slight decrease followed by D97K. Platelet spreading on F98I was similar to WT. However, all these effects were statistically non-significant. D97N again had lower percent area of spreading than the WT, but this was from a single experiment and did not reach statistical significance.

7.2.2 Studies of the Integrin-bound Fibrinogen on the RBC Surface

Fibrinogen-Red Blood Cells Plate-Binding Assay

Inhibitor of $\alpha_{IIb}\beta_3$; anti-$\alpha_V\beta_3$ and the anti-α_V antibodies were used to identify which of these integrins are responsible for the binding of fibrinogen to RBCs using plate-binding assay, solution binding assay and flow cytometry. The antibodies were first used in the plate binding assay as described in the method section 2.10. Briefly 100 µl per well of RBCs (5% final concentration) were incubated for 1 hr. The plate was then washed and each antibody (10 µg/ml final concentrations) was added to 100 µl of 20 µg/ml of recombinant WT final concentration, incubated for 1 hr before washing. RBCs were haemolysed with 100 µl of water and the supernatant was stored at -80°C for quantification of bound fibrinogen by ELISA.

The results of this experiment are shown in Figure 7-3. Strangely, the addition of the antibodies marginally enhanced fibrinogen-RBC binding instead of decreasing it but, this effect was minimal and failed to reach statistical significance. According to the previous study by Guedes *et al.* (2016), the integrin $\alpha_V\beta_3$ is the binding site of fibrinogen on the RBC membrane. Increased binding should not be the case if this integrin is the site of interaction of fibrinogen on the RBCs.

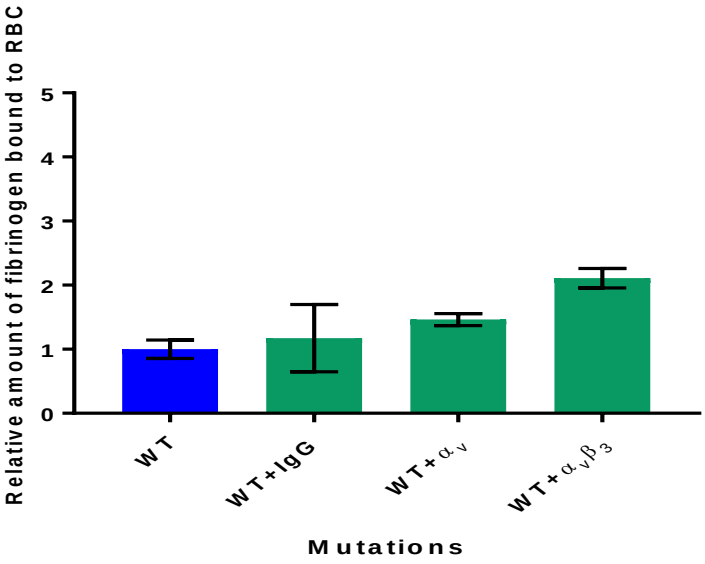


Figure 7-3 Effect of antibodies on fibrinogen-RBCs binding using WT fibrinogen. The results showed unexpected but statistically non-significant increase in binding. Total of 10 µg/ml of antibodies, 5% RBC, 20 µg/ml fibrinogen. Error bars represent Standard Error of Mean (SEM). n=3. One-way ANOVA multiple comparisons in GraphPad prism, Dunnett's post hoc test were used. **WT:** wild type, **WT+IgG:** wild type + anti-IgG antibodies, **WT+α_V:** wild type + anti-α_V antibodies, **WT+$\alpha_V\beta_3$:** wild type + anti-$\alpha_V\beta_3$ antibodies.

Fibrinogen-Red Blood Cells Binding Assay in Solution

The same antibodies (10 µg/ml final concentrations each), as well as Eptifibatide (an $\alpha_{IIb}\beta_3$ inhibitor, 5 µg/ml), were used in the solution assay presented in chapter 5. This was to verify whether the small effects of the antibodies on the fibrinogen-RBCs binding in the plate assay were caused by the static condition of the experiment. Briefly, 20 µg/ml of WT fibrinogen, 10 µg/ml of each antibodies (except Eptifibatide, 5 µg/ml), and 5% RBCs final concentrations were mixed together in a final volume 300 µl. Fibrinogen-RBCs were then transferred to a fibrinogen antibodies pre-coated plate in a volume of 100 µl per well for 2 hrs incubation as described in the methods section 2.11. The plate was then washed and the cells were haemolysed in water and released haemoglobin was measured at 405 nm as a reflection of RBCs that bound to fibrinogen. The results of this experiment are shown in Figure 7-4. Again there was no significant

difference after the addition of the antibodies and they did not block the interaction as suggested by Guedes *et al* (2016).

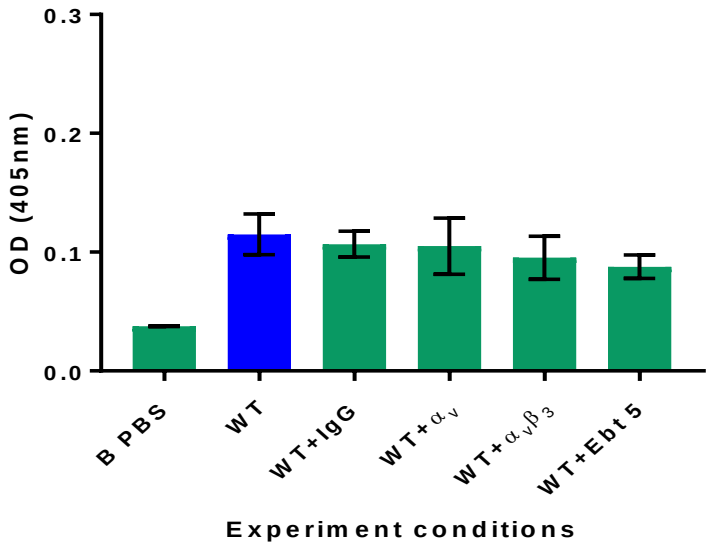


Figure 7-4 Effect of antibodies on WT fibrinogen binding to RBCs by solution-interaction assay. No clear effect on the fibrinogen-RBCs binding was observed from all the inhibitory antibodies tested. Error bars represent Standard Error of Mean (SEM). (n=3). One-way ANOVA multiple comparisons in GraphPad prism, Dunnett's post hoc test were used. **B PBS:** blank PBS. **Ept 5:** eptifibatide 5 µg/ml. **WT:** wild type, **WT+IgG:** wild type + anti-IgG antibodies, **WT+α_V:** wild type + anti-α_V antibodies, **WT+$\alpha_V\beta_3$:** wild type + anti-$\alpha_V\beta_3$ antibodies

Flow Cytometric Assay

Using flow cytometry was the last attempt to use the antibodies to study the binding between fibrinogen and RBCs, using the method that was relying on counting the cells as described in methods section 2.12. Briefly, 5 µg/ml of AlexaFluor^{488}-labelled WT fibrinogen was combined with 10^4 RBC/ml and 20 µl of CD235a^{PE} + 10 µg/ml of each type of antibodies, final volume 420 µl. The mixture was incubated for 30 min then washed, centrifuged, re-suspended, and taken to the flow cytometer. The results in Figure 7-5 are presented as median fluorescent intensity (MFI) of the bound fibrinogen and show a statistically non-significant difference of the binding between WT and RBCs interactions being slightly higher when the antibodies were present. This was similar to what have been shown by the previous two techniques (plate-binding assay and solution

plate-binding assay) confirming that the binding is not through this integrin $\alpha_V\beta_3$ so the experiment was not repeated and statistical analysis was not applied.

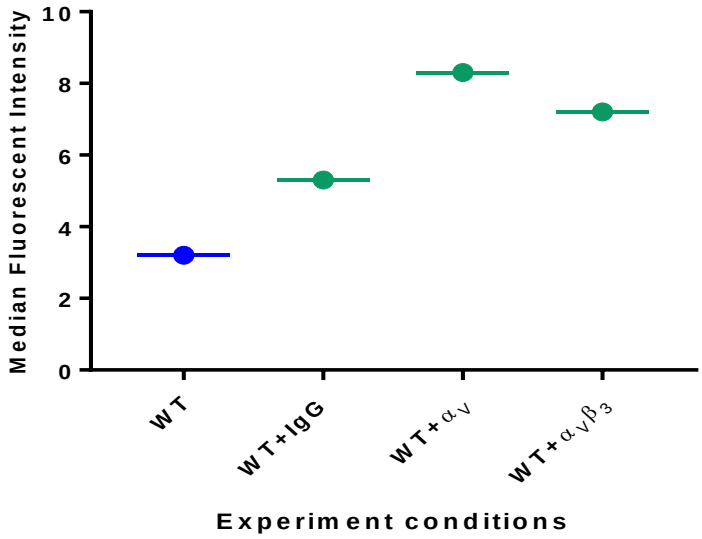


Figure 7-5 Effect of different antibodies added to the WT on the binding between fibrinogen and RBCs measured as MFI. Total of 5 µg/ml AlexaFluor^{488}-labelled WT + 10^4 RBC/ml + 20 µl CD235a^{PE} + 10 µg/ml antibodies. All antibodies showed increased the binding of fibrinogen WT to RBC, n=1. **WT:** wild type, **WT+IgG:** wild type + anti-IgG antibodies, **WT+α_V:** wild type + anti-α_V antibodies, **WT+$\alpha_V\beta_3$:** wild type + anti-$\alpha_V\beta_3$ antibodies.

7.3 Discussion

7.3.1 Effect of Fibrinogen RGD Mutations on Platelet Adhesion and Spreading

The data generated in this chapter showed no significant difference in the spreading of platelets on coverslips coated with mutated fibrinogens. All the mutants showed different degrees of presence of lamellipodia and some densely stained cells, which may be retracted cells. In general for all mutants, the platelets showed almost similar sizes in the images, which may reflect that the degree of spreading was similar to the WT; this may be due to strong interaction with fibrinogen. The similarity in size of platelets to the WT may suggest that mutations in the Aα- $R_{95}GDF_{98}$ sequence did not affect the spreading. The

amount of available D97N was enough to be used only once whereas G96V was not used in this experiment because this mutant was no longer available.

In the current project, the effect of mutating fibrinogen Aα-95-97 RGD sequence on its interaction with platelets was studied to evaluate whether this RGD sequence is involved in the binding site of the $\alpha_{IIb}\beta_3$ integrin on platelets since no effect of these mutations was observed in the interaction of fibrinogen with RBCs. This would support the probability to target this sequence as a therapeutic strategy for thrombotic disease.

Looking at the results in this chapter, the spreading of platelets on fibrinogen in response to the mutations in the fibrinogen Aα-95-97 RGD sequence reflected that the binding of fibrinogen to the integrin $\alpha_{IIb}\beta_3$ was not affected by the mutations and they did not block or decrease the interaction between fibrinogen and platelets. These results are similar to what has been found in the binding between fibrinogen and RBCs in that those mutations did not have a significant effect on the binding between fibrinogen and platelets. In this project, the hypothesis was that mutations in the RGD sequence would inhibit the binding by blocking the binding site of fibrinogen to the RBC integrin or $\alpha_{IIb}\beta_3$ if it is the binding site of fibrinogen on RBCs in addition to platelets. The role of Aα-95-97 RGD in the binding was tested, but mutating the RGD site did not significantly affect the spreading of platelets on fibrinogen or inhibit the binding between fibrinogen and $\alpha_{IIb}\beta_3$ integrin.

When spreading, platelets change their dendritic appearance to a fully spread form with a smooth membrane (Sandmann and Koster, 2016, White and Escolar, 1993). Fully spread platelets should have a regular shape. Sandmann and Köster, 2016 have found that platelets retracted when spreading on a structured, but not on a smooth surface (Sandmann and Koster, 2016). Accordingly, some cells appeared in the figurers as densely stained retracted cells. Other platelets in our assays showed projections that could be lamellipodia, which allow platelets to adhere to underlying surfaces and help to cover the wound, rather than the filopodia which act to recruit other platelets and help in the spreading (Sandmann and Koster, 2016).

Looking at platelet spreading on the mutated fibrinogens by microscopy, it seems that the mutations in the RGD sequence had influenced the spreading (fully spread appearance with lamellipodia). However, this effect of mutations on the spreading was statistically non-significant compared to the WT, indicating that the mutations did not affect the binding between the $\alpha_{IIb}\beta_3$ integrin of the platelets and the RGD sequence on fibrinogen.

In conclusion, no significant differences were observed, these data showed that the Aα-95-97 RGD sequence does not play a significant role in the binding between fibrinogen and $\alpha_{IIb}\beta_3$ of platelets excluding this site from the binding between fibrinogen and RBCs and that the binding site could be γ-400-411 sequence or the Aα-572-574 RGD sequence (Cheresh et al., 1989b). These results were mostly in agreement with the effects of the mutations on the RBC binding to fibrinogen by the plate binding assay and the flow cytometry reflecting that the Aα-95-97 RGD seems not to have a role in the interaction of fibrinogen with either RBCs or platelets. However, some of the results are only preliminary as some experiments could not be repeated due to the limited amounts of recombinant protein available.

7.3.2 Effect of Using Integrin Antibodies and Inhibitor on the Binding between Fibrinogen and RBC Surface

In addition to studying the effect of the mutations in fibrinogen Aα-95-97 RGD sequence on the binding between fibrinogen and RBCs, the expected binding site of fibrinogen on the RBC membrane was investigated using some antibodies. The choice of antibodies was based on a study by Guedes *et al.* (2016) suggesting that integrin $\alpha_V\beta_3$ is involved in the binding of fibrinogen on the RBC membrane. Eptifibatide, an inhibitor of the platelets integrin $\alpha_{IIb}\beta_3$, was used as well in the solution binding assay to find out if this integrin is the binding site of fibrinogen on the RBC membrane. Integrin studies were performed in this project using all the functional methods of fibrinogen-RBC binding studies; plate binding assay, solution binding assay and flow cytometry.

The results of all experiments were consistent showing that neither $\alpha_V\beta_3$ nor $\alpha_{IIB}\beta_3$ are the integrin receptors for fibrinogen on the RBC membrane. This is

because there was a non-significant enhancement in the binding between fibrinogen and RBCs with the addition of the antibodies or the inhibitor. Antibodies blocking the $\alpha_V\beta_3$ integrin either exposed an unknown integrin on the RBC surface and increased RBC binding or induce conformational changes in the WT fibrinogen exposing the hidden RGD sequence for more binding when it is exposed. The non-specific binding of the RBCs with the plate may block the $\alpha_V\beta_3$ integrin site preventing its binding to the antibodies and subsequently inhibit their effect.

Lominadze and Dean 2002, have suggested that the binding between fibrinogen and erythrocytes, which may induce erythrocytes aggregation, is via an integrin binding site and is inhibited by RGDS peptide; the integrin receptor antagonist (Lominadze and Dean, 2002). They confirmed the presence of specific fibrinogen binding sites on the surface of the RBC, which they suggested to be through an unidentified integrin type receptor (Lominadze and Dean, 2002). They suggested that RBC receptor has low affinity to RGDS, however, they did not exclude the presence of a non-integrin type binding through the RGD as well (Lominadze and Dean, 2002). High concentrations of RGDS was required to show significant inhibition of the binding reflecting low affinity of the fibrinogen receptor on the erythrocytes surface whilst lower concentrations failed to reach significant effects (Lominadze and Dean, 2002). Unlike Lominadze and Dean, in this project RGDF was investigated rather than the RGDS, and studying the interaction site was via antibodies against the integrin rather than using antagonist peptide to the RGD. The statistically non-significant effects of RGDF mutations on the binding between fibrinogen and RBCs may be because higher concentrations of fibrinogen were required to provide statistical significant.

Addition of eptifibatide in my assays was found to not affect the binding between fibrinogen and RBCs. This finding could not confirm the previous results by Carvalho *et al* where RBC-fibrinogen interaction was studied in comparison to platelets interaction with fibrinogen using atomic force microscopy (AFM) (Carvalho et al., 2010). In this study they indicated that the interaction between fibrinogen and RBCs is through β_3-related integrin (Carvalho et al., 2010). The authors have found that this interaction is inhibited by the $\alpha_{IIb}\beta_3$ integrin inhibitor;

eptifibatide, but to a lower degree than platelets, which excluded the platelet integrin from being the receptor for fibrinogen on the RBC membrane. The authors found that the RBC receptor is calcium-dependant and has lower binding force than the platelet integrin (Carvalho et al., 2010).

By using AFM with anti-α_V and anti-$\alpha_V\beta_3$ antibodies, Guedes *et al,* showed that the integrin $\alpha_V\beta_3$ is the specific receptor of the fibrinogen on the RBC membrane (Guedes et al., 2016). The use of these antibodies in the current project did not inhibit the binding between fibrinogen and RBCs. Nonetheless, they non-significantly enhanced this binding, excluding these two integrins from being the binding site of fibrinogen on the RBCs. The inconsistency observed between these studies may be due to difference in the techniques or the type of fibrinogen used. On the other hand, both studies lack any biochemical evidence of the presence of the integrin on the RBCs such as a western blotting results or knockout mouse studies. However, it should not be ignored that the results of the integrin studies in the current project were consistent among all the techniques used, suggesting more studies to confirm either one of the findings.

In conclusion, the consistency of the results obtained using two inhibitory antibodies and three different binding methods, in causing a slight enhancement of the binding between fibrinogen and RBCs rather than inhibiting it, might exclude the integrin $\alpha_V\beta_3$ from being the binding site for fibrinogen on the RBCs.

Chapter 8 Discussion

In this project the focus was on fibrinogen binding and interaction with RBCs, aiming to establish the site of this interaction on both fibrinogen and RBCs. The experimental approach to achieve this aim was by producing recombinant fibrinogens with mutations in the integrin binding site, and to study their effect(s) on the binding of fibrinogen with RBCs and platelets, and on clot structure and function.

8.1 Overview on the results

In order to achieve the aims of this project, six mutants of fibrinogen in the Aα-chain $R_{95}G_{96}D_{97}F_{98}$ integrin binding site were produced using recombinant technology in βγ-CHO cells (R95E, R95Q, G96V, D97K, D97N, F98I), and studied for their effect(s) on clot structure as well as on the binding between fibrinogen and RBCs. The recombinant fibrinogens showed some changes in clot structure, polymerisation profile and fibrinolysis. The selection of these mutations was based on the side-chain sizes of the new residues being similar to the original residues, to avoid disruptions to the molecular structure of the fibrinogen with a factor other than charge. The data from all experiments including clot structure and fibrinogen-RBC binding suggested that the original charged residues provide stability to the fibrinogen molecule. Data with neutral mutants showed more variability compared to the WT, especially regarding clot structure.

The new recombinant fibrinogens were tested for presence of any changes in their secondary structures which may be caused by conformational changes in the protein folding that can affect the biological function of the protein (Kelly et al., 2005). The CD-spectra curves showed a slight decrease in the α-helix for both R95Q and D97N compared to the WT. This decrease was found to be minimal and had no effect on the biological functions of fibrinogen; however, it cannot be excluded that the mutations may have induced conformational changes in the tertiary structure of the protein. All fibrinogen mutants resulted in significant effects on clot structure showing thinner fibres and longer clotting times that were significant for R95E, R95Q and D97N.

The overall results of the effect of the mutations on clot structure and the binding between fibrinogen and RBCs are summarised in Table 8-1. Recombinant mutated fibrinogens were tested for their effect on the binding between fibrinogen and RBCs. This effect was first tested in static conditions using a plate-binding assay. The results from this experiment showed no significant changes of mutants compared to the WT with regards to the binding between the fibrinogen and RBCs. Next, the effect on binding was tested in solution, and evaluated by haemoglobin release from RBCs bound to fibrinogen that was captured with fibrinogen antibodies. The results were again minimal and statistically non-significant. Finally the effect was tested in full solution state by using flow cytometer and the results again showed no significant differences between the mutants. For the sake of conformation of the role of Aα-chain $R_{95}G_{96}D_{97}$ on the binding between fibrinogen and RBCs, this was tested by different methods and all of them suggested that this site does not participate in this binding.

The use of antibodies in this project has revealed that $\alpha_v\beta_3$ was not the binding site of fibrinogen on the RBC surface, because antibodies against this integrin induced the binding of fibrinogen to the RBCs instead of inhibiting this binding. The platelet spreading experiment has confirmed that Aα-95-97 RGD is not the main binding site for $\alpha_{IIb}\beta_3$ on platelets surface.

8.2 Variable Effects on Clot Structure and Function among Mutants

The R95E, D97K, and F98I mutants showed similar clotability to the WT followed by R95Q and D97N, whilst G96V had a significantly lower clotability compared to the WT. All mutants showed significantly thinner fibres when converted to fibrin and to a lesser extent of significance for D97K than the WT. Although thinner fibres were formed, R95E and R95Q had significantly longer lag phase whereas the G96V and D97N had only slightly longer clotting time that did not reach statistical significance. According to Collet et al., clots that are made up of a large number of thin fibres lyse slower than clots made up of a smaller number of thicker fibres (Collet et al., 2003). Even when mutants lysed slower than the WT, they either had a smaller number of thin fibres (R95E) or a larger number of thin

fibres (D97K). Some mutants produced clots that were made up of a large number of thin fibres, but still lysed faster than the WT (R95Q and F98I), or a similar number of fibres as the WT and lysed faster (D97N). There were no lysis results for G96V due to low availability.

8.2.1 Summary

In summary, all mutants were found to produce clots with thinner, shorter fibres that were highly branched compared to the WT. When it came to the binding between the recombinant fibrinogens and RBCs, there were non-significant differences between the different methods used (plate-binding assay, plate-based solution assay and flow cytometry in solution). However, overall, the effects of the mutations on RBC binding were not significantly different for any of the mutants when compared with the WT taking all three methods into consideration.

Table 8-1 Summary of the results of the effect of the mutations on clot structure and properties, compared to the WT. Arrows represent the increase or decrease in each particular parameter. One arrow represents statistically non-significant effect, two arrows represent statistical significant effects. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

	R95E	R95Q	G96V	D97K	D97N	F98I
Clotability	93.7%	88.4%	68.8% **	96.4%	84.9%	94.2%
Max OD	↓↓ ***	↓↓ ****	↓↓ ****	↓↓ *	↓↓ ****	↓↓ ***
Lag phase	↑↑ ***	↑↑ **	↑	↓	↑	↓
Time to ½ lysis	↑	↓	-	↑	↓	↓
Clot structure	Shorter fibres and slightly less branched	Shorter fibres and highly branched	-	Shorter fibres and highly branched	Much shorter fibres and highly branched	Shorter fibres and highly branched
Fibre density	↓↓ *	↑↑ *	-	↑↑ ****	≈	↑↑ ****
α-helix count	≈	↓	≈	↑	↓	≈
Plate-binding assay of RBC interaction	↑	↑	↓	↓	↑	↑
Mice plate-binding assay	≈	≈	≈	≈	≈	↑
Solution-binding assay	↑	↑	≈	↑	↑	≈
Flow cytometry	↓	↑	↓	≈	-	≈
Platelets spreading	≈	↓	-	↓	↓	↓

8.3 General discussion

8.3.1 RGD Aα-95-97 Involvement in Fibrinogen-RBC Binding

The choice of making mutations in the RGD Aα-95-97 was supported by a naturally occurring mutation just after this site (F98I) which was a heterozygous point mutation found in a woman with multiple miscarriages, but no bleeding or thrombotic symptoms (Riedelova-Reicheltova et al., 2014). The turbidity and confocal microscopy experiments in the present study showed that recombinant F98I has lower maximum absorbency, with thinner, shorter and more branched fibres, lower fibrinolysis and a denser clot structure. This was in agreement with the laboratory investigations of the natural F98I, which revealed some changes in clot structure showing thin, short, and curly fibres with a lower final turbidity, impaired fibrinolysis, and greater platelet aggregation (Riedelova-Reicheltova et al., 2014). This mutation was suggested to cause conformational changes that causes disturbances to the folding of the coiled-coil region on the Aα-chain of fibrinogen (Riedelova-Reicheltova et al., 2014). The mutation was produced as a recombinant mutation for the first time in the laboratory in the present project to study its effect on clot structure and the binding with RBCs.

Similar to F98I, the G96V mutation was selected based on previous studies. The results which were obtained for the binding between fibrinogen and RBCs showed that this mutant did not affect this binding, despite a previous report showing an effect on the binding between fibronectin and fibroblasts (Pierschbacher and Ruoslahti, 1984). The presence and involvement of the RGD sequence in cell attachment was first discovered in fibronectin by Pierschbacher and Ruoslahti, 1984. Substitution of Glycine with Valine (G96V) was found to diminish the binding of fibroblasts to fibronectin (Pierschbacher and Ruoslahti, 1984). However, it was found that in an Arg-Gly-Asp-Ser (RGDS) sequence, when Ser was replaced with Cys (RGDC), no loss of the binding was observed. This indicated that the Serine residue was not required for the binding activity of the molecule, which therefore totally relied on the Arg-Gly-Asp (RGD) sequence (Pierschbacher and Ruoslahti, 1984).

Other mutations which were selected in the present study to test the binding between fibrinogen and RBCs were also based on synthetic peptides tested in the Pierschbacher and Ruoslahti study of interactions between fibronectin and fibroblasts. The authors found that deletion of Arginine (R) or Aspartic acid (D) lost binding activity of the molecule, and substitution of Aspartic acid with Asparagine (D97N) diminished the binding between fibronectin and fibroblasts (Pierschbacher and Ruoslahti, 1984). The results of similar mutations in fibrinogen in this project showed that D97N did not have significant effect on the binding between fibrinogen and RBCs compared to the WT, although it did show somewhat slightly lower binding in all experiments compared to other mutants that failed to reach statistical significance.

Not many studies have been performed on the RGD sequence which was the focus of this project or on its interaction with RBCs. Mutations in fibrinogen to inhibit its binding with different cells were previously used to confirm which of the three sites was involved in the binding of fibrinogen to platelets. Farrell et al. 1992, produced three different mutations in the Aα and γ chains of fibrinogen. In the Aα-chain, the D97E, which was one of the sites that were mutated in this project but with different residues. The other mutant in the Aα-chain was D574E whereas in the γ-chain, the $\alpha_2\beta_2\gamma'_2$ recombinant fibrinogen was produced replacing the two γ-chains by two γ'-chains (Farrell et al., 1992). In similar results to what we have achieved with Aα-95-98 RGD, the authors found that mutations in either RGD binding sites of fibrinogen did not show a great effect on the aggregation. On the other hand, the γ-carboxylic binding site was able to inhibit the aggregation when it was mutated showing its role in the adjacent platelet bridging during the aggregation; so it was possible to confirm the site of fibrinogen-platelet binding as the γ-chain C-terminus of the common γ-chain splice variant (Farrell et al., 1992). These results agreed with Kloczewiak *et al* 1984 that γ-chain is the main interaction site of platelets with fibrinogen (Kloczewiak et al., 1984) but contradicted Hawiger *et al*, 1989 who suggested a role for both alpha RGD sites and gamma chain in the binding between platelet integrin and fibrinogen (Hawiger et al., 1989). This controversy keeps the door open for more research in the area of allocating the site of interaction on fibrinogen with different cell types.

The involvement of the Aα-95-97 RGD in the binding of fibrinogen to $\alpha_{IIb}\beta_3$ platelet integrin was investigated recently, in addition to the other two sites (Aα-572-574 RGD and γC-12 AGDV-containing dodecapeptide), in a study by Kononova *et al.* (2017). The authors found that a single molecule of both the Aα and γ chains contributed almost the same in the binding with the platelet integrin. However, with high concentrations of fibrinogen, it was found that both the Aα-RGDs bind significantly more than the γ-chain dodecapeptide most probably because there are two different RGD sites compared to one γC-12 (Kononova et al., 2017). According to their results more fibrinogen may be required to show significant results. Although the amount of fibrinogen which was used in the platelet spreading assay (100 µg/ml) was relatively high in the method developed by the Naseem Laboratory, the results failed to show a statistically significant effect. This may mean that any change in the binding between fibrinogen and RBCs reflects the effects of the mutations with no influence of the amount of fibrinogen used. In another study, it was found that both RGD and γ-chain synthetic peptides are able to inhibit the binding of $\alpha_{IIb}\beta_3$ on platelets to different adhesive proteins such as fibronectin and vWF (Tranqui et al., 1989). However, mutations in the Aα-95-97 RGD in present study did not show any inhibition of the binding between fibrinogen and platelets**, excluding this RGD from being the site of interaction between fibrinogen and platelets**.

8.3.2 RGD Site on Fibrinogen

While the use of synthetic peptides by some studies was aimed at inhibiting interactions between fibrinogen and platelets, and thereby subsequent platelet activation, in the present study the focus was on abolishing the interaction between fibrinogen and RBCs by mutating the proposed site(s) of interaction. Introducing mutations was used in early and more recent studies to evaluate the effect of these mutations on fibrinogen binding. To confirm the involvement of the both RGD sequences in the binding of fibrinogen and other adhesive proteins to different cells, Pytela *et al* (1986) found that the amino acid substitution of aspartic acid by glutamic acid was inactive in synthetic peptide and had no effect on the binding to platelets, which was confirmed later by other studies as stated above (Pytela et al., 1986).

In a more recent study on the involvement of the Aα-95-97 RGD in the binding between RBCs and fibrinogen, Carvalho *et al*, 2018 investigated its involvement in a way similar to our route of mutating the RGD site to abolish this interaction. The authors compared the relative roles of the two RGD sites on the Aα-chain in the binding between fibrinogen and RBCs (Carvalho et al., 2018). Three mutations were studied, namely D97E, D574E and a D97E/D574E double mutant and compared to WT fibrinogen. Using AFM, they found that the D97E had a lower binding force to the RBCs and a lower rupture force compared to the WT followed by the double mutant, however, the D574E showed a stronger binding force and a higher rupture force than the WT, suggesting that Aα-95-97 RGD provides the main binding site for RBCs on fibrinogen (Carvalho et al., 2018).

The results in which D97E inhibited the binding contradict the results presented in this thesis, which showed no significant effect of mutations at this site. In addition, Pytela *et al*, 1986 also showed that there was no effect of this specific mutation on the binding of platelets with fibrinogen. The difference between the results of Carvalho *et al* and the present study may relate to differences in the techniques that were used in the studies. Another reason may be the electromagnetic charge of the mutation, since in the Carvalho *et al* the authors replaced the negative aspartic acid with a negative glutamic acid (Carvalho et al., 2018). So in their case, they replaced an amino acid with a similar size and charge, which means that the degree of any disruption to the molecule was not as much as when we changed the charge to its opposite (positive lysine: K) or to neutral (asparagine: D). The disturbances in the structure of the molecule in the present study may have led to increased exposure of the cryptic Aα-95-97 RGD for enhanced binding with RBCs (Riedelova-Reicheltova et al., 2014)

The involvement of F98I in thrombosis (Riedelova-Reicheltova et al., 2014) drew our attention to investigate this residue which is located right next to the RGD. In the present study, the RGDF mutant was found to slightly enhance the binding between fibrinogen and RBCs, supporting thrombotic rather than inhibitory effects. It was found previously that the tripeptide RGD is not enough to inhibit the binding between fibrinogen and platelets or endothelial cells. There was a

requirement for the presence of a fourth amino acid in position X (RGDX) for the synthetic peptide to be able to inhibit the binding of both cells to fibrinogen (Tranqui et al., 1989). This is not in agreement with the suggestion from synthetic peptide studies about the non-involvement of serine residue in the binding between fibronectin and fibroblast above (Pierschbacher and Ruoslahti, 1984) This disagreement may be due to the difference in the proteins in which the synthetic peptide was located in either fibrinogen or fibronectin. In a more recent study, RGD containing disintegrins of snake venom, which are able to inhibit ADP- and collagen-mediated platelet activation and aggregation were investigated (Oyama et al., 2017). The authors confirmed that the composition of the amino acid residues in the RGD loop was very important for the binding, and that the selectivity of these disintegrins was influenced by the amino acids nearby the RGD sequence (Oyama et al., 2017).

The results of this project suggested the presence of a discrepancy in the effect of the mutations on the binding of fibrinogen to RBCs, which may be related to the charge and biophysical nature of the newly introduced residue. The replacement of an Arginine by neutral or oppositely charged residue would encourage the new fibrinogen to favour the binding with RBCs rather than to inhibit it. Previously in the case of platelets, this inhibitory effect was found to be increased with the increase of hydrophobicity of amino acid X for example RGDS had less inhibitory potential than RGDF (Tranqui et al., 1989). However, when the Arginine residue was changed by a more acidic residue such as Histidine (HGDF) or neutral such as glutamine (QGDF), a discrepancy effect was detected with opposite effects on the binding of fibrinogen to both cells (Tranqui et al., 1989).

8.3.3 Integrin Studies

In addition to investigating the involvement of the Aα-95-98 RGDF in the binding between fibrinogen and RBCs, the binding site on the RBC is equally important in participating in this binding. The way that the RGD is recognised by its ligand was found to depend on the conformation of the sequence, which subsequently depends on any substitution of the residues and the chemistry of the residue that follows the tripeptide (Pierschbacher and Ruoslahti, 1987). This suggests that

the specificity of the receptor relies on the conformation of the RGD, which is affected by its neighbouring residues (Park et al., 2002). In the present study, this conformation may be affected by the mutations as well. In the platelet spreading method presented in this thesis, the Aα-95-98 RGD may be excluded from being the site of interaction between fibrinogen and $\alpha_{IIb}\beta_3$ using adsorbed fibrinogen on the coverslips. A previous study excluded any role of the platelet glycoprotein $\alpha_{IIb}\beta_3$ in the binding between RBCs and fibrinogen which may occur through the adherence of the platelets or platelet micro-particles (MP) in the RBCs (De Oliveira et al., 2012). No staining for the RBCs was detected as a result of the platelet marker CD41 as per Carvalho *et al*, 2010. In spite of using different methods, there was agreement between the results of the adsorbing fibrinogen on the AFM functionalised tip in the Carvalho *et al.* (2010) and the soluble fibrinogen in De Oliveira *et al*. study because immobilising fibrinogen could cause conformational changes in fibrinogen structure (De Oliveira et al., 2012, Carvalho et al., 2010). Adsorbed fibrinogen in this project was used in solution assay and plate-binding assay as well. By using RGD and RGDS peptides, De Oliveira *et al.* contradicted the results that suggested the involvement of the RGD sequence in fibrinogen-RBC interaction and the presence of integrin-like receptor involvement of the interaction between soluble fibrinogen and RBCs (De Oliveira et al., 2012). However, it was shown that this interaction is age dependant and higher in young RBCs than older ones (De Oliveira et al., 2012). Finally the authors assign the CD47 surface receptor on the RBC membrane as a mediator for their interaction with fibrinogen, because the antibodies against this receptor decreased binding of soluble fibrinogen to the RBCs (De Oliveira et al., 2012). Our results may agree with the findings of De Oliveira *et al*. of excluding the RGD sequence from the binding of fibrinogen with RBCs, because by mutating this sequence there was no significant effect on fibrinogen-RBC binding.

Using antibodies against α_V and $\alpha_V\beta_3$; the endothelial receptor that bound fibrinogen via RGD, Guedes *et al*, 2016 showed that this receptor may be the integrin on the RBC membrane that is responsible for its binding with fibrinogen at the single-molecule level using AFM (Guedes et al., 2016). Our results do not confirm these findings, because by all techniques in the present study, using

either of these two antibodies slightly enhanced the binding compared to the WT. This may exclude this integrin as the site of binding of fibrinogen on the surface of the RBCs. The discrepancy in the results between Guedes *et al.* and the present study may be caused by the nature of the techniques that were used in the two studies. According to De Oliveira *et al.* fibrinogen adsorbed on the AFM tip may cause conformational changes that affect the binding of fibrinogen with the RBCs. This could be the case in fibrinogen-RBCs solution and plate-binding assays, because fibrinogen was adsorbed on the plate. Nonetheless, the results of these two techniques were consistent with the results of the flow cytometer, which involved the presence of fibrinogen in solution and not adsorbed to any surface.

In this project antibodies against $\alpha_V\beta_3$ and α_V integrins were used to establish if the $\alpha_V\beta_3$ is the binding site of the fibrinogen on the RBCs. Using antibodies against the glycoproteins $\alpha_{IIb}\beta_3$ and GPIb in addition to RGDS peptides, Wick *et al.* suggested the presence of similar proteins to these integrins on the surface of the young RBCs facilitate their interaction with the vWF (Wick et al., 1993). These results were contradicted by the results of another group who used well-characterised monoclonal antibodies against several integrins. The authors found GPIV and $\alpha_4\beta_1$ non-RGD recognised integrins on the surface of reticulocytes of anaemic patients. These integrins were decreased as the reticulocyte maturated to erythrocytes (Joneckis et al., 1993). Their results demonstrated the absence of the adhesion receptors GPIb, $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$ and $\alpha_V\beta_5$ for vWF and receptors $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$ and $\alpha_M\beta_2$ for fibronectin on the reticulocyte (Joneckis et al., 1993).

In addition to the interaction between fibrinogen and RBCs, the effect of the mutations in the Aα-95-98 RGD on the binding between fibrinogen and platelets was also studied in this project to elucidate if this sequence is involved in this binding interaction. However, results from the present study did not show inhibition in the binding between fibrinogen and platelets in response to mutating the Aα-95-98 RGD, which may exclude this site from being the site of interaction with the $\alpha_{IIb}\beta_3$ integrin on platelets.

8.4 Project limitations

Difficulties with this project started from the first step of mutagenesis, a series of experimental repeats were performed in a long process of method optimisation before the successful mutants were produced. Next, production of the protein was a very long and costly process that concluded with a low yield of fibrinogen for most of the mutants. The low amount of available fibrinogen was the main limiting factor in this project.

Some experiments such as scanning electron microscopy and clot retraction, which were initially planned to study the effect of RBCs on the clot structure were cancelled. This was because these particular experiments required higher amounts of fibrinogen than the other methods, which were not sufficiently available due to the low yield and concentrations of the recombinant fibrinogens. When planning the SEM and clot retraction experiments, the calculations revealed that the amount of recombinant fibrinogen which was required for the experiment for some of the mutants was about 50% of the total volume of the reaction mixture. Adding recombinant fibrinogen up to 50% of the clot volume would highly dilute the clot preparation mixture, which contains blood to the extent that may prevent clotting. On the other hand, to study the effect of mutants on clot structure by SEM, the required fibrinogen concentration was too high compared to the available recombinant fibrinogens.

For fibrinogen-RBCs binding under static conditions of the plate-binding assay, the experiment was run under non-physiological conditions, since binding was affected by the static conditions which might affect the conformation of fibrinogen. This condition is different from the physiological binding conditions which might be seen if the experiment was perform under shear stress (i.e. under flow conditions). Addition of thrombin would convert fibrinogen to fibrin, which would resemble the physiological condition in case of injury, and might lead to activation of RBCs that is similar to platelet activation and subsequently enhance the binding of RBCs and fibrinogen. At the time of experiment a suitable positive control such as platelets was not available to test the sensitivity of the assay.

For fibrinogen-RBC binding in solution by flow cytometry, AlexaFluor^{488}-labelled recombinant fibrinogen was required. Labelling the fibrinogen by itself caused a

respectful amount of fibrinogen to be lost and the concentration of the labelled recombinant fibrinogen was lower than unlabelled ones. This limited the amount of available fibrinogen even more than what was available for the other methods. The flow cytometry experiment was performed by two different ways; firstly by using haematocrit as the measurement tool for the amount of RBCs required for the experiment. Secondly by counting RBCs in a step to improve the binding signal between fibrinogen and RBCs. Repeating the experiment was not possible for all the mutants because some of them were not available for the updated method of flow cytometry.

One of the most important limiting factors in this project was the availability of volunteers for blood donation. The initial plan of the project was to study the effect of the mutations on binding between fibrinogen and RBCs on different blood Rh-groups, but this was omitted because of the difficulty of getting volunteers.

Some other techniques were proposed to be used in this project, but the nature of the RBC prevented us from proceeding with these techniques. RBCs were found to be difficult to be processed in the lab; for example Carr and Hardin, mentioned that clots made with RBCs were difficult to be studied for their structure. For light scattering the gels of whole blood were too turbid and electron microscope showed two dimensional images instead of three dimensional networks, which made the measurements difficult to perform (Carr and Hardin, 1987). Gersh *et al* had confirmed this difficulty of processing the RBCs in clots by describing their incorporation in the clot as uncontrollable and variable. They added that presence of RBCs in the clot would interfer with laboratory techniques such as turbidity (Gersh et al., 2009).

8.5 Clinical relevance and Future direction

Red blood cells interactions with fibrinogen are potentially a very important area of research due to the effect this binding may have on erythrocyte hyper-aggregation with major implications for cardiovascular disease and thrombus formation (Brust et al., 2014). Working on the binding to find the exact site of fibrinogen interaction on the RBCs can be a therapeutic target to find an inhibitor

for the integrin site on RBCs that resembles the effect of the eptifibatide inhibition on platelet integrin $\alpha_{IIb}\beta_3$. The new RBC integrin inhibitor would be able to block the binding between RBCs and fibrinogen and subsequently decrease the tendency of thrombosis.

Proceeding with the study of the binding to specify the site of interaction both in fibrinogen and RBC would be of great benefit. To find the binding site on fibrinogen and based on the results presented in this thesis, the Aα-95-98 RGD should be excluded in relation to RBC binding and the attention should be focused on the other two sites of Aα-272-274 RGD and γC-12 dodecapeptide. However, it was clearly established that mutating the Aα-95-98 RGDF site changed the clot structure, which may still be a cause of thrombosis or bleeding that may be taken into account for any naturally occurring mutations in this sequence.

In conclusion, Aα-95-98 RGDF has no obvious effect on the binding between red blood cells and fibrinogen, or platelets and fibrinogen. Nonetheless, mutations at this site changed clot structure and polymerisation profile, as well as the susceptibility to lysis. These alterations may be of importance in the occurrence and risk of thrombosis and consequently cardiovascular disease, so should be taken into account in the diagnosis of suspected thrombosis with no clear cause, which might be achieved by *in vivo* studies in mice.

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Chapter 9 Appendices

Appendix A Sequencing

>pMLP+FbgAa WT with AA to be mutated

CATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGGGGTGGAGTTTGTGAC
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Key of the colouring primers

MiniPrep sequencing primer

GATTCAGACTGGCCCTTCTG

MaxiPrep sequencing primer

Forward:

AaF1: TTCTCATTCGTTGACCACTAATATA

AaF3: GATGAAGCTGCCTTCTTCGA

pMLP-F1: AAGTGTGGCGGAACACATG

pMLP-F3: CCTTTCTCTCCACAGGTGTC

F4: TGCTGGACACTGGACTTCTG

F5: AGTGGTGACCTCCGAAGATG

Reverse:

pMLP-R1: TTGTTGTTAACTTGTTTATTGCAGC

R5: CATCTTCGGAGGTCACCACT

R6: AGCTCTTGCTTTCAAATGTGG

AGA: Arginine (R)

GGC: Glycine (G)

GAT: Aspartate (D)

TTT: Phenylalanine (F)

ATG: Start codon

TAG: Stop codon

>pMLP+FbgAa R95E

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TTGTCCAAACTCATCA

GAA is the mutated Glutamate (E).

>pMLP+FbgAa R95Q

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CAA is the mutated Glutamine (Q).

>pMLP+FbgAa G96V

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GTC is the mutated Valine (V).

>pMLP+FbgAa D97K

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AAA is the mutated Lysine (K).

>pMLP+FbgAa D97N

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ATT is the mutated Asparagine (N)

>pMLP+FbgAa F98I

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T**ATT**TCCTCAGCCAATAACCGTGATAATACCTACAACCGAGTGTCAGAGGATCTGAGAAGCAGAATTG
AAGTCCTGAAGCGCAAAGTCATAGAAAAAGTACAGCATATCCAGCTTCTGCAAAAAAATGTTAGGGCCCA
GTTGGTTGATATGAAACGACTGGAGGTGGACATTGATATTAAGATCCGATCTTGTCGAGGGTCATGCAGT
AGGGCTTTAGCTCGTGAAGTAGATCTGAAGGACTATGAAGATCAGCAGAAGCAACTTGAACAGGTCATTG
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GGTTCCCGGAAATTTTAAGAGCCAGCTTCAGAAGGTACCCCCAGAGTGGAAGGCATTAACAGACATGCCG
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CCGGATCAGAGACGGAAAGCCCCAGGAACCCTAGCAGTGCTGGAAGCTGGAACTCTGGGAGCTCTGGACC
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CTGGACCTCTGAGAGCTCTGTATCTGGTAGTACTGGACAATGGCACTCTGAATCTGGAAGTTTTAGGCCA
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GCAATTCTGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACC
TCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGG
TTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGT
TTGTCCAAACTCATCA

ATT is the mutated Isoleucine (I)

Appendix B Ethics

UNIVERSITY OF LEEDS RESEARCH ETHICS COMMITTEE APPLICATION FORM [1]

UNIVERSITY OF LEEDS

Please read each question carefully, taking note of instructions and completing all parts. If a question is not applicable please indicate so. The superscripted numbers refer to sections of the guidance notes, available at www.leeds.ac.uk/ethics. Where a question asks for information which you have previously provided in answer to another question, please just refer to your earlier answer rather than repeating information.

Research ethics training courses: http://www.sddu.leeds.ac.uk/sddu-research-ethics-courses.html

To help us process your application enter the following reference numbers, if known and if applicable:

Ethics reference number:	**HSLTLM12045**
Grant reference and/ or student number:	

PART A: Summary

A.1 Which Faculty Research Ethics Committee would you like to consider this application?[2]

- ○ Arts and PVAC (PVAR)
- ○ Biological Sciences (BIOSCI)
- ○ ESSL/ Environment/ LUBS (AREA)
- ○ MaPS and Engineering (MEEC)
- ○ Medicine and Health (Please specify a subcommittee):
  - ○ Leeds Dental Institute (DREC)
  - ◉ Health Sciences/ LIGHT/ LIMM
  - ○ School of Healthcare (SHREC)
  - ○ Medical and Dental Educational Research (EdREC)
  - ○ Institute of Psychological Sciences (IPSREC)

A.2 Title of the research[3]

Role of different components on fibrin clot structure and function

A.3 Principal investigator's contact details[4]

Name *(Title, first name, surname)*	Prof Helen Philippou
Position	Professor
Department/ School/ Institute	Division of Cardiovascular and Diabetes Research LIGHT LICAMM

Faculty	Medicine
Work address *(including postcode)*	LIGHT Laboratories, Clarendon Way, University of Leeds, Leeds, LS2 9NL
Telephone number	01133437768
University of Leeds email address	h.philippou@leeds.ac.uk

Name *(Title, first name, surname)*	Prof Robert Ariëns
Position	Professor
Department/ School/ Institute	Division of Cardiovascular and Diabetes Research, LIGHT LICAMM
Faculty	Medicine
Work address *(including postcode)*	LIGHT Laboratories, Clarendon Way, University of Leeds, Leeds, LS2 9NL
Telephone number	01133437734
University of Leeds email address	r.a.s.ariens@leeds.ac.uk

A.4 Purpose of the research:[5] (Tick as appropriate)

☑ Research

☐ Educational qualification: ***Please specify:*** _____

☐ Educational Research & Evaluation[6]

☐ Medical Audit or Health Service Evaluation[7]

☐ Other

A.5 Select from the list below to describe your research: (You may select more than one)

☐ Research on or with human participants

☐ Research with has potential significant environmental impact.[8] If yes, please give details:

_____

☐ Research working with data of human participants

- ☐ New data collected by questionnaires/interviews
- ☐ New data collected by qualitative methods
- ☐ New data collected from observing individuals or populations
- ☐ Research working with aggregated or population data
- ☐ Research using already published data or data in the public domain

☑ Research working with human tissue samples[9]

A.6 Will the research involve any of the following:[10] (You may select more than one)

If your research involves any of the following an application must be made to the National Research Ethics Service (NRES) via IRAS www.myresearchproject.org.uk as NHS ethical approval will be required. There is no need to complete any more of this form. Contact governance-ethics@leeds.ac.uk for advice.

- ☐ Patients and users of the NHS (including NHS patients treated in the private sector)[11]
- ☐ Individuals identified as potential participants because of their status as relatives or carers of patients and users of the NHS
- ☐ Research involving adults in Scotland, Wales or England who lack the capacity to consent for themselves[12]
- ☐ A prison or a young offender institution in England and Wales (and is health related)[14]
- ☐ Clinical trial of a medicinal product or medical device[15]
- ☐ Access to data, organs or other bodily material of past and present NHS patients[9]
- ☐ Use of human tissue (including non-NHS sources) where the collection is not covered by a Human Tissue Authority licence[9]
- ☐ Foetal material and IVF involving NHS patients
- ☐ The recently deceased under NHS care
- ☑ None of the above

You must inform the Research Ethics Administrator of your NRES number and approval date once approval has been obtained.

If the University of Leeds is not the Lead Institution, or approval has been granted elsewhere (e.g. NHS) then you should contact the local Research Ethics Committee for guidance. The UoL Ethics Committee need to be assured that any relevant local ethical issues have been addressed.

A.7 Will the research involve NHS staff recruited as potential research participants (by virtue of their professional role) or NHS premises/ facilities?

☐ Yes ☑ No

If yes, ethical approval must be sought from the University of Leeds. Please note that NHS R&D approval is needed in addition, and can be applied for concurrently: www.myresearchproject.org.uk.

Contact governance-ethics@leeds.ac.uk for advice.

A.8 Will the participants be from any of the following groups? (Tick as appropriate)

- ☐ Children under 16[16]
- ☐ Adults with learning disabilities[12]
- ☐ Adults with other forms of mental incapacity or mental illness
- ☐ Adults in emergency situations
- ☐ Prisoners or young offenders[14]
- ☐ Those who could be considered to have a particularly dependent relationship with the investigator, eg members of staff, students[17]
- ☐ Other vulnerable groups
- ☑ No participants from any of the above groups

Please justify the inclusion of the above groups, explaining why the research cannot be conducted on non vulnerable groups.

A Criminal Record Bureau (CRB) check will be needed for researchers working with children or vulnerable adults (see www.crb.gov.uk and http://store.leeds.ac.uk/browse/extra_info.asp?modid=1&prodid=2162&deptid=34&compid=1&prodvarid=0&catid=243)

A.9 Give a short summary of the research[18]

*This section must be completed in **language comprehensible to the lay person**. Do not simply reproduce or refer to the protocol, although the protocol can also be submitted to provide any technical information that you think the ethics committee may require. This section should cover the main parts of the proposal.*

People at risk of cardiovascular problems, e.g. heart attack, can have differences in their components (proteins) of the blood clotting system. These differences can be beneficial, detrimental, or not have any effect. We hope to understand the contribution of these differences to cardiovascular problems, and thus aid future drug development against these diseases. The purpose of this study is to investigate the effect of different components (proteins) on blood coagulation, for instance factor XIII which is known to strengthen the blood clot. This will involve adding the proteins to the donated sample, and using standard clotting assays (e.g. aPTT, PT, turbidity, thrombin generation, ROTEM, platelet aggregometry, permeation, confocal and electron microscopy imaging, binding assay; protocols attached) to determine how much longer the sample takes to clot compared to whole blood/plasma/cells/proteins without any extra components.

Each volunteer will be asked to donate up to 54 mls of blood in a single visit to a private patient room in the LIGHT laboratories, with a maximum of 2 visits per week. The blood will then either

- be used as is and tested on the same day, except where preparation of the sample for testing requires multiple days. In this case, samples will be prepared as expediently as possible and discarded once testing is complete. This will be within a maximum of 3 days. A log will be taken of each sample together with the timeline of the test(s) being performed and the date the cellular specimen is discarded.
- separated to isolate biological cells (e.g. platelets, red blood cells) for testing. These will be tested and then discarded on the same day, except where preparation of the sample for testing requires multiple days. In this case, samples will be prepared as expediently as possible and discarded once testing is complete. This will be within a

maximum of 3 days. A log will be taken of each sample together with the timeline of the test(s) being performed and the date the cellular specimen is discarded.

- Separating the donated blood into platelet poor plasma (decellularised) and either testing it on the same day or storing it for future use. To produce decellularised plasma, samples will be centrifuged at room temperature for 20 mins at 3,000xg. The cells are pelleted by this centrifugation and we carefully pipette the plasma from the top half of the tube). Regular blood smears will be performed to confirm that the plasma does not contain any cells.
- used to isolate a particular protein from the donated blood (e.g. coagulation or complement proteins which are of interest to our research group) and either testing it on the same day or storing it for future use
- separated into plasma and combined with other volunteers donated plasma to produce a normal pool and stored for future use

For the individual samples, we anticipate getting up to 100 volunteers per year.

For the normal pool, we will aim to get up to 25 volunteers to reduce person-to-person variability and anticipate doing this annually.

In addition, volunteers may be asked to complete a questionnaire detailing basic information (age, height, weight, gender, medical history of cardiovascular disease or diabetes, smoking history, exercise and dietary habits, plus any agents taken from a list of known agents that may alter blood coagulation).

A.10 What are the main ethical issues with the research and how will these be addressed?[19]

Indicate any issues on which you would welcome advice from the ethics committee.

People who have a dependent relationship with the investigators will not be recruited.

Volunteers may be asked to complete a questionnaire of basic information (age, height, weight, gender, medical history of cardiovascular disease or diabetes, smoking history, exercise and dietary habits, plus any agents taken from a list of known agents that may alter blood coagulation). They can choose not to complete any or all of this questionnaire. Volunteer name and contact details will be kept solely on the consent form and an encrypted file on a private folder on the secure university network. A volunteer ID code will be used as the only link between the volunteer name and contact details, and any stored samples and questionnaires. Volunteer consent forms and questionnaires will be stored in a locked cupboard on university premises. This will allow the researchers to go back to the same volunteer on subsequent occasions if required. Information from the questionnaire may be input into an encrypted database which will be stored in a private folder on secure university network.

PART B: About the research team

B.1 To be completed by students only[20]

Name *(Title, first name, surname)*	
Qualification working towards (eg Masters, PhD)	
Supervisor's name (Title, first name, surname)	
Department/ School/ Institute	
Faculty	
Work address (including postcode)	
Supervisor's telephone number	
Supervisor's email address	
Module name and number (if applicable)	

B.2 Other members of the research team (eg co-investigators, co-supervisors) [21]	
Name *(Title, first name, surname)*	Dr Emma Hethershaw
Position	Post-doctoral research fellow

Department/ School/ Institute	Division of Cardiovascular and Diabetes Research, LIGHT LICAMM
Faculty	Medicine
Work address *(including postcode)*	LIGHT Laboratories, Clarendon Way, University of Leeds, Leeds, LS2 9NL
Telephone number	01133437743
Email address	e.hethershaw@leeds.ac.uk

Name *(Title, first name, surname)*	Dr Cedric Duval
Position	Post-doctoral research fellow
Department/ School/ Institute	Division of Cardiovascular and Diabetes Research, LIGHT LICAMM
Faculty	Medicine
Work address *(including postcode)*	LIGHT Laboratories, Clarendon Way, University of Leeds, Leeds, LS2 9NL
Telephone number	01133433235
Email address	c.duval@leeds.ac.uk

Part C: The research

C.1 What are the aims of the study?[22] (Must be in language comprehensible to a lay person.)

The aim of this study is to identify the effect, if any, of different components (proteins) on blood clot structure and function.

C.2 Describe the design of the research. Qualitative methods as well as quantitative methods should be included. (Must be in language comprehensible to a lay person.)

It is important that the study can provide information about the aims that it intends to address. If a study cannot answer the questions/ add to the knowledge base that it intends to, due to the way that it is designed, then wasting participants' time could be an ethical issue.

A number of different components (proteins) will be tested to see what effect they have on blood clot structure and function. This will be achieved by mixing different components with the donated whole blood, plasma, purified blood cells, or purified blood proteins and using standard clotting assays (e.g. aPTT, PT, turbidity, thrombin generation, ROTEM, platelet aggregometry, permeation, confocal and electron microscopy imaging, binding assay; protocols attached) to determine any differences in clotting time, rate of clotting, structure of the clot formed, and the breakdown of the clot.

Each volunteer will be asked to donate up to 54 mls of blood in a single visit to a private patient room in the LIGHT laboratories, with a maximum of 2 visits per week. Volunteers blood samples will either

- be used as is and tested on the same day, except where preparation of the sample for testing requires multiple days. In this case, samples will be prepared as expediently as possible and discarded once testing is complete. This will be within a maximum of 3 days. A log will be

taken of each sample together with the timeline of the test(s) being performed and the date the cellular specimen is discarded.

- separated to isolate biological cells (e.g. platelets, red blood cells) for testing. These will be tested and then discarded on the same day, except where preparation of the sample for testing requires multiple days. In this case, samples will be prepared as expediently as possible and discarded once testing is complete. This will be within a maximum of 3 days. A log will be taken of each sample together with the timeline of the test(s) being performed and the date the cellular specimen is discarded.
- Separating the donated blood into platelet poor plasma (decellularised) and either testing it on the same day or storing it for future use. To produce decellularised plasma, samples will be centrifuged at room temperature for 20 mins at 3,000xg. The cells are pelleted by this centrifugation and we carefully pipette the plasma from the top half of the tube). Regular blood smears will be performed to confirm that the plasma does not contain any cells.
- used to isolate a particular protein from the donated blood (e.g. coagulation or complement proteins which are of interest to our research group) and either testing it on the same day or storing it for future use
- separated into plasma and combined with other volunteers donated plasma to produce a normal pool and stored for future use

Additionally, volunteers may be asked to complete a questionnaire of basic information (age, height, weight, gender, any known cardiovascular disease, smoking history, exercise and dietary habits) which may be used to check for correlations with laboratory data obtained.

C.3 What will participants be asked to do in the study?[23] *(e.g. number of visits, time, travel required, interviews)*

Provide a maximum of 54 mls of blood in a single visit to a private patient room in the LIGHT laboratories, with a maximum of 2 visits per week. Additionally, the volunteer may be asked to provide basic information including age, height, weight, gender, medical history of cardiovascular disease or diabetes, smoking history, exercise and dietary habits, plus any agents taken from a list of known agents that may alter blood coagulation (questionnaire attached). This

questionnaire will not have the volunteers name or contact details on it, only the volunteer ID code. The only places linking the volunteer name and contact details with the volunteer ID code will be the paper consent forms, which will be kept in a locked cupboard in a locked office on university premises, and an encrypted file on a private folder on the secure university network. These will be discarded once no longer required, or if the respondent requested that they no longer wanted to take part.

C.4 Does the research involve an international collaborator or research conducted overseas:[24]

(Tick as appropriate)

☐ Yes ☑ No

If yes, describe any ethical review procedures that you will need to comply with in that country:

Describe the measures you have taken to comply with these:

Include copies of any ethical approval letters/ certificates with your application.

C.5 Proposed study dates and duration

Research start date (DD/MM/YY): 14/02/13 Research end date (DD/MM/YY): 13/02/18

Fieldwork start date (DD/MM/YY): _____ Fieldwork end date (DD/MM/YY): _____

C.6. Where will the research be undertaken? (i.e. in the street, on UoL premises, in schools)[25]

The LIGHT laboratories, University of Leeds

RECRUITMENT & CONSENT PROCESSES

How participants are recruited is important to ensure that they are not induced or coerced into participation. The way participants are identified may have a bearing on whether the results can be generalised. Explain each point and give details for subgroups separately if appropriate.

C.7 How will potential participants in the study be:

(i) identified?

N/A

(ii) approached?

N/A

(iii) recruited?[26]

Volunteers will be found by putting a notice up in the building and asking people to contact the relevant member of the research team (e.g. Cedric Duval or Emma Hethershaw). An item will also be included the weekly institute newsletter

C.8 Will you be excluding any groups of people, and if so what is the rationale for that?[27]

Excluding certain groups of people, intentionally or unintentionally may be unethical in some circumstances. It may be wholly appropriate to exclude groups of people in other cases

N/A

C.9 How many participants will be recruited and how was the number decided upon?[28]

It is important to ensure that enough participants are recruited to be able to answer the aims of the research.

For the individual samples, we anticipate getting up to 100 volunteers per year, based on predicted experimental requirements.

For the normal pool, we will aim to get up to 25 volunteers to reduce person-to-person variability and anticipate doing this annually, based on usage of previous normal pools.

If you have a formal power calculation please replicate it here.

N/A

Remember to include all advertising material (posters, emails etc) as part of your application

C10 Will the research involve any element of deception?[29]

If yes, please describe why this is necessary and whether participants will be informed at the end of the study.

No

C.11 Will informed consent be obtained from the research participants?[30]

☑ Yes ☐ No

If yes, <u>give details</u> of how it will be done. Give details of any particular steps to provide information (in addition to a written information sheet) e.g. videos, interactive material. If you are not going to be obtaining informed consent you will need to justify this.

A verbal description of what is required (i.e. up to 54 mls of blood and basic information on the volunteer questionnaire) will be discussed with the volunteers with a description of what their blood will be used for. A written information sheet will also be provided containing the same information.

If ***participants are to be recruited from any of potentially vulnerable groups, give details of extra steps taken to assure their protection. Describe any arrangements to be made for obtaining consent from a legal representative.***

Not applicable

Copies of any written consent form, written information and all other explanatory material should accompany this application*. The information sheet should make explicit that participants can withdrawn from the research at any time, if the research design permits.*

Sample information sheets and consent forms are available from the University ethical review webpage at http://researchsupport.leeds.ac.uk/index.php/academic_staff/good_practice/ethical_review_process/university_ethical_review-1.

C.12 Describe whether participants will be able to withdraw from the study, and up to what point (eg if data is to be anonymised). If withdrawal is not possible, explain why not.

Volunteers are able to withdraw from the study at any point if they are donating individual samples. Those volunteers who donate blood to be spun down to

plasma and pooled with other people's plasma and frozen will not be able to withdraw from the study once they have donated the blood. This will be explained to them at the time.

C.13 How long will the participant have to decide whether to take part in the research?[31]

It may be appropriate to recruit participants on the spot for low risk research; however consideration is usually necessary for riskier projects.

Participants will be given 2 weeks to decide if they wish to participate.

C.14 What arrangements have been made for participants who might not adequately understand verbal explanations or written information, or who have special communication needs?[32] *(e.g. translation, use of interpreters etc. It is important that groups of people are not excluded due to language barriers or disabilities, where assistance can be given.)*

Not applicable

C.15 Will individual or group interviews/ questionnaires discuss any topics or issues that might be sensitive, embarrassing or upsetting, or is it possible that criminal or other disclosures requiring action could take place during the study (e.g. during interviews or group discussions)?[33]

The information sheet should explain under what circumstances action may be taken.

☐ Yes ☑ No *If yes, give details of procedures in place to deal with these issues.*

C.16 Will individual research participants receive any payments, fees, reimbursement of expenses or any other incentives or benefits for taking part in this research?[34]

☐ Yes ☑ No

If Yes, please describe the amount, number and size of incentives and on what basis this was decided.

RISKS OF THE STUDY

C.17 What are the potential benefits and/ or risks for research participants?[35]

There are no other benefits from being involved in this study than the knowledge that the provided sample will further scientific research in

haemostasis and thrombosis, and ultimately may help to develop better screening assays and new therapeutics. There is a slight risk of bruising from the puncture site of blood being drawn.

C.18 Does the research involve any risks to the researchers themselves, or people not directly involved in the research? *Eg lone working*[36]

☐ Yes ☑ No

If yes, please describe:

_____

Is a risk assessment necessary for this research?

NB: Risk assessments are a University requirement for all fieldwork taking place off campus. For guidance contact your Faculty Health and Safety Manager or visit http://www.leeds.ac.uk/safety/fieldwork/index.htm.

☐ Yes ☑ No If yes, please include a copy of your risk assessment form with your application.

DATA ISSUES

C.19 Will the research involve any of the following activities at any stage (including identification of potential research participants)? (Tick as appropriate)

☐ Examination of personal records by those who would not normally have access

☐ Access to research data on individuals by people from outside the research team

☐ Electronic transfer of data

☐ Sharing data with other organisations

☐ Exporting data outside the European Union

☐ Use of personal addresses, postcodes, faxes, e-mails or telephone numbers

☐ Publication of direct quotations from respondents

☐ Publication of data that might allow identification of individuals to be identified

☐ Use of audio/visual recording devices

☐ FLASH memory or other portable storage devices

Storage of personal data on or including any of the following:

☐ Manual files

☐ Home or other personal computers

☐ Private company computers

☐ Laptop computers

C.20. How will the research team ensure confidentiality and security of personal data? E.g. anonymisation procedures, secure storage and coding of data.[37] You may wish to refer to the data protection and research webpage.

The questionnaire is confidential and anonymous and the generic nature of the information gathered does not allow for individual respondents to be identified. The only issue here relates to the option for respondents to provide their contact details on the consent form if they volunteer for subsequent blood donations. Volunteer name and contact details will be kept solely on the consent form and an encrypted file on a private folder on the secure university network. Contact details are purely to access the volunteers for subsequent blood donations and will not be used in conjunction with analysing any questionnaire or laboratory data. Paper questionnaires and consent forms will be kept in a locked cupboard in a locked office on secured university premises. A volunteer ID code will be used as the only link between the volunteer name and contact details, and any stored samples and questionnaires.

The nature of donating the blood means that the responses are not anonymous to the researcher, but procedures have been put in place to ensure the confidentiality of the individual.

C.21 For how long will data from the study be stored? Please explain why this length of time has been chosen.[38]

RCUK guidance states that data should normally be preserved and accessible for ten years, but for some projects it may be 20 years or longer.

***Students**: It would be reasonable to retain data for at least 2 years after publication or three years after the end of data collection, whichever is longer.*

Records will be kept for a minimum of 10 years, in accordance with RCUK guidance.

_10 years, _0 months

CONFLICTS OF INTEREST

C.22 Will any of the researchers or their institutions receive any other benefits or incentives for taking part in this research over and above normal salary or the costs of undertaking the research?[39]

☐ **Yes** ☑ **No**

If yes, indicate how much and on what basis this has been decided

_____

_____

C.23 Is there scope for any other conflict of interest?[40] *For example will the research funder have control of publication of research findings?*

☐ Yes ☑ No ***If yes, please explain***

_____

C.24 Does the research involve external funding? (Tick as appropriate)

☑ Yes ☐ No ***If yes, what is the source of this funding?*** Funding is primarily obtained from the Medical Research Council, British Heart Foundation and the Wellcome Trust

PART D: Declarations

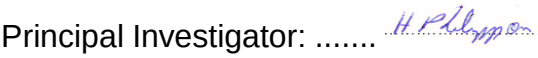
Declaration by Chief Investigators

1. The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.
2. I undertake to abide by the University's ethical and health & safety guidelines, and the ethical principles underlying good practice guidelines appropriate to my discipline.
3. If the research is approved I undertake to adhere to the study protocol, the terms of this application and any conditions set out by the Research Ethics Committee.
4. I undertake to seek an ethical opinion from the REC before implementing substantial amendments to the protocol.
5. I undertake to submit progress reports if required.
6. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register when necessary with the appropriate Data Protection Officer.
7. I understand that research records/ data may be subject to inspection for audit purposes if required in future.
8. I understand that personal data about me as a researcher in this application will be held by the relevant RECs and that this will be managed according to the principles established in the Data Protection Act.
9. I understand that the Ethics Committee may choose to audit this project at any point after approval.

Sharing information for training purposes: Optional – please tick as appropriate:

☐ I would be content for members of other Research Ethics Committees to have access to the information in the application in confidence for training purposes. All personal identifiers and references to researchers, funders and research units would be removed.

Principal Investigator

Signature of Principal Investigator: .....  .....(This needs to be an actual signature rather than just typed. Electronic signatures are acceptable)

Print name: ..Helen Philippou Date: (dd/mm/yyyy): 17/11/2016

Supervisor of student research: I have read, edited and agree with the form above.

Supervisor's signature: ..... (This needs to be an actual signature rather than just typed. Electronic signatures are acceptable)

Print name: ..... Date: (dd/mm/yyyy): .....

Please submit your form **by email** to J.M.Blaikie@leeds.ac.uk or if you are in the Faculty of Medicine and Health FMHUniEthics@leeds.ac.uk. **Remember to include any supporting material** such as your participant information sheet, consent form, interview questions and recruitment material with your application.

Checklist:

- ❑ I have used layman's terms to describe my research (applications are reviewed by lay members of the committee as well).
- ❑ I have answered all the questions on the form, including those with several parts (refer to the guidance if you're not sure how to answer a question or how much detail is required)
- ❑ I have included any relevant supplementary materials such as
    - ❑ Recruitment material (posters, emails etc)
    - ❑ Sample participant information sheet
    - ❑ Sample consent form. Include different versions for different groups of participants eg for children and adults.
- ❑ If I am not going to be using participant information sheets or consent forms I have explained why not and how informed consent will be otherwise obtained.
- ❑ If you are a student have you discussed your application with your supervisor and are they satisfied that you have completed the form correctly? (This will speed up your application).
- ❑ I have submitted a signed copy of my application. (If you are a student your supervisor also needs to sign the form).

Role of different components on fibrin clot structure and function

Volunteer Information Sheet

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it if you wish. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Purpose of the study
People at risk of cardiovascular problems, e.g. heart attack, can have differences in their components (proteins) of the blood clotting system. These differences can be beneficial, detrimental, or not have any effect. We hope to understand the contribution of these differences to cardiovascular problems, and thus aid future drug development against these diseases.

Why have I been chosen?
You have been chosen because you are between 18 and 70 years old and are generally healthy.

Do I have to take part?
It is up to you whether or not to take part. If you do decide to participate, you will be given this information sheet to keep, asked to sign a consent form, may be asked to give telephone or email address (so we can contact you again asking if you would be willing to provide subsequent blood donations), and may be asked to complete a questionnaire detailing basic information. Completing any part of this questionnaire is voluntary. You do not have to provide any information if you do not want to. Your answers will be treated in confidence and will not be identifiable. We will hold your information securely in accordance with the Data Protection Act (1998). Your name and contact details (if you choose to provide these) will be kept solely on the consent form. Your volunteer ID code will be the only link between your name and contact details, and any stored samples and questionnaires. If you do decide to take part, you are still free to withdraw at any time and without giving a reason. If you do decide to participate, your blood sample will either:

- be tested within 6 hours of being taken and any remaining discarded after 6 hours
- separated into different components (e.g. plasma, cells, proteins) and either tested on the same day or stored for future use

If you do decide to participate, it is accepted that your sample is a gift and may be retained for use in future ethically approved research.

What will happen to me if I take part?
The study will take approximately 15 minutes of your time on one or more occasions when you will be asked to come to the LIGHT laboratories in the University of Leeds where you will see one of our research nurses or Dr Hethershaw. Up to 54 mls of blood (which is the equivalent of about 4 tablespoons) will be taken from a vein on the inside of your arm. Additionally, you may be asked to complete a questionnaire detailing basic information.

What will happen to any samples I give?
The blood sample provided will be labelled with your volunteer ID code only so that you can not be identified. The whole blood will then either:

- be used as is and tested on the same day
- separated into different components (e.g. plasma, cells, proteins) and either tested on the same day or stored for future use

Standard blood clotting assays will be used to test the effects of the different components of blood clotting system with your sample. Any stored samples will also be labelled with your volunteer ID code as the only identifier and so you can not be identified.

What are the possible disadvantages and risks of taking part?
Giving a blood sample may be uncomfortable and there is a slight risk of bruising from the needle piercing the vein to take the blood.

What is there is a problem?
If you have a concern about any aspect of this study, you should contact one of the researchers who will do their best to answer your questions. Our contact numbers are at the end of this information sheet. If you are harmed by taking part in this study, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for this.

Will my taking part in this study be kept confidential?
All information which is collected about you during the course of this research will be kept strictly confidential.

Contact Details
If you require any further information please contact us on the telephone numbers below. Thank-you for reading this information sheet and for considering participating in the research.

Dr Emma Hethershaw
0113 343 7743
e.hethershaw@leeds.ac.uk

Julie Bailey – Research Nurse
0113 343 7702

Role of different components on fibrin clot structure and function

Questionnaire

Volunteer ID code	
Gender	
Age	
Height (cm)	
Weight (kg)	
Medical history of cardiovascular disease or diabetes	
Smoking history	
Exercise habits per week (where your heart rate is elevated)	None <1 hour 1-5 hours >5 hours
Eating habits	Poor Mild Moderate Healthy Very Healthy
Intense physical exercise in last 24 hours	No Yes
Any agents from table 1?	

Completing any part of this questionnaire is voluntary. You do not have to provide any information if you do not want to. Your answers will be treated in confidence and will not be identifiable. We will hold your information securely in accordance with the Data Protection Act (1998).

TABLE 1

Cyclo-oxygenase (COX)-1 inhibitors (irreversible)

Aspirin and all proprietary or over-the-counter preparations containing acetylsalicylic acid.

COX-1 and COX-2 inhibitors (reversible) (Nonsteroidal anti-inflammatory drugs (NSAIDs)

Ibuprofen
Indomethacin, naproxen
Mefenamic acid

Inhibitors of Platelet Receptors

Abciximab, tirofiban, eptifibatide (αIIbβ3)
Ticlopidine, clopidogrel, prasugrel (irreversible), cangrelor (reversible), ticagrelor (reversible) (P2Y12)

Phosphodiesterase Inhibitors

Dipyridamole
Cilostazole

Anticoagulants

Heparinoids, vitamin K antagonists and direct thrombin inhibitors may indirectly influence platelet function due to inhibition of thrombin.

Cardiovascular Agents

β-adrenergic blockers (propranolol)
Vasodilators (nitroprusside, nitroglycerin)
Diuretics (furosemide)
Calcium channel blockers

Antimicrobials

B-lactams (penicillins, cephalosporins)
Amphotericin (antifungal)
Hydroxychloroquine (antimalarial)
Nitrofurantoin

Chemotherapeutic agents

Asparaginase
Plicamycin
Vincristine

Miscellaneous

Clofibrate
Dextrans
Guaifenesin (expectorant)
Radiographic contrast media
Food/Herbs (at high concentrations)
Alcohol

Caffeine (methylxanthine)
Cumin
Dong quai

Psychotropics and Anaesthetics

Tricyclic antidepressants (imipramine)
Phenothiazines (chloropromazine)
Local and general anaesthesia (halothane)
Thrombolytic Agents
Streptokinase
Urokinase
Tissue Plasminogen Activator (TPA)
Benzodiazepines (diazepam, temazepam)

Fenugreek
Garlic, onion, ginger

Ginseng
Fish Oil
Tamarind
Turmeric
Willow
Vitamins C and E
Black Tree Fungus ("Chinese mushroom")

Consent Form

Role of different components on fibrin clot structure and function Blood Donation

Volunteer ID code:
Date sample taken: **Date test(s) performed:**
Date sample discarded:

	Please initial box
1. I confirm that I have read and understood the volunteer information sheet for the above study and have had the opportunity to ask questions.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without any legal rights being affected.	☐
3. I agree that my sample is a gift and may be retained for use in future ethically approved research.	☐
4. I understand that any samples and questionnaires will be labelled with my volunteer code only so that I can not be identified from them.	☐

Name of volunteer Date
Signature

Contact details (optional)

Name of person taking consent Date
Signature

Healthy Volunteers Wanted

Role of different components on fibrin clot structure and function

Volunteers are wanted for a study to the red blood cells binding to fibrinogen. We are looking for adults of all ages who are generally healthy. The commitment will take between 10-15 minutes and involves donating approximately 10 mls of blood in the LIGHT laboratories. You may also be asked to complete a questionnaire of basic information. This is voluntary.

If you feel you would be available for this study, or have any questions regarding the experiment, please contact Aliaa Sabban (umas@leeds.ac.uk) (0113 43) 31142.

This study has received ethical approval from the School of Medicine Research Ethics Committee, reference number HSLTLM12045.