Aspirin in diabetes mellitus:

mechanisms and clinical implications

Zeyad Shafiq Kurdee

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

University of Leeds
School of Medicine

March 2015

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Some of the work in Chapter 6 (flow cytometry technique) was done by the candidate in Professor Khalid Naseem's lab, Hull University with the help of Dr. Simba Magwenzi. Radioactive aspirin work was done with the help of my colleague Dr. Saad Alzahrani.

Acknowledgments

It is only right that the opening gambit of my thesis is to thank the person that has made all this possible, my supervisor Dr Ramzi Ajjan. I am indebted to him for allowing me to start this journey and for all his considerable support along the way. With Ramzi's guidance I have greatly increased my confidence in basic science, and research. It has been a privilege to work with him and I hope this is only the start of a long-term working relationship.

I am also grateful for my second supervisor Dr. Peter J Grant who was always providing invaluable advice and support throughout my project.

Numerous members of LICAMM laboratories have been instrumental in getting me this far and I am hugely thankful to Fladia, Natalie, Saad, Katie and Rhodri in helping me. I am also hugely grateful to those outside the LICAMM for their expertise most notably Professor Khalid Naseem and Dr.Simba Magwenzi from the University of Hull for their assistance in getting training in their lab on static platelet fibrinogen interaction technique, and in doing platelet fibrinogen binding by flow cytometry technique.

I would like to thank members of the Division of Cardiovascular and Diabetes Research with whom I spent a great time during my studies.

Finally, and most importantly, to my parents, and special thanks to my mother Haneefa, my wife Hanadi and my children Hanan, Tareq, Nada and Yousef. Thank you for your unfaltering love and support in every way possible and helping me to get to this stage in my career. I really could not have done it without you.

Abstract

Diabetes is characterised by increased activation of platelets and coagulation factors, contributing to the high risk of cardiovascular disease in these individuals.

Aspirin is a classic antiplatelet agent used for the protection from atherothrombotic disease. Clinical evidence suggests the efficacy of this agent is reduced in diabetes, by mechanisms that are not entirely clear. I hypothesise that the reduced clinical efficacy of aspirin in diabetes is related to a compromise in the effects of this agent on both the cellular and fluid phase of coagulation, secondary to elevated blood glucose levels.

Using a combination of ex vivo/in vivo aspirin experiments in patients with type 1 diabetes and healthy controls, I demonstrate reduced platelet inhibition by low aspirin concentration in diabetes, which appears to be related to both medium term as well as instantaneous hyperglycaemia. This suggests that current treatment strategies of administering aspirin once daily in diabetes are inadequate. Moreover, I show that the fibrinolytic properties of aspirin are reduced in diabetes, which may be related to reduce acetylation of one or more plasma protein other than fibrinogen. Finally, I describe a potential mechanism for aspirin, related to the effect of this agent on platelet-fibrinogen interaction, an effect that is also compromised in patients with diabetes.

In conclusion, my data indicate that both medium term and immediate hyperglycaemia modulate the effects of aspirin on the cellular and protein arm

of coagulation. Future work is required to understand the best glycaemic indices that optimise response to aspirin in diabetes. Moreover, additional studies are necessary to clarify the role of alternative dosing strategies for aspirin in patients with diabetes

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List of abbreviations used in the project report

Abbreviation	Definition
AA	Arachidonic acid
ASA	Acetyl salicylic acid
AU	Aggregation unit
AUC	Area under the curve
CaCl2	Calcium chloride
COX-1	Cyclo-oxygenase-1
EC	Endothelial cell
FVIII	Factor VIII
FX	Factor X
FXIIIa	Activated factor XIII
GP	Glycoprotein
GPCRs	G-protein-coupled receptor
IHD	Ischemic heart disease
LDL	Low density lipoprotein
MA	Maximum absorbance
mg	Milligram
Mg/l	Milligram per litre
mM	Millimolar
Mmol/l	Millimole per litre
mRNA	Messenger ribonucleic acid

NaCl	**Sodium chloride**
NO	**Nitric oxide**
PAI	**Plasmingen activator inhibitor**
PBS	**Phosphate buffer solution**
PPP	**Platelet poor plasma**
PRP	**Platelet rich plasma**
RGD	**Arg-LysAsp-x**
SDS-PAGE	**Sodium dodecyl sulphate-polyacrylamide gel electrophoresis**
T1DM	**Type 1 diabetes mellitus**
T2DM	**Type 2 diabetes mellitus**
tPA	**Tissue plasminogen activator**
TRAP	**Thrombin receptor activating peptide**
Tris	**Tris(hydoxymethyl) aminomethan**
TXA2	**Thromboxane A2**

Publications and presentations

Publications

Søs Neergaard-Petersen1,2; Anne-Mette Hvas3; Steen D. Kristensen1; Erik L. Grove1; Sanne B. Larsen1; Fladia Phoenix2; Zeyad Kurdee2; Peter J. Grant2; Ramzi A. Ajjan2. The influence of type 2 diabetes on fibrin clot properties in patients with coronary artery disease. Blood coagulation and fibrinolysis. September 4, 2014 112.6: 1142-1150.

Zeyad Kurdee, Rhodri King, Ramzi A. Ajjan. The fibrin network in diabetes: its role in thrombosis risk Polskie Archiwum Medycyny Wewnetrznej November 7, 2014: 124 (11).

Oral presentations

Aspirin treatment failure in diabetes: the role of glycaemia Kurdee.Z , Grant P, Ajjan R Leeds Institute Postgraduate Symposium, Leeds, 26th March 2013.

Diabetes modulates the fibrinolytic properties of aspirin without altering the platelet inhibitory action: a possible mechanism for aspirin treatment failure Z Kurdee, A Mamaniat, F Phoenix, P Rice, PJ Grant and RA Ajjan The sth Saudi Scientific International Conference, London, 11-14 October 2012.

Diabetes modulates the fibrinolytic properties of aspirin without altering the platelet inhibitory action: a possible mechanism for aspirin treatment failure XXIInd International Fibrinogen Workshop, Brighton, July 2012

Poster presentations

Molecular mechanisms for the reduced clinical efficacy of aspirin in diabetes mellitus Kurdee.Z , Grant P, Ajjan R
Leeds Institute Postgraduate Symposium, Leeds, March 2012

Diabetes modulates the fibrinolytic properties of aspirin without altering the platelet inhibitory actions: a possible mechanism for aspirin treatment failure Z Kurdee, A Mamaniat, F Phoenix, P Rice, RF Storey, PJ Grant and RA Ajjan

XXIV Congress of The international Society on Thrombosis and Haemostasis, Amestredam 29th Jun- 4th July 2013

Prize

Prize for the Best Oral Presentation at the Institute Postgraduate Symposium 26th March 2013.

Chapter 1

Introduction

1.1 Cardiovascular disease

Cardiovascular disease (CVD) remains a major cause of mortality and morbidity worldwide, a trend that is likely to continue in the near future (Franco et al., 2011). Coronary artery disease (CAD) is predicted to be the most common cause of death globally by 2020 (Murray and Lopez, 1997) despite efforts to control various risk factors (Padmanabhan et al., 2010). In the UK, 33% of death in 2008 was due to CVD(BHFreport, 2010), with an increased risk in some ethnic minorities, including South Asians (Wild and McKeigue, 1997). The higher risk in South Asians may be secondary to the higher prevalence of insulin resistance and diabetes (Dhawan et al., 1994), which contribute to vascular pathology.

An enhanced thrombotic milieu is associated with increased risk of CVD, and this has been the focus of many therapeutic agents aiming to reduce the thrombotic environment. This is discussed in details below (1.6).

1.2 Mechanisms of vascular pathology that enhance thrombosis risk

1.2.1 Atherosclerotic plaque formation

Atherosclerosis is an inflammatory disease affecting blood vessels. Endothelial denudation was proposed to be the first step in the atherosclerotic process (Ross and Glomset, 1973), but more recently endothelial dysfunction was found to be the key initial event. Endothelial dysfunction could be caused by a large number of factors including hyperglycaemia, elevated LDL, smoking and hypertension (Ross and Glomset, 1973, Ross, 1986, Ross, 1981).

Endothelial dysfunction occurs as a compensatory response to injury in order to maintain normal homeostatic properties of the blood vessel (Ross, 1999). With

prolonged endothelial dysfunction, cells become less tight, allowing LDL-cholesterol to diffuse into the vessel wall, which is subsequently transformed into atherogenic oxidised LDL (ox-LDL). Endothelial dysfunction also causes the expression of adhesion molecules, allowing the attachment of inflammatory cells and subsequent migration into the vessel wall. Migrated monocytes become macrophages, which take up ox-LDL and are then transformed into foam cells. A collection of foam cells forms the fatty streak, the earliest abnormality in the atherosclerotic process and which can be found at a very early age, as young as children (Stary et al., 1994, Napoli et al., 1997, Simionescu et al., 1986, Ajjan and Grant, 2006b).

The presence of fatty streaks creates an inflammatory reaction with inflammatory mediators, vasoactive molecules, cytokines and growth factors drawn into the site of lesion. The inflammatory infiltrate further results in migration and proliferation of smooth-muscle cells, which contribute to narrowing the lumen of the vessel. At this stage, the artery compensates by dilation in order to maintain the lumen of the artery and to ensure healthy blood flow(Glagov et al., 1987). If the inflammation continues, it increases migration of inflammatory cells (Jonasson et al., 1986, van der Wal et al., 1989, Ross, 1999) and the fatty streak is gradually transformed into the atherosclerotic plaque through the deposition of collagen (Libby, 2009). This results in the development of an advanced atherosclerotic lesion that is composed of a lipid core and a fibrous cap covering the lesion. In the late stages, the atherosclerotic lesion ruptures, exposing a prothrombotic core which activates platelets and coagulation factors resulting in the formation of an obstructive thrombus that

may block the lumen of the blood vessel, consequently causing organ damage (Ross, 1999).

1.2 Thrombus formation

Thrombus forms secondary to a complex interaction between the cellular arm of coagulation, represented by platelets (A), and the fluid phase of coagulation that includes a large number of plasma proteins (B).

1.2.3 Role of platelets in thrombus formation

Platelets play a key role in the formation and growth of thrombus. Platelets are anucleate cells produced by megakaryocytes in the bone marrow and released into the circulating blood at about 85 million cells per day. Around 70% of the platelets are present in circulating blood and 30% are stored in the spleen. The average size of platelets is 2-3 μm, which makes them the smallest component of circulating blood cells, and have a half-life of 7-10 days $(150\text{-}400)\times10^{9}$/L (Wong, 2013, Walsh et al., 2014, Smyth, 2010).

Platelets activation, adhesion, spreading and degranulation

The main role of platelet is to prevent bleeding by haemostatic thrombi formation. It is very important that a balance is maintained between platelet activation and inhibition, as dysregulation of this process may lead to inappropriate thrombus formationor conversely it may increase bleeding risk. Plateletsin blood arein their resting state and get activated following vascular injury (Li et al., 2010). Platelets undergo a very organised series of functional response (activation) starting with adhesion to the site of injury followed by spreading, release reaction, aggregation, exposure of procoagulant surface,

culminating in mature clot formation (Harrison, 2005). Platelet receptor glycoprotein GP or integrins such as $\alpha_{IIb}\beta3$ “fibrinogen receptor”, $\alpha_2\beta1$ “collagen receptor” and $\alpha_5\beta1$ “fibronectin receptor” play key roles in platelet adhesion and aggregation (Li et al., 2010).

Following vascular damage, the subendothelial tissue is exposed which contains collagen fibrils (Brass, 2000). Subsequently, collagen comes into contact with vWF, which then adheres to platelet glycoprotein receptor GP Ib/IX/V complex. As platelet primarily exist in a high shear stress environment and need to “settle” at the site of injury, vWF forms the so-called “flex bond” or “catch bond” with the receptor Gb Ib-IX (Kim et al., 2010).This slows down movement of platelets, allowing them to adhere to the vessel wall. Furthermore, platelet receptor GbVI directly binds to collagen leading to cellular activation, further ensuring firm adhesion.Also plateletsare activated by thrombin, which can maintain platelet activation at low concentrations. Thrombin is generated from prothrombin following activation of the coagulation cascade secondary to TF release from subendothelial tissue (Ruggeri and Landolfi, 2001).

During the adhesion step, platelet glycoprotein receptor (GP) interacts with extracellular matrix proteins which triggers a complex cascade of signals transmitted from platelet membrane into the cytoplasm, consequently leading to platelet activation. This process is referred to as outside–in signalling (Jurk and Kehrel, 2005). The signalling events occur following interactions between collagen and GPVI/GPIbα, Adenosine diphosphate (ADP), an organic molecule that involved in blood platelet activation and P2Y1/P2Y12, thrombin and PAR1/PAR4 and thromboxane A2 TxA2 and thromboxane receptor (TP) (Brass, 2010). As a result of activation of receptors GpIIb/IIIa and α2β1

(Harrison, 2005) and the presence of other platelet activators such as thrombin, ADP and TxA2, activated platelets change their shape secondary to conformational alterations of cytoskeleton proteins and the formation of pseudopodia in cell membrane, in which new actin filaments are formed (Offermanns, 2006, Fox, 1993). This helps the platelets to act as a “plug” at sites of injury as further detailed below.

Platelet adhesion and activation trigger platelet intracellular signalling and subsequent degranulation (Harrison, 2005). Platelets secrete granule components that modulate the coagulation process through a number of mechanisms. The dense granules in activated platelet are responsible for ADP production, one of the most important platelet agonist, particularly in the early stages of platelet aggregation (Gachet, 2001). Also, dense granules produce serotonin (5-hydroxtrryptamine, 5-HT), a vasoconstrictor that works together with ADP to increase platelet activation (Jurk and Kehrel, 2005). Furthermore, there is a calcium pool in dense tubular system which causes calcium fluxes that are necessary for platelet activation and aggregation (Nesbitt et al., 2003). The second type of platelet prothrombotic factors are stored in the α-granules that contain various proteins including vWF, vitronectin and some coagulation factors, including factors V, VII, XI, and XIII as well as protein C and PAI-1. The more advanced step in platelet activation is platelet aggregation which occurs rapidly and leads to the formation of platelet plug. With platelet receptor GPIIb/IIIa (α_{IIb}/β_3-integrin) having a key role in this process. Fibrinogen acts as a bridge between platelets by interacting with GPIIb/IIIa ($\alpha_{IIb}\beta3$) (Bennett et al., 2009). In the resting stage, $\alpha_{IIb}\beta3$is not able to bind to soluble ligands such as fibrinogen or vWF but following platelet stimulation,$\alpha_{IIb}\beta3$undergoes a

conformational change that enable binding to soluble ligands. Platelet-fibrinogen interaction is further discussed in detail in section 1.2.5 and 1.3.7

Adhesion and degranulation of platelet result in liberation of ADP, thromboxane, serotonin, calcium, which help in starting fibrin network formation by immobilising fibrinogen, through interaction via $\alpha_{IIb}\beta 3$ and activation of the coagulation cascade as detailed below. Finally, the orientation of platelet membrane phospholipids changes and negatively charged phospholipids such as phosphatidylserine are expressed on the surface of platelets. These negatively charged surfaces in turn increase binding activity of plasma coagulation factors such as V, VIIIa, IXa and Xa which are essential for thrombin production. The various phases of platelet activation are summarised in Figure 1.1

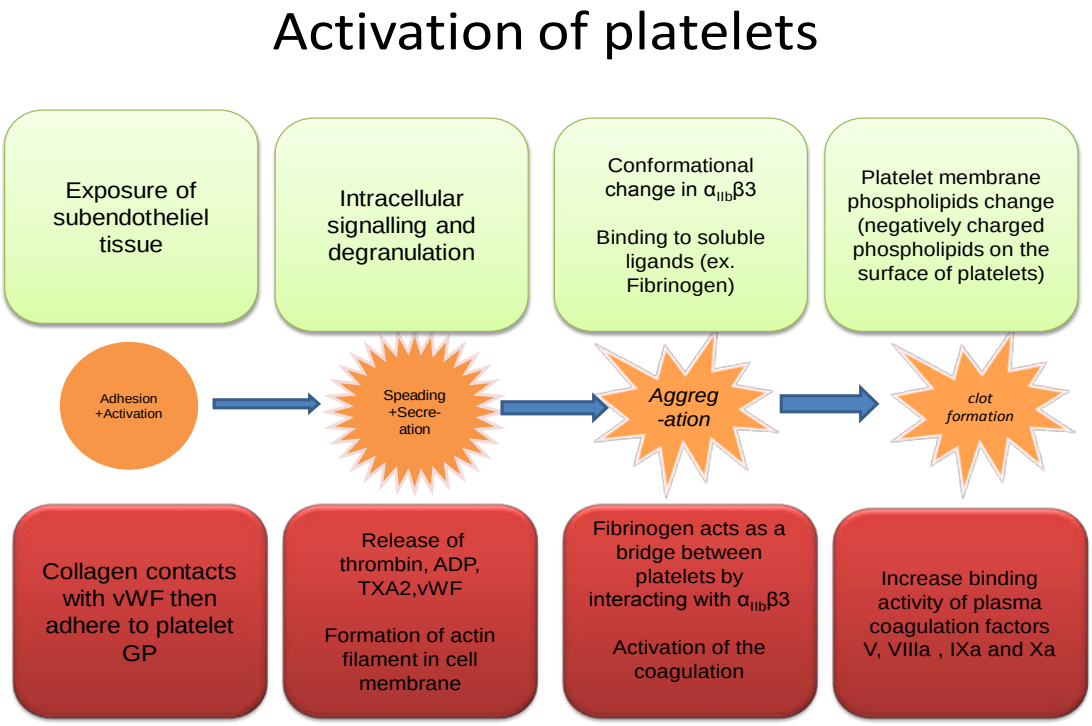

Figure 1.1 Platelet activation steps. At the beginning adhesion and activation, secondary to vascular damage, platelet comes in contact with exposedsubendothelial tissue that contains collagen fibrils. Platelet adheres to thevessel wall and subsequently spreading occurs together with release of thrombin, ADP, TXA2, vWF. In the aggregation step, $\alpha_{IIb}\beta3$undergoes a conformational change that enables binding to soluble ligandslike fibrinogenand then binding toanother platelet. Finally, the orientation of platelet membrane phospholipids changes and negatively charged phospholipids are expressed on the surface of platelets. This in turn increases binding activity of plasma coagulation factors such as V, VIIIa, IXa and Xa.

Platelet and coagulation

It is well recognised that platelets play a key role in controlling the coagulation process by producing two types of proteins:intrinsic platelet coagulation proteins and platelet surface-related proteins. As discussed before, platelet α granules are the main storage of intrinsic platelet coagulation proteins which are released when platelets are activated (Bevers et al., 1991). The formed platelet plug that produced in the aggregation stage is unstable until it is enforced by the fibrin

strands. The formation of fibrin depends on the conversion of prothrombin to thrombin.

Platelet signalling

On platelet membrane, there are different integrins: $\alpha_{IIb}\beta_3$ (fibrinogen receptor), $\alpha_2\beta_1$ (collagen receptor), and $\alpha_5\beta1$ (fibronectin receptor). The most important integrin in platelet is$\alpha_{IIb}\beta3$ that mediates the aggregation of platelets. The formation of stable thrombus is mediated by the process of outside-in and inside-out signalling (Ma et al., 2007). Several receptors have different functions and signalling pathways. As the platelet integrin $\alpha_{IIb}\beta3$ binds to VWF and fibrinogen, an integrin-proximal intracellular signalling process triggers changes in extracellular ligand binding domain of the integrin leading to transformation from “low-affinity” to “activated” state, which is referred to as inside-out signalling (Coller and Shattil, 2008). Meanwhile, after ligand binding to integrin $\alpha_{IIb}\beta3$, a ligand–induced conformational change of integrin occurs leading to a series of intracellular signalling process which are called “outside-in” signalling (Li et al., 2010).

In what can be called “the intermediate common signalling”, a mixture of soluble platelet agonists are liberated from activated platelets such as TXA2, ADP and serotonin. (Offermanns, 2006). Platelet stimulation by these agonists occurs by binding to G-protein-coupled receptors (GPCRs) in which the signals are transmitted from integrin via heterotrimeric G proteins seven transmembrane receptors (Li et al., 2010).

1.2.4 Fibrin clot formation and lysis

Fibrin clot formation

As alluded to earlier, the fibrin network is the skeleton of the blood clot and is formed from fibrinogen, an abundant plasma protein. Fibrinogen is a trinodular 340 KDa glycoprotein, produced by the liver and consists of three pairs of polypeptide chains, linked by 29 disulfide bond (Wolberg and Campbell, 2008, Guthold et al., 2007). All six polypeptide chains are supercoiled as α-helices (Standeven et al., 2005) to form the molecule which consists of central domain (E) and two lateral domains (D). N-termini of all six chains end in E domain, while C-termini of Bβ and γ chains expand into the lateral 2D domain and C-termini of Aα chains are extended outside D domain (Wolberg and Campbell, 2008). The basic structure of fibrinogen is summarised in Figure 1.2

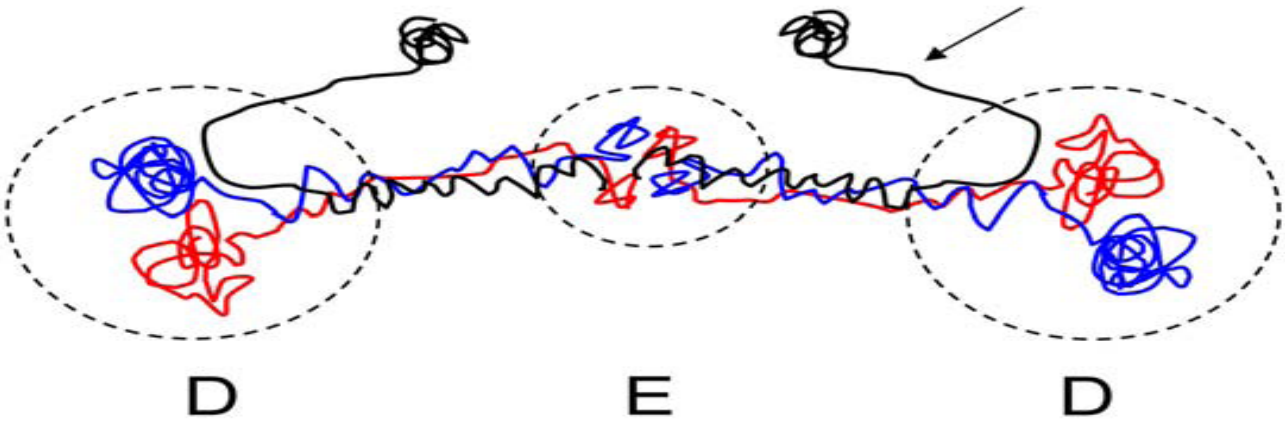

Figure 1.2 Schematic draw of the fibrinogen structure showing the E and D domains. Bβ chain is shown in blue, γ chain in red and Aα chain in black. N-termini of all six chains end in E domain. Carboxyl end of Bβ and γ chains expand into the lateral 2D domain. The arrow indicates the terminal of one of the Aα chains [(adapted from (Weisel et al., 1985)].

As a result of plaque rupture, a prothrombotic core is exposed and plasma comes in contact with tissue factor (TF). Simultaneously, platelets attach to the site of injury and get activated as described above. TF activates FVII and activated FVII/TF complexes, then activates FIX and FX, which in turn activate FX and FV. Activated FX and FV mediate prothrombin cleavage to produce small amount of thrombin sufficient to maintain platelet activation but not enough for full activation of the coagulation cascade(Kazama et al., 1995). The contact of partially activated platelet with collagen and thrombin leads to full activation and degranulation of platelet followed by FV release which is then activated by thrombin and FX. Platelet surface FXa/Va complex leads to the production of enough amount of thrombin suitable to convert fibrinogen into fibrin and form a stable plug (Ajjan and Grant, 2006a, Medved and Weisel, 2009).

Thrombin cleaves small fibrinopeptides (A and B), from aminotermini of Aα and Bβ chains allowing interaction with cleaved chains from another molecule resulting in the formation of insoluble fibrin fibres that further complex together to form the cross-linked fibrin network (Ariens et al., 2002). At first, Fibrinopeptide A (FpA) is cleaved and this exposes an N-terminal α-chain motif Gly-Pro-Arg (GPR), called knob A (Medved and Weisel, 2009). Knob A is then joined with a hole (a) in the γ-chain of an adjacent fibrin molecule. This process produces A:a bond which is both strong and stable (Litvinov et al., 2005), and regarded as the cornerstone in fibrin polymerization (Weisel and Litvinov, 2013). The cleavage of FpB exposes N-terminal β-chain motif Gly-His-Arg-Pro (GHRP), called knob B, which binds to hole b in an adjacent fibrinogen β-chain. The affinity of knob B is relatively low compared with knob A (Everse et al.,

1998). However, cleavage of FpA without the cleavage of FpB leads to theformation of clots made up of thinner fibres than if it is produced by the cleavage of both fibrinopeptides (Weisel and Litvinov, 2013). The binding of knob A from fibrin monomer with a hole from another monomer (Knob-hole interaction) occurs in a half-staggered manner to hold the two monomers together (Weisel and Medved, 2001).

In addition to knob–hole interaction, there is D-D interface that is similar to end-to-end junction between monomers (Everse et al., 1998, Zhmurov et al., 2011). More fibrin monomers can be added to form a longitudinal chain of oligomers and then protofibrils then join with each other side by side (lateral aggregation) to increase fibre thickness (Weisel and Litvinov, 2013)

To stabilise the produced clot, thrombin-activated FXIIIforms several cross-links within α and β chain. Furthermore, FXIII-mediated crosslinking of various proteins into the clot, such as plasminogen inhibitor, increases the clot strength and resistance to fibrinolysis. (Ajjan and Ariens, 2009).

Fibrin clot lysis

There is a fine balance between clot formation and breakdown (lysis) *in vivo*, in order to avoid widespread vascular occlusion following an external injury. Analogous to thrombin, plasmin is the pivotal enzyme in the fibrinolytic cascade. Plasmin is generated following cleavage of plasminogen by a serine protease, tissue plasminogen activator (tPA) and this reaction occurs 1000 fold faster in the presence of fibrin. Plasmin cleaves arginine and lysine sites on a range of molecules and its activity is tightly controlled by plasmin inhibitor (PI) to prevent systemic proteolysis (Weisel and Litvinov, 2008). Cleavage of fibrin by plasmin

leads to the generation of fibrin degradation products, which can be clinically used as an indicator of a thrombotic condition. In addition to PI, other inhibitors of this pathway include plasminogen activator inhibitor-1 (PAI-1) and thrombin activatable fibrinolysis inhibitor (TAFI). PAI-1 is the fast acting inhibitor of tPA and is produced by endothelial cells, platelets and adipose tissue. TAFI is found in large quantities in platelets and plasma, is activated by thrombin, a cleavage event that is much enhanced when thrombin is bound to thrombomodulin. Activated TAFI cleaves the N-terminal lysine residues from degrading fibrin fibres to prevent binding of plasminogen and tPA to fibrin which results in inhibition of plasmin generation and clot lysis (Antovic, 2003). The main steps in clot formation and lysis are summarized in Figure 1.3

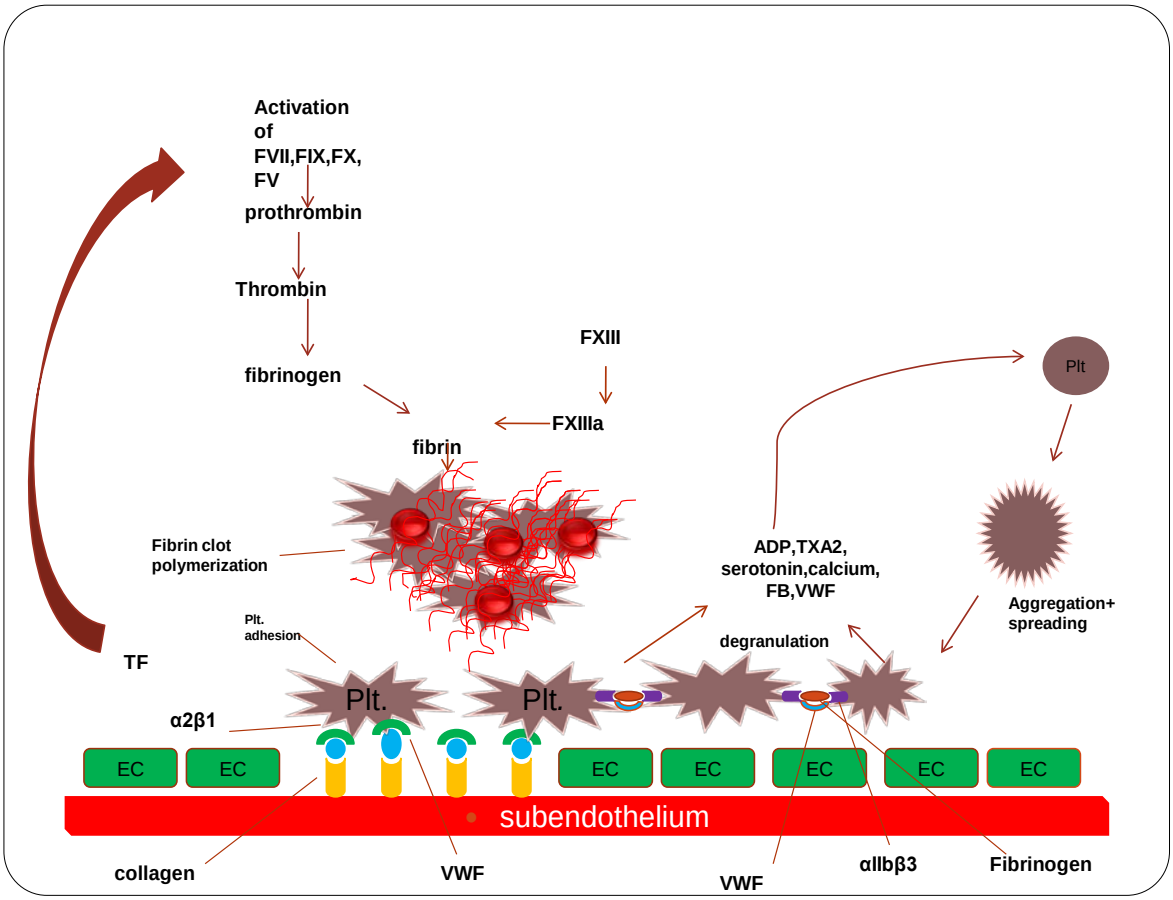

Figure 1.3 Diagram summarising blood clot formation secondary to activation of the cellular and fluid phase of coagulation. Following plaque rupture, platelets interact with collagen, which leads to initial and partial platelet activation and degranulation, resulting in the secretion of various agonists. These agonists further enhance platelet activation and facilitate platelet spreading and platelet fibrinogen-interaction. Simultaneously, TF release results in activation of the coagulation cascade, including FV, FVII, FIX and FX, resulting in thrombin generation and conversion of soluble fibrinogen into insoluble fibrin networks. A number of interactions take place between coagulation proteins and platelets, which are detailed in the text. EC: endothelial cells, ADP: adenosine diphosphate, Plt: platelets, TF: tissue factor, TXA2: thromboxane A2, VWF: Von Willebrand factor, FXIII: factor FXIII.

1.2.5 Platelet-fibrinogen interaction

In the platelet aggregation process, fibrinogen binds to platelet glycoprotein IIb-IIIa (integrin $\alpha_{IIb}\beta3$) complex, which is the most abundant integrin on platelet membrane and represents about 50000-80000 copies per platelet (Jennings, 2009, Kouns et al., 1994).The integrin $\alpha_{IIb}\beta3$ receptor is formed from two calcium-dependent heterodimers (Jennings and Phillips, 1982, Wencel-Drake et al., 1986). These are encoded on chromosome 17q21.32 (O'Toole et al., 1989, Thornton et al., 1999) leading to the production of two glycoprotein subunits GPIIb/GPIIIa (integrin $\alpha_{IIb}\beta3$) that are either transported to the cell surface or stored within platelets alpha granules (Phillips and Agin, 1977, Bray et al., 1986, Rosa and McEver, 1989).

Resting platelet integrin $\alpha_{IIb}\beta3$ does not interact with soluble fibrinogen but when platelets attach to extracellular wall and get activated, conformational changes in $\alpha_{IIb}\beta3$ occur making it able to bind to fibrinogen (Sims et al., 1991) as well as fibrin (Falati et al., 2002, Hayashi et al., 2008, Hechler et al., 2010). The connection between fibrinogen and integrins occurs in thecarboxyl-terminus of α-chain and carboxyl-terminus on γ-chains (Kloczewiak et al., 1984, Springer, 2009, Kloczewiak et al., 1989). The initial interaction between fibrinogen and $\alpha_{IIb}\beta3$happens in the carboxyl-terminal sequence in γ central domain which is highly specific (Springer et al., 2008, Kloczewiak et al., 1984, Kloczewiak et al., 1989). By means of specific binding sites present in the carboxy terminus of α- and γ-chains, one fibrinogen molecule can attach with two $\alpha_{IIb}\beta3$ molecules in which each one is located on a different platelet, thereby having a bridging effect between the cellular components of thrombosis.Integrins have the affinity to adsorb proteins and peptides containing the tetrapeptide sequence Arg-Gly-

Asp-X (RGD) in which x could be a different residue such as valine, serine, phenylalanine or alanine (Pytela et al., 1986).

The binding between $\alpha_{IIb}\beta3$and fibrinogen is a multi-step process: First, the reversible step, within second to minutes, followed by the step where the bound ligand is no longer able to dissociate, making the process irreversible (Marguerie et al., 1980, Peerschke and Wainer, 1985). $\alpha_{IIb}\beta3$is a receptor that requires signalling events to be in the active state. ADP is one of the agonists that activate $\alpha_{IIb}\beta3$, which leads to rearrangement of the ligand-binding site, transforming the integrin from closed low-affinity conformation to an open high affinity conformation (Kamata et al., 2010, Floyd and Ferro, 2012). Savage and Ruggeri demonstrated that unstimulated platelets do not attach to immobilizedfragment E of fibrinogen, which contains an Arg-Gly-Asp sequence at Aα and adhere to fragment D γ-chain. In contrast, activated platelets adhere to immobilised fibrinogen and fragments D, and E in time–dependent and equivalent manner. Thus unstimulated platelets partially interact with fibrinogen, and the initial attachment is followed by spreading and irreversible adhesion (Savage and Ruggeri, 1991). Platelet-fibrinogen interaction steps are summarised in Figure1.4

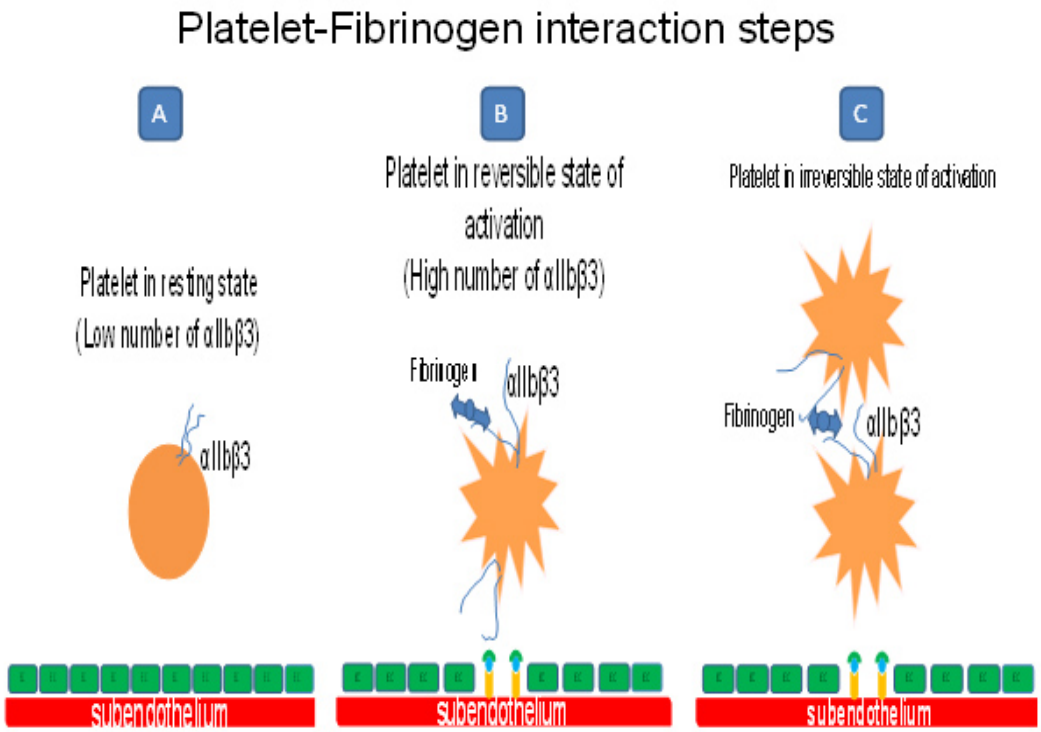

Figure 1.4 Platelet fibrinogen interaction steps. A. Resting platelets express low copies of integrin $\alpha_{IIb}\beta_3$ which do not interact with soluble fibrinogen. B. When platelets attach to extracellular wall and get activated, conformational changes in $\alpha_{IIb}\beta_3$ occur making it able to bind to fibrinogen. This active state is a reversible step that extends from second to minutes. C. The final and irreversible step in which bound ligand can no longer dissociate.

1.3 Diabetes mellitus

Diabetes mellitus (DM) is defined by the American Diabetes Association as "group of metabolic diseases characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both". Diabetes mellitus is becoming the epidemic of the 21st century and the complications associated with this condition result in significant personal and financial burden. The percentage of diabetes for all age-groups globally was predicted to reach 2.8% in 2002 and 4.4% in 2030. The total number people having diabetes worldwide were estimated to increase from 171 million in 2000 to 366 million in 2030 (Wild

et al., 2004). Diabetes leads to both macrovascular and microvascular complications, consequently resulting in high degree of morbidity and mortality.

Type 1diabetes, evident in around 5% of diabetes patients and previously called insulin-dependent diabetes (IDDM), develops as a result of pancreatic β-cells destruction secondary to an autoimmune process. In contrast, type 2 diabetes (T2DM), previously known as non-insulin dependent diabetes, is far more common representing over 90% of patients with diabetes. T2DM develops secondary to obesity and insulin resistance, following the failure of the pancreatic β-cells to keep up with the increasing demand for insulin secretion as a result of resistance to this hormone (NDFS, 2007). There are some rare forms of diabetes, including maturity onset diabetes of adults (MODY). This is an autosomal dominant condition due to various mutations in transcription factors that are involved in glucose haemostasis. Diabetes can also occur secondary to pancreatic destruction (due to alcohol excess for example) or rare endocrine conditions including acromegaly and Cushing's syndrome (American Diabetes Association, 2004).

1.3.1 Diabetes and cardiovascular risk

Despite advances in therapy, cardiovascular disease (CVD) remains the main cause of morbidity and mortality in patients with diabetes. Globally, 281 million men and 317 million women died of diabetes related complications in 2011, the majority from CVD (www.diabetesatlas.org/content). Patients with DM have 2-4 times higher mortality than the general population and up to 80% of patients of diabetes subjects die secondary to cardiovascular disease (CVD). The risk of first ischaemic event in subjects with diabetes and no clinical CVD is similar to

the non-diabetic population with a previous history of ischaemic heart disease (IHD) (Haffner et al., 1998, Wild et al., 2004). It is not only that diabetes increases the risk of first cardiac event, but the prognosis of these patients following coronary ischaemia remains poor despite advances in management. Our Division investigated the efficacy of modern treatment strategies on clinical outcome following acute coronary syndrome (ACS). Comparing 2003 with 1995, a 15% reduction in mortality at 18 months was evident in individuals without diabetes ($p<0.01$), but only a non-significant 4% reduction was documented in those with diabetes ($p=0.71$) (Cubbon et al., 2007). It could be argued that treatment has further improved over the past decade, and more up-to-date strategies may have narrowed the difference between diabetes and non-diabetes subjects. However, recent work on individuals with myocardial infarction (MI), undergoing percutaneous coronary artery intervention, has shown higher mortality in patients with diabetes compared to those without diabetes at 30 days (7.4% vs 3.8%, respectively; $p<0.01$), and at 12 months (13.9% and 6.5%, respectively; $p<0.0001$) (Kahn et al., 2012). The same applies to individuals treated with coronary artery bypass graft (CABG). In 10626 patients who have undergone CABG, mortality in individuals with diabetes remained significantly higher at 1, 5 and 10 years of follow-up (van Straten et al., 2010). A separate study has shown that drug-eluting stents or CABG, as a treatment of multi-vessel coronary artery disease in diabetes, do not improve clinical outcome after a median follow-up of 5.6 years, indicating that type of revascularisation has little effect on longer term prognosis in diabetes (Kim et al., 2012). The increase in cardiovascular mortality in patients with diabetes is due to a number of factors including more extensive vascular

disease and increased thrombosis potential due to changes in clotting factor plasma level/activity, impaired fibrinolysis and increased platelet reactivity (Alzahrani and Ajjan, 2010, Angiolillo et al., 2005).

The close link between diabetes and CVD formed the basis for the “common soil” hypothesis. This postulates thatDM and CVD share common environmental and genetic risk factors that lead to vascular pathology (Ajjan and Grant, 2006b). These common risk factors are discussedabove and clinically targeted modulation of these risk factors result in asignificant reduction in vascular risk (Mccalum and Fisher, 2005).

Insulin resistance does not only lead to the development of diabetes but is also an important factor in the development of vascular disease. Insulin resistance results in deranged glucose and lipid metabolism, both of which results in endothelial dysfunction, one of the earliest abnormalities in the atherosclerotic process as described above. Therefore, insulin resistance provides an important link between diabetes and CVD.

1.3.2 Hypoglycaemia

In people with T2DM, severe hypoglycaemia is a potential risk factor for cardiovascular disease (Zoungas et al., 2010, Duckworth et al., 2011). Intensive glucose control was shown to reduce the risk of non-fatal myocardial infarction in T2DM (Kelly et al., 2009, Boussageon et al., 2011). However, some studies have demonstrated an increase in cardiovascular mortality following hypoglycaemia, which is becoming an important clinical topic (Zoungas et al., 2010). The mechanisms for increased cardiovascular mortality following hypoglycaemia may be related to cardiac arrhythmia and/or the predisposition

to an inflammatory and thrombotic environment (Stahn et al., 2014). The link between hypoglycaemia in cardiovascular disease has been recently emphasised in a meta-analysis covering six studies with 903510 participants and the authors concluded that avoiding hypoglycaemia may help in preventing vascular ischaemia (Goto et al., 2013).

1.3.3 Diabetes-specific atherothrombotic risk factors and extensive vascular pathology

Diabetes-specific mechanisms for increased atherothrombosis include more extensive vascular pathology and an enhanced thrombotic environment. Individuals with diabetes display a prothrombotic environment characterised by increased prothrombotic coagulation factor level/activity coupled with hypofibrinolysis, as well as increased platelet activation. For example, TF expression by endothelial cells is upregulated in diabetes resulting in higher plasma protein levels (Breitenstein et al., 2010). Furthermore, there is enhanced thrombin production in diabetes and elevated levels of fibrinogen, related, at least in part, to elevated glucose levels, adding to the prothrombotic milieu (Ceriello et al., 2007, Boden et al., 2007, Klein et al., 2003). Hypofibrinolysis is documented by increased levels of plasminogen activator inhibitor (PAI)-1 levels and enhanced incorporation of plasminogen inhibitor into the clot (Dunn et al., 2004).

Medium to long term increased plasma glucose levels result in various protein undergoing post-translational modifications, and in the case of coagulation proteins, this can increase thrombosis risk as further detailed below (Ajjan et al., 2013).

It is well accepted now that chronic inflammation plays a key role in the pathogenesis of vascular pathology. Diabetes is associated with raised plasma levels of inflammatory markers including C-reactive protein (CRP) and complement C3, both of which have a predictive value for future vascular events (Hess et al., 2012). The rise in inflammatory markers is more pronounced in patients with type 2 than type 1 diabetes, consistent with earlier vascular disease in the former group. This may further explain the reasons behindyouth with T2DM having increased damage of the vascular endothelium compared with age-matchedT1DM subjects (Ohsugi et al., 2014).

1.3.4 Role of plateletsin increased thrombosis potential

The elevated turnover of circulating platelets results in high numbers of young platelets which are characterised by increased reactivity compared with the older platelet population. These young circulating reticulated platelets are increased in patients with acute coronary syndrome and stroke (Lakkis et al., 2004). Similarly, platelets from patients with diabetes display similar characteristics as they are larger in size and hyperactive, thereby increasing thrombosis risk (Arjomand et al., 2003).

A number of mechanisms could be behind the increase in platelet activity in diabetes. One is related to non-enzymatic glycation of platelet surface proteins that decreases membrane fluidity, which in turn enhances platelet adhesion (Winocour et al., 1992). Also in hyperglycaemic environment, there is increased platelet-surface expression of $\alpha_{IIb}\beta3$ (GPIIb/IIIa) and GP Ib (Vinik et al., 2001). Furthermore, thromboxane A2 (TxA2) production is increased in diabetes due to the reduction in theintracellular level of antioxidants such as glutathione

(Thomas et al., 1985). Davi and his group have shown that tight metabolic control in patients with diabetes result in asignificant decrease in TxA2 biosenthysis, suggesting that controlling glycaemia reduces thrombosis risk (Davi et al., 1990). In addition to various factors that increase platelet activation in diabetes, there is a reduction in factors that inhibit the activity of these cells. Nitric oxide (NO), produced by endothelial cells, is a potent inhibitor of platelet activation. In the setting of endothelial dysfunction, secondary to metabolic changes in diabetes, NO production is reduced, thereby liberating the platelets from NO-induced inhibition (Queen et al., 2003). Moreover, Li and his group have assessed the ability of platelets to release or promote Ca^{2+} influx. Platelets from healthy controls released Ca^{2+} more effectively than platelets from patients with diabetes. In contrast, diabetic platelets showed a more active Ca^{2+} influx process compared with controls (Li et al., 2001). Finally, the P2Y receptor, one of ADP agonists receptors, is suggested to be upregulated in platelets of patients with diabetes (Yamagishi et al., 2005), further contributing to enhanced platelet activation. The main mechanisms leading to increased platelet activation in diabetes are summarised in Table 1.1

	Effect of diabetes	Platelet response
1	Non-enzymatic glycation of platelet surface (Winocour et al., 1992)	Reduced membrane fluidity and increased platelet adhesion
2	Increasedplatelet-surface expression of $\alpha_{IIb}\beta3$(Vinik et al., 2001)	Increasedplatelet-fibrinogen binding
3	Reduced intracellular level of antioxidants Ex: Glutathione (Thomas et al., 1985, Davi et al., 1990)	Increased TXA2 production and hence increased platelet activation
4	Hyperglycaemia and insulin resistance(Queen et al., 2003)	Reduced Nitric oxide production by endothelial cells
5	Na^{+}/Ca^{2+} exchange process working in reverse mode(Li et al., 2001)	Increased Ca2+ influx
6	P2Y receptor upregulation (Yamagishi et al., 2005)	Increased platelet activation to ADP

Table 1.1 Potential mechanisms that are affecting increased platelet reactivity in diabetes. ADP: adenosine diphosphate, TXA2: thromboxane A2, P2Y.

1.3.5 Role of fibrin structure and fibrinolysis in diabetes

Studies have shown that both compact fibrin networks and clots that are resistant to fibrinolysis are associated with increased risk of CVD. I discuss below the changes observed in fibrin network properties in individuals with diabetes with special emphasis on the role of glycaemia.

Fibrin network structure in diabetes

A number of studies have shown an increase in fibrin network density in diabetes. Jörneskog and colleagues were the first to report changes in the fibrin network in individuals with type 1 diabetes (T1DM) (Jorneskog et al., 1996). Work from our laboratory further documented that alterations in fibrin network structure can occur at a very early age in patients with T1DM (Hess et al., 2012). The advantage of studying T1DM patients is the ability to address the role of glycaemia on fibrin clot structure whilst keeping the effects of confounding factors, frequently seen in type 2 diabetes (T2DM) patients, to a minimum. Indeed, the relationship between glycaemia and fibrin clot properties in T2DM is less clear. One study of 20 T2DM patients and 18 controls did not show significant differences in plasma clots comparing the two groups, which may be due to the small number of samples studied and the heterogeneity observed in T2DM (Pieters et al., 2008). The latter concept is particularly important as we have shown that the presence of micro and/or macrovascular complications is associated with altered fibrin clot properties in T2DM (Alzahrani et al., 2012).

In order to dissect out the mechanistic pathways, work from our laboratory studied the characteristics of clots made from plasma purified fibrinogen of 150 T2DM patients and 50 healthy controls. Diabetes clots had smaller pore size, increased fibre thickness and number of branch points compared with controls, indicating that post-translational modifications in fibrinogen are directly responsible for altered clot structure in diabetes (Dunn et al., 2005).Furthermore, HbA1c levels showed a negative correlation with pore size and a positive correlation with number of branch points within the clots,

suggesting that glycaemic control has an effect on clot structure in this population (Dunn et al., 2006). Therefore, current studies indicate the formation of more compact fibrin networks in individuals with both T1DM and T2DM. Altered clot structure in diabetes is evident from childhood and glycaemic control appears to modulate the characteristics of the fibrin network. However, there is a great variability in fibrin networks in patients with T2DM with additional changes in clot characteristics in the presence of complications. This indicates that altered clot structure in diabetes is present at various stages of this condition, which may have future clinical implications.

Fibrinolysis in diabetes

Several studies have shown impaired clot lysis in diabetes and these included patients with both T1DM and T2DM (Hess et al., 2012, Neergaard-Petersen et al., 2014b, Alzahrani and Ajjan, 2010). We have shown that fibrinolytic alterations in diabetes are detected at a young age and can also be found in those with advanced vascular complications (Hess et al., 2012, Alzahrani et al., 2012, Neergaard-Petersen et al., 2014b). Whilst impaired fibrinolysis is generally associated with more compact fibrin networks (Collet et al., 2000), other factors also play a role such as increased incorporation of antifibrinolytic proteins into the clot and direct impairment in the fibrinolytic system, which are discussed in detail below.

1.3.6 Mechanisms for altered clot structure and fibrinolysis in diabetes

There are a number of factors that affect clot structure and fibrinolytic efficiency in diabetes, and these include qualitative and quantitative changes in the coagulation proteins.

Clot structure

Quantitative changes in coagulation factors modulate the final ultrastructure of the clot. Most importantly, increased plasma levels of fibrinogen, commonly found in diabetes, result in the formation of more compact clots (Alzahrani and Ajjan, 2010). In addition to quantitative changes, qualitative alterations in clotting factors can influence the structure of the fibrin network. High plasma glucose mediates glycation of fibrinogen (Pieters et al., 2007) and clots made from glycated protein are more tightly packed. Moreover, the by-product of protein glycation, glycolaldehyde, induces post-translational modifications in fibrinogen, which impairs the fibrinolytic process (Andrades et al., 2009). It should be remembered that diabetes is also associated with increased oxidative stress and fibrinogen oxidation has been shown to modulate clot structure, adding another mechanism for altered clot structure in diabetes (Henschen-Edman, 2001).

If hyperglycaemia has a deleterious effect on clot structure, then improving glycaemic control should result in the production of less compact clots. Initial attempts at optimising glucose control with continuous subcutaneous insulin infusion (CSII) were associated with increased plasma fibrin gel porosity but, unexpectedly, this was not related to improvement in glycaemic control as the fall in HbA1c was minor and non-significant (Jorneskog et al., 2003).

Subsequent work from our laboratory has shown that a mean drop in HbA1c by 13 mmol/mol is associated with a decrease in plasma clot final turbidity, indicating the formation of less compact clots (Hess et al., 2012).Others have demonstrated that improving glycaemic control in 20 T2DM subjects has no effect on fibrinogen levels, plasma clot porosity or turbidity curves but it results in a small reduction in clot compaction (Pieters et al., 2007). Investigating clots made from plasma-purified fibrinogen from the same patients has shown that improving glycaemia results in variable changes in clot structure, an effect that was more obvious in a small group of 7 individuals with larger drop in fibrinogen glycation (Pieters et al., 2008).

Fibrinolysis

Changes in fibrinolysis in diabetes can be secondary to: i) Altered clot structure, ii) Increased incorporation of antifibrinolytic proteins into the clot and iii) Deranged activity of the fibrinolytic system.

More compact clots display increased resistance to fibrinolysis as previously demonstrated (Collet et al., 2000, Collet et al., 2006). Therefore, the changes in clot structure outlined above can indirectly affect theefficiency of the fibrinolytic process in diabetes. More recently, an additional mechanism has been described that is particularly interesting as it may have future therapeutic implications. FXIII mediates the cross-linking of various proteins into the clot in order to strengthen the fibrin and increase its resistance to fibrinolysis. PI represents a classic protein that is cross-linked into the fibrin clot in order to make it more resistant to lysis (Schaller and Gerber, 2011). Recent work has shown that diabetes clots are characterised by increased PI incorporation into

the fibrin network, representing an additional mechanism for impaired clot lysis in diabetes (Agren et al., 2014). Another protein that may also be diabetes-specific is complement C3. Work from our laboratory has shown that complement C3 can be detected in the fibrin clot using proteomics techniques (Howes et al., 2012, Distelmaier et al., 2009). Interestingly, C3 incorporation into the clot impairs the fibrinolytic process, which may be due to the mechanical inhibition of clot lysis secondary to presence of C3 but may also be related to C3 acting as a substrate for plasmin (Seya et al., 1985). Our studies in patients with T1DM have shown increased incorporation of C3 into diabetes clots, explaining the exaggerated anti-fibrinolytic response to C3 in this population (Hess et al., 2012). Further work in 822 patients with type 2 diabetes has shown that C3 plasma are at least as good a predictor of fibrin clot lysis as PAI-1 (Hess et al., 2014). Interestingly, there was no correlation between C3 and PAI-1 plasma levels, consistent with the two proteins affecting different pathways in the fibrinolytic system. Increased incorporation of PI and C3 into the fibrin clot in diabetes offers the exciting possibility of developing diabetes-specific therapies that target fibrin-PI or fibrin-C3 interactions as a means of improving the fibrinolytic efficiency in this condition, whilst keeping bleeding risk to a minimum.

Finally, diabetes can directly modulate the efficiency of the fibrinolytic system. It has been known for a long time that increased plasma levels of PAI-1, a characteristic of insulin-resistant states and T2DM, represent a key mechanism for impaired fibrin clot lysis in these conditions (Trost et al., 2006). PAI-1 inhibits plasmin generation and levels of this protein have been repeatedly used as a surrogate marker for fibrinolytic efficiency in various research studies. More

recently, however, a new mechanism for impaired fibrin clot lysis in diabetes has been described that is related to increased glycation of plasminogen. Nε-fructosyl-lysine residue on plasminogen from T1DM individuals was increased three-fold compared with protein purified from healthy controls, and a preferential glycation of lysine 107 and 557 within the protein was demonstrated. These sites are believed to be involved in fibrin binding and plasmin(ogen) cleavage, offering mechanistic explanations for our findings (Ajjan et al., 2013). Indeed, reduced conversion of diabetic plasminogen to plasmin together with decreased protein activity were evident, with improvement documented following better control of glycaemia. Mechanisms for hypofibrinolysis in diabetes are summarised in Figure 1.5

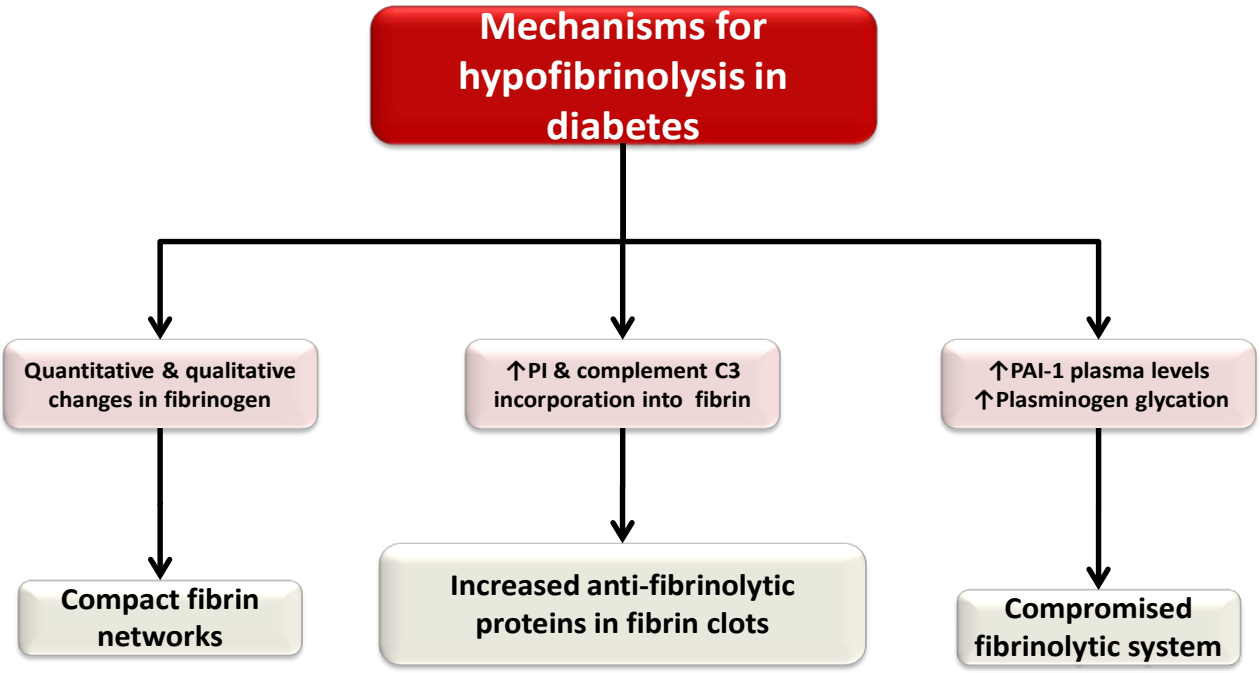

Figure 1.5 Mechanisms responsible for hypofibrinolysis in diabetes. The three main mechanisms responsible for hypofibrinolysis in diabetes include: 1) the formation of more compact clots, which increases mechanical resistance to lysis; 2) increased incorporation of antifibrinolytic proteins into the fibrin network, including plasmin inhibitor and complement C3; and 3) compromised efficacy of the fibrinolytic system through increased plasma levels of plasminogen activator inhibitor and compromised plasmin production and function secondary to increased protein glycation. Abbreviations: PA: Plasminogen activator inhibitor-1, C3: complement 3, PI: plasmin inhibitor.

1.3.7 Role of fibrinogen-platelet interactions in diabetes

The stickiness of platelets from both patients with diabetes and healthy individuals is increased in the presence of excess glucose (Bridges et al., 1965). Comparing 41 patients with diabetes (27 type I and 14 type II) and 23 healthy controls, Tschoepe and his group have shown that the average number of glycoproteins per platelet (including $\alpha_{IIb}\beta 3$), is significantly increased in diabetes.Additionally, they found that there is apositive relationship between vWF plasma levels and HbA1c.This may further contribute to increased platelet fibrinogen binding capacity (Tschoepe et al., 1990).

Glycation of the fibrinogen affects both its conformation and function. Due to the reaction between the free amino group in the fibrinogen molecule and the carbonyl group of a reducing sugar, amadori product is produced, which can alter protein conformation (Mirmiranpour et al., 2012). These changes in fibrinogen may in turn lead to different interaction with platelets, thereby modulating the thrombotic process. However, this concept is not supported by other studies, demonstrating that fibrinogen binding to ADP-activated platelets from patients with diabetes and healthy control have the same pattern (Niewiarowski et al., 1987).

On the other hand, clinical studies indicate that disrupting platelet-fibrinogen interaction, as part of the treatment for unstable coronary artery disease, appears to be particularly beneficial in individuals with diabetes. This suggests that platelet-fibrinogen interaction may be different in diabetes compared to normoglycaemicindividuals, or it may simply indicate that manipulating this interaction has a superior role in protecting from thrombotic events in this population.

1.3.8 Glycaemia and clinical studies

Given that high glucose levels have a deleterious effect on clot structure/fibrinolysis, it is expected that improving glycaemia reduces the risk of atherothrombotic disease. However, this has not been shown in all studies and remains an area of constant debate. The Diabetes Control and Complications Trial (DCCT) and further extension in the Epidemiology of Diabetes Interventions and Complications (EDIC) trial has shown that early glycaemic control in type 1 diabetes reduces longer-term atherothrombotic complications(Nathan et al., 2005). Largely similar findings were documented in the UK Prospective Diabetes Study (UKPDS) with long-term follow-up of patients with newly diagnosed type 2 diabetes (Holman et al., 2008). The Diabetes mellitus Insulin Glucose infusion in Acute Myocardial Infarction (DIGAMI)-1 study demonstrated that improving glycaemic control following a cardiac event improves clinical outcome (Malmberg et al., 1996). In contrast, the DIGAMI-2 study, investigating the best strategy to lower blood glucose levels following MI, failed to show a beneficial effect of tight glycaemic control (Malmberg et al., 2005). This was hardly surprising given the similar HbA1c values in three study arms and the fact that the study was underpowered. Two large studies investigating the medium-term vascular effects of tight glycaemic control have shown either an absence of an effect, or quite worryingly, an increase in mortality (Patel et al., 2008, Gerstein et al., 2008). There are a number of explanations for these contradictory findings when addressing fibrin-related thrombosis risk. First, the effect of glycaemia on fibrin network structure is only apparent with very high glucose levels, and perhaps only a modest improvement in glycaemia is required for the reduction of fibrin-related

thrombosis risk. Therefore, a drop in HbA1c from 75 to 65 mmol/l will have a significant effect on thrombosis risk that is not seen following a reduction in HbA1c levels from 65 to 55 mmol/mol. Second, HbA1c is a poor marker of acute glycaemia as it fails to take into account fluctuations in glucose levels, which can be prothrombotic (Ceriello et al., 2014). Third, several pieces of evidence indicate that hypoglycaemia, which HbA1c fails to capture, is prothrombotic and may have a role in the adverse clinical outcome in diabetes. Previous work has shown that acute hypoglycaemia is associated with a rise in PAI-1 levels (Gogitidze Joy et al., 2010) and an increase in factor VIII coagulation activity coupled with accelerated thrombin generation (Ibbotson et al., 1995). Our *ex-vivo* studies using hypoglycaemia clamp studies in patients with diabetes have shown that low blood glucose levels are associated with hypofibrinolysis, measured using the validated turbidimetric assays. Moreover, this thrombotic effect of hypoglycaemia can persist for one week following the event, adding an important dimension to our understanding of the impact of hypoglycemia on thrombosis risk (Kurdee et al., 2014). The above findings may explain improved clot porosity after continuous subcutaneous insulin infusion, despite no improvement in HbA1c, as this treatment is typically associated with less hypoglycaemic episodes (Jorneskog et al., 2003). Therefore, clinical studies investigating the effects of glycaemia on cardiovascular event and thrombosis risk should not only rely on HbA1c but should take into account glucose variability and hypoglycaemia (Ajjan and Owen, 2014), which will only be possible using continuous glucose monitoring. With the development of new continuous glucose devices, a more sophisticated approach to monitoring

glycaemia is now a real possibility that should be explored in future cardiovascular studies in diabetes.

1.4 *Ex vivo* tests of thrombosis potential (Platelet function tests)

Assessing bleeding potential is regularly used to tailor therapy in a large number of medical conditions. With the same token, assessing thrombosis may help to tailor anti-thrombotic therapy according to the need of each individual. However, the latter remains largely a research tool and clinical applicability has been somewhat limited mainly due to the large variability encountered with these tests and the relative inability to define a “normal range”. A number of *ex vivo* tests can be performed to assess thrombosis risk and these are briefly described in this section.

Platelets function tests were mainly used to determine bleeding risk (Peerschke, 2002) but more recent studies are calling for their use to predict risk of thrombosis and to monitor the efficacy of antiplatelet therapy (Paniccia et al., 2015). One of the earliest platelet function tests is *in vivo*bleeding time that was used for decades (Guidelines on platelet function testing (BCSH, 1988)). The principle of the test depends on measuring the time between thestart of bleeding, after an incision is made in the skin, until the bleeding stops (Harrison, 2005). The disadvantages of this test are that it is invasive, time-consumingwith poor reproducibility (Rodgers and Levin, 1990). Therefore, platelet aggregation tests were subsequently developed, which are less invasive and more accurate.

1.4.1 Measurement of platelet aggregation (LTA and multiplate)

Turbidimetric platelet aggregometryor light transmission aggregometry (LTA) is the *in vitro* test for platelet aggregation in response to different agonists such as ADP, Collagen and AA using platelet rich plasma (PRP). It is still regarded by many as the “gold standard” when assessing platelet reactivity (Seyfert et al., 2007, Harrison, 2004). However, it has several limitations, including sample preparation to obtain platelet rich plasma (PRP), which is both times consuming and requires laboratory expertise. Moreover, platelets tested are not in their normal physiological environment, casting doubt about the clinical validity of such an approach (Valles et al., 1993, Valles et al., 2002).

Perhaps a more “physiological” test is whole blood impedance aggregometry (Cardinal and Flower, 1980). This technique offers several advantages over LTA, including: i) only a small blood volume is needed, ii) there is no need for samples preparation, iii) platelets are tested in their normal environment (Jarvis, 2004).

1.4.2 VerifyNow system

The verifyNow system (ITC, Edison, NJ, USA) is a technique that assesses platelet aggregation using turbidimetric-based optical detection. The system uses cartridges that contain fibrinogen-coated beads and different platelet agonists. As platelets aggregate upon the fibrinogen-coated beads within the assay cartridge, the optical signal increases, which is then measured (Paniccia et al., 2015). There is moderate agreement between VerifyNow system and other platelet function tests (Paniccia et al., 2015). However, variability remains high and the early enthusiasm for the use of this technique has been quickly

fading. However, it remains a useful clinical tool to predict the post-operative haemorrhage (Rosengart et al., 2013, Yu et al., 2014).

1.4.3 Platelet function analyser (PFA)-100

The platelet Function Analyser PFA-100 tests the capacity of platelets to adhere under shear stress conditions in response to agonist present in the system. Citrated whole blood is flows under high shear stress through a capillary containing collagen-coated membrane, treated with either ADP or epinephrine (EPI). Once the platelet are activated and clump together, the capillary is blocked and blood flow stops, which is then measured as closure time (CT). Shorter CT is associated with increased platelet aggregation (Paniccia et al., 2015). Limitation of this technique includes low platelet count, a decrease in haematocrit as well as conditions with inherited platelet dysfunction (Chakroun et al., 2004, Mannini et al., 2006). Given these limitations, PFA-100 has fallen out of favour and it is now less commonly used to test platelet function.

In double-blind, placebo-controlled, randomised, three-period cross-over study in healthy volunteers, Chen and his group have compared different techniques assessing platelet reactivity aggregation using LTA, Multiplate®, Platelet function analyser-100®, and VerfyNow®. They have found that any of the simple to use technique can reliably test aspirin effect on platelet function (Chen et al., 2012).

1.4.4 Markers of platelet activation (FACS)

One of therecent advances is testing platelet activation using flow cytometry. Quantitation of glycoprotein receptor density, to diagnose disease like Bernard Soulier disease and Glansmann's thrombosthenia, is one of the most commonly used platelets tests (Cohn et al., 1997, Lindahl et al., 1992). A clear advantage of this technique is studying the platelets in their natural environment with little sample manipulation (Harrison, 2005). The main disadvantage, however, is the need for expensive equipment and considerable expertise (Harrison, 2005).

1.5 *Ex vivo* tests of thrombosis potential (fibrin structure and fibrinolysis)

The fibrin network forms the backbone of the fibrin clot and a number of tests are routinely used in research studies to investigate structure of the fibrin clot and resistance to fibrinolysis.

1.5.1 Permeation assays

In fibrin permeation test, plasma samples incubated with human thrombin and calcium in an open tube at room temperature for 2 hours, resulting in the formation of a fibrin clot. A neutral buffer is then dropped under pressure through the fibrin clot and flow rate through the fibrin clots is measured. In this technique the Darcy constant Ks or permeation coefficient is calculated, which measures the porosity of the clot (less porous clots are more thrombotic) (Mills et al., 2002).

1.5.2 Turbidimetric assays

In turbidity measurement technique, plasma is incubated with human thrombin and calcium in a microtiter plate followed by reading of absorptivity every couple

of seconds using spectrophotometric plate reader. In this technique, lag phase (the time required for the fibrin clot to start forming) maximum absorbance (a measure of clot density and fibre thickness) and lysis time (the time required for fibrin clot to be degraded) (Mills et al., 2002, Weisel and Nagaswami, 1992).

1.5.3 Clot visualisation

In scanning electron microscopy (SEM),the clot is prepared after the addition of thrombin and calcium followed by dehydration and sputter coating before visualisation under electron microscopy (Langer et al., 1988). The power of the electron microscope (x5000-100000 magnification) allows detailed investigation of clot ultrastructure includingfibre thickness and clot pore size. The other helpful clot imaging technique is confocal microscopy in which the fibrin clot of fluorescently labelled fibrinogen is analysed (Collet et al., 2005). The main advantage of confocal microscopy over SEM is visualisation of fully hydrated clots and the ability to conduct clot lysis experiments. On the other hand, confocal microscopy is unable to provide the resolution necessary to study clot ultrastructure. Therefore, the two techniques are complementary.

1.5.4 Platelet fibrinogen interaction tests

In order to determine the adhesion of human platelets to fibrinogen and other proteins, Bellavite et al 1994 developed a protein-coated microwell system. Isolated and washed platelets are added to 96-well microtiter plate coated with the fibrinogen. Unbound platelets are washed off and acid phosphatase activity in adherent platelet is subsequently measured (Bellavite, 1993). Another technique to study platelet fibrinogen interaction is flow cytometry, whereby

fibrinogen is labelled with fluorescent dye that enables detection of platelet binding (Heilmann et al., 1994). This technique has the advantages of being sensitive and is able to test interactions in solution without the need to immobilise fibrinogen.

1.6 The role of hypoglycaemic agents in Prevention of thrombus formation and thrombosis risk in diabetes

As mentioned above, type 2 diabetes is a heterogeneous condition with a large number of variables that may affect CVD risk. One set of these variables is introduced by the type of treatment used to treat hyperglycaemia. Metformin is usually used as first-line therapy in subjects with type 2 diabetes and the UKPDS demonstrated that use of this agent is associated with reduced risk of ischemic heart disease in overweight patients (UK Prospective Diabetes Study(UKPDS, 1998)). This is further supported by observational data from the REACH registry including a large number of 19,691 patients (Roussel et al., 2010). The mechanisms for reduced cardiovascular events in metformin users may be partly related to the effects of this agent on the fibrin network, reviewed elsewhere (Alzahrani and Ajjan, 2010).

Thiazolidinediones are peroxisome proliferator-activated receptor γ stimulators that directly modulate insulin resistance, the key pathogenic mechanism in diabetes. Treatment with thiazolidinediones is associated with lower fibrinogen and PAI-1 levels, which reduces thrombosis potential and improves fibrinolysis (Haffner et al., 2002, Buckingham, 2005, Chen et al., 2010, Perriello et al., 2007). Furthermore, these agents can delay intra-arterial thrombus formation and reduceprogression of atherothrombotic lesions (Li et al., 2005,

Mieszczanska et al., 2007, Marx et al., 2005). In the PROactive trial, pioglitazone treatment resulted in reduction in the composite end point of all-cause mortality, nonfatal MI, and stroke (2006, Dormandy et al., 2005). However, there was an increase in heart failure making the cardiac protective role of this agent debatable. In a controversial meta-analysis, rosiglitazone was found to increase the risk of cardiovascular events in diabetes (Nissen and Wolski, 2007), which subsequently resulted in withdrawal of this agent from the European market. The promising effects of glitazones on various cardiovascular risk factors did not translate clinically into meaningful vascular protection and the use of rosiglitazone was associated with apparent harm, although the data are far from conclusive. This clearly demonstrates that reliance on surrogate markers is not enough to assess the clinical efficacy of a drug, for which outcome studies are required.

Gliptins and glucagon-like peptide 1 (GLP-1) analogues are relatively new hypoglycemic agents and current evidence suggests that gliptins have a neutral effect on cardiovascular risk, whereas studies with GLP-1 analogues are yet to be reported (De Caterina et al., 2010, Scirica et al., 2013, White et al., 2013). Finally, insulin-treated patients with type 2 diabetes are at greater risk of cardiovascular events compared with noninsulin-treated subjects, which may simply reflect longer disease duration with a consequent increase in the risk of complications (Margolis et al., 2008).

1.7 The role of antiplatelet therapy in prevention of thrombus formation in diabetes

Anti-platelet therapy remains a cornerstone of the management of individuals with established CVD. Following an acute ischaemic event, dual antiplatelet therapy is used to inhibit two pathways of platelet activation usually for one year. This is then followed by anti-platelet monotherapy that continues indefinitely (Carreras and Mega, 2014).

1.7.1 P2Y12 receptor blockers

The receptor P2Y12 plays an important role in platelet aggregation and activation by the agonist ADP (Dorsam and Kunapuli, 2004). Inhibition of P2Y12 reduces platelet aggregation, decreases thrombin production, and modulates production of several pro-inflammatory markers such as TNF-α, p-selectin and CRP (Gurbel et al., 2006) There are different generations of ADP P2Y12 receptor inhibiting drug. The first generation is represented by the thienopyridine, ticlopidine. This drug is banned from the market because of unfavourable side effect profile. Clopidogrel is the second generation of thienopyridines and has been extensively used in the past two decades. Prasugrel is the newer version of irreversible P2Y12 inhibitors. Whilst prasugrel is largely similar to clopidogrel, a key difference is related to metabolism of the active drug that requires a single step, in contrast to clopidogrel that requires two steps, a process that may influence its clinical efficacy. The last generation of thienopyridines is ticagrelor, which differs from clopidogrel and prasugrel by being an active compound (no metabolism is necessary) and characterised by

reversibility of action, which may increase safety of this agent (Mega and Simon, 2015, Bonaca et al., 2015).

It is worth noting that platelets are more active in T2DM and have higher resistance to P2Y12 inhibitors, which is particularly evident with clopidogrel (Angiolillo et al., 2005, Angiolillo et al., 2006, Mangiacapra et al., 2010).

1.7.2 Inhibitors of platelet-fibrinogen interaction

As mentioned previously, platelet $\alpha_{IIb}\beta3$ receptor is the receptor that bindsfibrinogenthereby forming a bridge between platelets. Integrin $\alpha_{IIb}\beta3$inhibitors suppress the aggregation of platelets by preventing fibrinogen binding to $\alpha_{IIb}\beta3$receptors. There are three common $\alpha_{IIb}\beta3$inhibitors (Abciximab, Tirofiban and Eptifibatide) (Judy W. M. Cheng, 2012). All of these drugs can only be used intravenously and therefore they are not suitable for chronic therapy (Ajjan and Grant, 2006b). The work of Roffi M and his group, three decades ago, indicated that $\alpha_{IIb}\beta3$ inhibitors are effective in reducing 30 day mortality after acute coronary syndromes (ACS), especially in patients with diabetes (Roffi et al., 2001). However, theIntracoronary Stenting and Antithrombotic Regimen: Is Abciximab a Superior Way to Eliminate Elevated Thrombotic Risk in Diabetics [ISAR-SWEET] trial conducted on 701 patients with diabetes and ACS undergoing percutaneous coronary intervention (PCI), showed there is no reduction in one year mortality after treatment with abciximab and high dose clopidogrel (600mg) compared with clopidogrel alone (Mehilli et al., 2004). In addition eptifibatide and tirofiban did not show a significant benefit in patients with diabetes (Schneider, 2011). This was not gone unchallenged as others have shown that individuals treated with

abciximab (both diabetes and non-diabetes subjects) have better clinical outcome. The reason for the contradictory results is almost certainly due to the heterogeneity of patients with ACS and, therefore, the large number of patients required to make concrete conclusions, which would allow subgroup analysis (Ndrepepa et al., 2006).

1.8 Aspirin (the Inhibitor of the thromboxane pathway): the evidence, mode of action and role of aspirin

Aspirin, or acetylsalicylic acid (ASA), is one of the salicylate drugs with a formula C9H8O4 (PubChem, 2004) It was first produced by Felix Hoffman in 1897 as an anti-pyretic and anti-inflammatory and it was not until 1971 that the antithrombotic properties of this drug were discovered by Sir John Vane (Tantry et al., 2009). Although the role of aspirin for primary CVD prevention is questionable, particularly in those with diabetes, it remainsan important agent for secondary prevention (Szczeklik et al., 2005).

1.8.1 Platelet and aspirin

Aspirin saved the lives of millions of CVD patients(Antithrombotic Trialists' Collaboration(ATT, 2002)). ASA acts by acetylation of platelet cyclooxygenase-1(COX-1) at position serine 529. This chemical modification inactivates the enzyme and as a result cyclic endoperoxide (prostaglandin G2 “PGG2” and “PGH2”) production from arachidonic acid is reduced. Consequently, this reduction in PGG2 and PGH2 leads to reduction of prostanoid thromboxane A2 (TXA2) synthesis, a potent vasoconstrictor and platelet activator (Roth and

Calverley, 1994). The action of aspirin is irreversible and lasts till the end of platelet life (Angiolillo and Suryadevara, 2009)

In addition to this “traditional” mode of action for aspirin, this agent can acetylate fibrinogen resulting in the formation of a more porous fibrin network that is easier to lyse. Earlier work has shown that acetylation of fibrinogen occursat both the D and E domains and on α, β and γ chains where N-acetyl-lysin formed (Bjornsson et al., 1989). However, a more recent study suggested that acetylation occurs preferentially in the α-chain of fibrinogen (Ajjan et al., 2009). Furthermore, aspirin can also affect other coagulation factors resulting in altered thrombin generation and FXIII activity (Undas et al., 2007).

1.8.2 Aspirin and the fibrin clot

The fibrin clot formed from purified fibrinogen after incubation with aspirin *ex vivo* showed increased gel porosity, making these clots less thrombotic (He et al., 2001). *In vivo* administration of aspirin in healthy controls also resulted in altered clot structure, that was particularly noticeable with lower aspirin dose (Antovic et al., 2005, Williams et al., 1998). More recent work employed a recombinant system to test the effect of aspirin on the fibrinogen molecule. Chinese Hamster Ovary (CHO) cells that have been transfected with the three chains of fibrinogen were grown in the presence and absence of increasing concentrations of aspirin. Fibrinogen was then purified from culture media and fibrin clots were made. Clots made from recombinant fibrinogen produced by CHO cells in the presence of aspirin showed less compact structure and reduced resistance to fibrinolysis, which was due, at least in part, to acetylation

of the fibrinogen molecule (Ajjan et al., 2009). The different modes of action of aspirin are summarised in Figure 1.6.

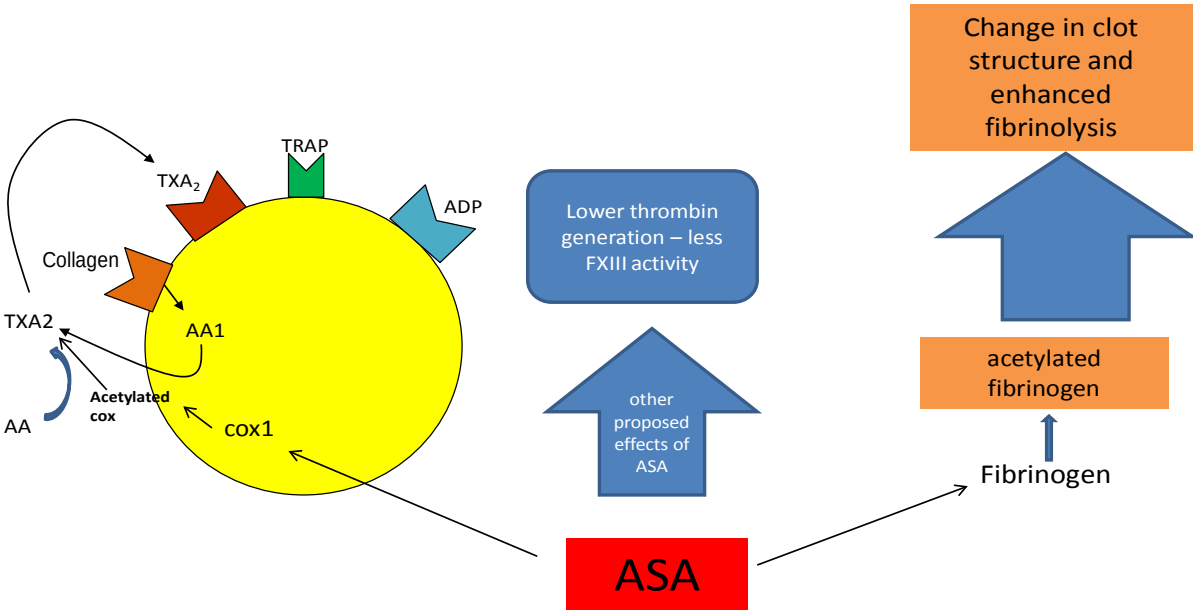

Figure 1.6 The effects of aspirin on platelet function, coagulation factor activity and clot structure/fibrinolysis. When aspirin acetylates cyclo-oxygenase (Cox)-1, the production of the platelet agonist thromboxane A2 (TXA2) is diminished, thereby inhibiting platelet reactivity. Aspirin acetylates fibrinogen and the resulting fibrin network is less compact and easier to lyse. Aspirin has another proposed effect on lowering thrombin generation and may also affect FXIII activity. ASA: acetyl salicylic acid, Cox: cyclo-oxygenase, TXA2: thromboxane A2, AA: arachidonic acid, TRAP: thrombin receptor-activating peptide ADP: adenosine diphosphate, a2B1, 5HT2a,P2Y12: Different platelet receptors.

1.8.3 Diabetes and aspirin treatment

The term aspirin variable response has recently appeared in the literature and generated significant interest. This can be divided into biochemical and clinical aspirin resistance. The former is defined as incomplete inhibition of platelet

function by aspirin, measured using various platelet function assays (Watala et al., 2004). Given the lack of a consensus over the best assay to measure biochemical aspirin resistance, the prevalence is hugely variable comparing different studies ranging from 5.5-45% (Pamukcu, 2007). Clinical aspirin resistance is defined by some as “the occurrence of an arterial thrombotic event despite aspirin therapy” (Michelson et al., 2005) However, others argue that occurrence of an ischaemic event despite aspirin treatment is perhaps better described as aspirin treatment failure.

The exact cause(s) for aspirin resistance is still not clear but likely to include a mixture of environmental and hereditary factors (Watala et al., 2004, Martin and Talbert, 2005). For example, cigarette smoking leads to increased platelet reactivity and aggregation, which is related, at least in part, to lower platelet NO production in long term smokers (Ichiki et al., 1996) secondary to inhibition of eNOS mRNA expression (Shimasaki et al., 2007).

In patients with diabetes, it is now generally accepted that the clinical effects of aspirin are compromised (reviewed by (Ferreiro et al., 2010). The exact mechanisms for this phenomenon are unknown, but there are a number of hypotheses, which are discussed in detail below.

1.8.4 Aspirin treatment failure

Early evidence for reduced clinical efficacy of aspirin in diabetes came froma meta-analysis including 287 studies (135000 patients). The authorsdocumented that antiplatelet treatment (aspirin in most studies) was associated with a significant 22% risk reduction in vascular event when used for primary prevention, but this figure was much smaller, and non-significant, at 7%,

(Antithrombotic Trialists' Collaboration (ATT, 2002)).

The Primary Prevention Project (PPP) is an early randomised open label trial that demonstrated that patients with diabetes do not benefit from aspirin when used for primary CVD prevention (Sacco et al., 2003). More recent work by Belch and colleagues (POPADAD trial) have also arrived at the same conclusions that low daily dose aspirin (100mg/day) fails to provide primary CVD protection (Belch et al., 2008). However, this study has been repeatedly criticised, mainly due to lack of adequate power and the inclusions of patients with evidence of peripheral vascular disease, making it a secondary prevention study (Elwood, 2008). Finally, the JPAD study using low-dose aspirin also concluded that this agent has little benefit in term of primary CVD prevention, at least in Japanese (Ogawa, 2011). Subgroup analysis suggested that some groups may benefit, further highlighting the difficulties encountered when studying a highly heterogeneous population such as individuals with type 2 diabetes.

Ongoing and adequately powered clinical studies such as ASCEND and ACCEPT-D, which will be reporting in the next 2-3 years, should be able to shed more light on the role of aspirin in primary prevention in diabetes and hopefully will also identify patient subgroup(s) that benefit the most from this form of therapy.

The reduced clinical efficacy of aspirin in diabetes is related to a number of factors. Some of these factors are non-specific, related to the general increase in thrombosis potential in diabetes, whereas others appear to be specific arising secondary to direct interactions between glycaemia and response to aspirin therapy.

1.8.5 High platelet reactivityrole in aspirin treatment failure

Even in the absence of vascular injury, platelets of subject with diabetes appear to be in a state of constant low-grade activation (Watala, 1991, Winocour, 1992).

The non-enzymatic glycation of platelet proteins can alter the physio-chemical properties of cell membranes, making platelet more “sticky” (Watala, 1991, Watala et al., 1998). Also, patients with diabetes show high platelet Ca^{2+} influx in the resting condition and higher Ca^{2+} mobilisation secondary to activation with ADP or thrombin (Mazzanti et al., 1990, Watala et al., 1998).

1.8.6 Interaction between glycation and acetylation role in aspirin treatment failure

In diabetes, increased glycation of platelets and coagulation proteins glycation may interfere with the acetylation process by aspirin (Rendell et al., 1986, Swamy-Mruthinti and Carter, 1999, Watala et al., 2005). Watala and his group have shown that in animal models of streptozotocin-induced diabetes platelet response to aspirin is blunted compared with non-diabetic animals, likely to be related to documented reduced acetylation of platelet protein (Watala et al., 2006). Given the concept of competition between glycation and acetylation in diabetes, it is valuable to analyse whether glycaemic control or increased aspirin dose enhance the efficacy of aspirin (Watala et al., 2004, Takahashi et al., 2007). Using optical platelet aggregation and platelet function analyzer (PFA)-100 techniques, Gum and colleagues found no differences in biochemical

aspirin resistance comparing individuals with and without diabetes with higher doses of 325 mg/day aspirin (Gum et al., 2001).

1.8.7 Increased platelet turnover role in aspirin treatment failure

In patients with diabetes, an increase in platelet turnover is represented by an increase in circulating reticulated platelets, which are more sensitive to activation (Guthikonda et al., 2008). Pharmacokinetic studies have shown that plasma concentration of aspirin after 80 mg/day oral dose reaches 1ug/ml after 30 minutes and declines rapidly witha terminal half-life of 0.4 hrs. Salicylic acid (SA), the aspirin metabolite,forms rapidly and peaks at ~4ug/ml within approximately 1hr with a terminal half-life of 2.1 hrs (Benedek et al., 1995). Given this relatively short half-life of aspirin and the documented increase in platelet turn-over in diabetes, some platelets may “escape” inhibition by aspirin over 24 hours. This concept is gaining momentum as a mechanistic explanation for the reduced clinical efficacy of aspirin in diabetes (Guthikonda et al., 2007).

1.8.8 Possible compromised fibrinolytic effects

As explained above, enhanced fibrinolysis by aspirin represents a non-platelet pathway for the antithrombotic action of this agent (He et al., 2001). It is possible that interaction between acetylation and glycation impairs the fibrinolytic effects of aspirin in diabetes, which may contribute to the reduced clinical efficacy. However, there is a lack of studies investigating this possibility.

1.9 Hypothesis and Aims

Although aspirin was previously recommended for primary cardiovascular protection in diabetes, this is not the case anymore due to the reasons outlined above. However, aspirin remains in use for secondary cardiovascular protection, although its efficacy in diabetes is questioned (Cubbon et al., 2008). Taken together, the antithrombotic effects of aspirin in diabetes appear to be significantly diminished. Several hypotheses have been forward to explain the reduced efficacy of this agent in diabetes, but no concrete conclusions have been made. Therefore, there is a need to better understand the mechanisms for this phenomenon, which will help to clinically tailor therapy thereby maximising benefits and reducing the risks associated with antiplatelet therapy.

Hypothesis

The reduced clinical efficacy of aspirin in diabetes is related to a compromise in the effects of this agent on both the cellular and fluid phase of coagulation, secondary to long term hyperglycaemia and increased protein glycation.

Aims of the project

The main aims of the project were to investigate the molecular basis for variable response to aspirin treatment failure in diabetes, which will hopefully help to identify those at risk and tailor therapy according to the need of each indevidual. Therefore, the main aim of this work was to investigate the *ex vivo* effects of aspirin on platelet function and on fibrin clot lysis in individuals with and without diabetes in the presence and absence of excess glucose. Secondary aims were to:

1. Study the *in vivo* effects of aspirin on platelet function and fibrin clot lysis in individuals with and without diabetes.

2. Investigate the effects of *ex vivo* and *in vivo* aspirin on platelet-fibrinogen interaction in individuals with and without diabetes.

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Chapter 2

General Methods

2.1 Sample collection and preparation

2.1.1 Patient recruitment

Blood samples from healthy donors were used to optimise the various experiments. Samples were then taken from patients with type 1 diabetes (T1DM) and sex/age-matched healthy controls after informed consent, see appendix.T1DM patients were chosen for the study rather than T2DM patients as the latter group represents a heterogeneous population of patients who are likely to be on a number of different treatments, thereby introducing confounding factors, making data analysis and interpretation problematic. Our T1DM patients were only on insulin treatment. A total of 29 T1DM patients and 29 controls were recruited after informed consent.

Exclusion criteria include: a history of acute coronary syndrome or stroke within 3 months of enrolment, prior treatment with aspirin, clopidogrel, warfarin or non-steroidal anti-inflammatory drugs, current treatment with any drug other than insulin, a history of deep venous thrombosis or pulmonary embolism, previous or current history of upper gastrointestinal pathology, malignancy or coagulation disorders. Any individual with abnormal LFTs (ALT>3 fold upper limit of normal) or abnormal TFTs are not included in the study. Finally, patients with proteinuria, advanced nephropathy, clinical signs of neuropathy or retinopathy (except for those with background changes) are excluded, to minimise the effects of confounding factors.

2.1.2 Sample collection and preparation

Blood samples were taken in the morning after a light breakfast, without a tourniquet. The first 5 ml of blood sample was used for clinical tests and the rest was collected for platelet experiments or to separate plasma samples. Samples were collected in tubes contain hirudin anticoagulant (Canyon, USA) for platelet aggregation test using platelet rich plasma light transimssionaggregometry and hirudin (Multiplate™, Germany) for platelet aggregation test using whole blood. For plasma fibrin clot analysis (turbidity analysis, scanning electron microscopy and confocal microscopy Images) blood was collected in tubes containing sodium citrate (Sigma). For plasma fibrinogen purification, blood samples were collected in lithium heparin tubes (Greiner, USA). For platelet fibrinogen interaction test, I tested binding of fibrinogen purified from patients and controls to platelets of healthy individuals collected in citrated dextrose tubes (Sigma). Citrate-based anticoagulants prevent the coagulation of blood by virtue of the citrate ion's ability to chelate ionized calcium present in the blood to form a non-ionized calcium-citrate complex. To investigate the *ex vivo* effects of glucose and aspirin [acetylsalicylic acid (ASA)]on platelet activity, tubes were prepared with 0 mg, 1 mg/l and 10 mg/l aspirin in the presence and absence of additional 20 mmol/l glucose. Samples were then incubated at 37°C for 15 minutes and then at RT for 15 minutes followed by the analysis of platelets activity in whole blood (Multiplate). For *in vivo* effect of aspirin on platelet activity using whole blood analysis, samples were collected at different visits (A, B, C, D) according the treatment plan, which will be discussed later and the test was done in the lab immediately after receiving the sample.

To investigate the *ex vivo* effects of glucose and aspirin on fibrin clot, whole blood was incubated with different concentration of aspirin (0, 1 and 10 mg/l) with and without extra glucose (20 mmol/l) for 30 minutes at 37°C and 30 minutes at RT, followed by separation of platelet poor plasma (PPP) that was aliquoted and stored at -80°C until analysis. To study the *in vivo* effects of aspirin on platelet function and fibrin clot structure, patients were randomised to 75 mg/d or 300 mg/d ASA for a period of 2 weeks with 3 weeks wash out period between the two treatments.Samples were collected at four different visits as detailed : In the first visit (visit A), no treatment was given; in the second visit (visit B), first half of the patients and controls were given 75 mg/day aspirin and the second half were given 300 mg/day aspirin for two weeks followed by three weeks wash out and sample collected in the third visit (Visit C). In the fourth visit (visit D), the first half of patients and controls were given 300 mg/day aspirin and the second half were given 75 mg/day for two weeks (i.e. cross-over study design). For fibrinogen purification, samples were collected in lithium heparin, PPP separated and stored at -80°C until analysis Sample collection, preparation and analysis is summarised in (Figure 2.1).

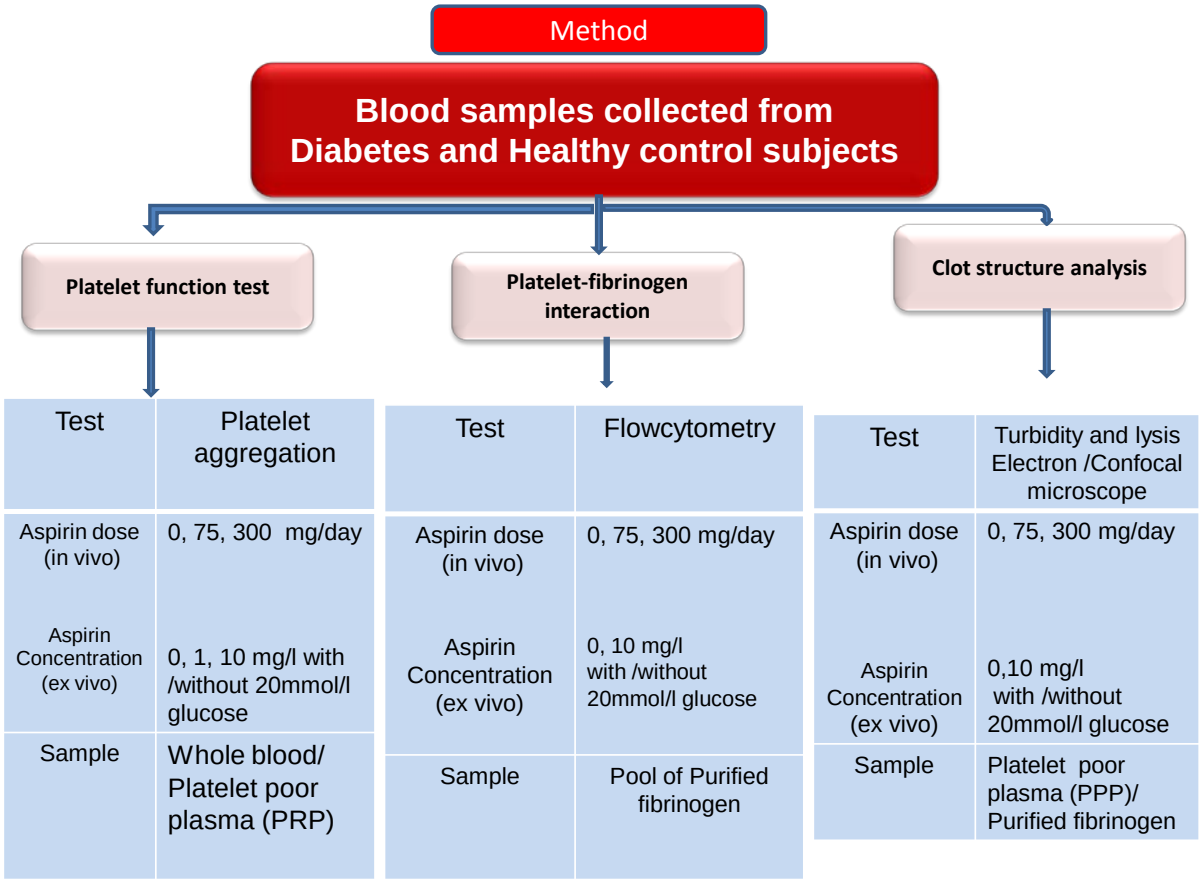


Figure 2.1 Sample collection, preparation and analysis. For platelet aggregation tests using whole blood, samples were collected in hirudin. For *ex vivo* studies, different concentrations of aspirin were added with and without additional glucose to whole blood. Platelet aggregations tests were performed using platelet rich plasma (PRP) as well as whole blood. Platelet fibrinogen binding assays, samples were collected in lithium heparin followed by fibrinogen purification and incubation with different concentrations of aspirin. In clot structure analysis, samples were collected in sodium citrate followed by incubation with different concentration of aspirin with and without extra glucose and clots developed from PPP were analysed. In testing the *in vivo* effect of aspirin, samples were collected from different visits (A-D).

2.1.3 Sample size calculation

Using previous data on platelet inhibition by aspirin treatment in patients with diabetes (Watala 2004 and 2005), a group size of n=26 is sufficient to detect a 15% difference in platelet activation, following arachidonic acid stimulation, comparing diabetes patients with controls, with a power of 80% (at p=0.05).

This is based on the assumption that within patient standard deviation of the response variable is 13%. This number of individuals is adequate to detect a 12% difference in turbidity analysis in response to antiplatelet agents with a power of 80% at p=0.05, based on the assumption that standard deviation for the response variable within patients is 10% as per our own data in 40 patients with type 1 diabetes. This number is also adequate to detect 14% difference in clot lysis time given the standard deviation of the response variable at 12% with a power of 80% ($p<0.05$). Power was calculated using approved online software (http://hedwig.mgh.harvard.edu/sample_size/js/js_parallel_quant.html) and it was double checked by a statistician. This software was developed by David Schoenfeld, Ph.D. with support from the MGH Mallinckrodt General Clinical Research Centre Massachusetts General Hospital, Boston.

Given these power calculations, the study is designed to test 30 patients to account for a drop out of 15%. Within group analysis will be carried out using paired *t*-test and between group analysis of mean changes in response to antiplatelet agents will be done using unpaired *t*-test for normally distributed data and the non-parametric Mann-Whitney test will be used for data that are not normally distributed.

2.2 Platelet aggregation

2.2.1 Platelet aggregation using light transmission aggregometry (LTA)

After incubation of blood samples for various time points with aspirin (Sigma), hirudin anticoagulated blood tubes were centrifuged for 10minutes at 200 rpm and 23^0C. Platelet rich plasma (PRP) was separated to be tested then the remaining red cell layer was centrifuged for 20 minutes at 1500 rpm and 23^0C.

500µl of the upper layer “platelet poor plasma” PPP was pipetted into glass tube and set as a baseline for the test. At 37°C, 240 µl of PRP was pipetted into glass tubes containing stir bars where 10 µl of one of the agonists: 10 µmol/l and 1 µmol/l ADP (Sigma), 1 mM AA (Chronolog) or 16 ug/ml collagen (Nycomed) was added and platelet aggregation recorded using LTAaccording to the standard operation protocol. The glass tube sits between a light source and a photocell. When an agonist is added the platelets aggregate and absorb less light and so the transmission increases and this is detected by the photocell and recorded as arbitrary Units (AU).

2.2.2 Platelet aggregation of blood using Multiplate

Aggregation of platelet collected in hirudin anticoagulated blood tube was performed within 90 mins of blood collection in case of testing the *in vivo* effects of aspirin. For *ex vivo* experiments, the test was conducted immediately after incubation with different concentrations of aspirin for the required period. The test was performed using Multiplate™. The response of platelet to different agonists was tested by adding 300 µl of pre-warmed 0.9% NaCl solution followed by adding 300 µl blood. After 3 minutes incubation time,, 20 µl of one of agonists, 6.4 µM ADP, 0.5 mM ASP or 3.2 µg/ml collagen (Multiplate) was added. When platelets aggregate, they accumulate on the electrodes and change the electrical resistance (or impedance). The change in resistance during the analysis is continuously recorded. The Multiplate®-system records a double determination that gives the average of two different readings recorded in Aggregation Unit (AU) (Figure 2.2)

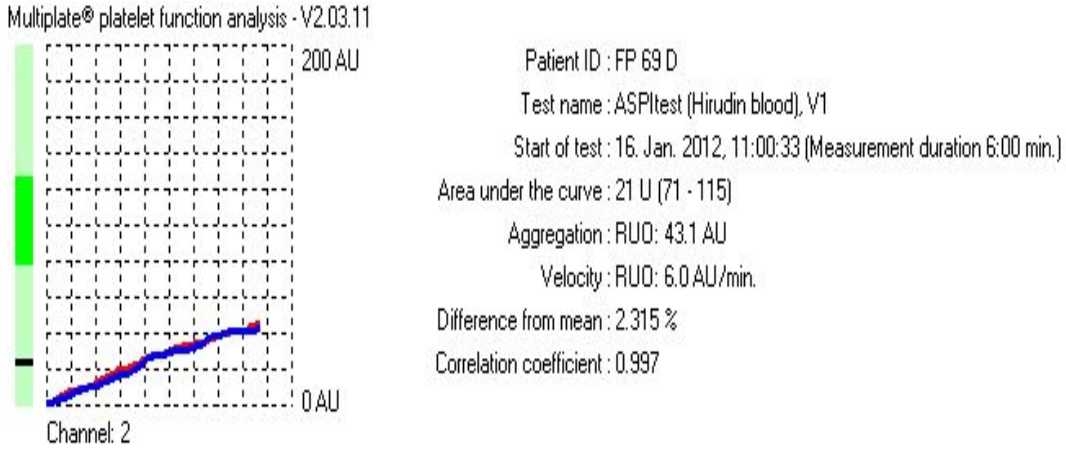


Figure 2.2 Example of the platelet aggregation analysis (Multiplate technique). I demonstrate platelet aggregationtest of one patientin response to aracidonic after *in vivo* treatment with 300 mg/day for two weeks. X axis represents time (S), Y axis represents aggregation unit (AU). Two readings are recoreded in the same time, redline and blue line. The final reading is the the verage between the two readings.

2.3 Fibrinogen purification

Purification of fibrinogen was performed by affinity chromatography using calcium-dependent IF-1 monoclonal antibody (Biomedical, Seattle, WA, USA) employing an automated chromatography system (Biocad sprint, Applied Biosystems Warrington, UK). One ml of heparinised plasma was mixed with 4 ml of equilibration buffer (0.02 M Tris, 0.3 NaCl and 1 mM $CaCl_2$, pH 7.4) and then filtered by a syringe driven filter unit (Millipore, Bedford, MA, USA) prior to loading onto the column. After sample application, the column underwent washing using 6 column volumes (CV) of 0.02 M Tris,1M NaCl,1 mM $CaCl_2$ pH 7.4 and 0.05 Na-acetate, 0.3 NaCl,1 mM $CaCl_2$, pH 6. Elution of fibrinogen was achieved by adding 6 CV of 20 mmol/LTris, 300 mmol/LNaCl, 5 mmol/Lethylenediaminetetraacetic acid (EDTA), pH 7.4 to the column. The chromatograph produced was used to estimate a peak of fibrinogen eluted. Serial tubes of elutant were tested using spectrophotometry by a ND-100 spectrophotometer (NanoDrop, Wilmington, DE USA), extinction coefficient

15.1. All those fragments with a protein concentration >0.05mg/ml were pooled and stored at -80°C.

2.3.1 Fibrinogen concentration

The eluted fibrinogen was pooled and centrifuged using Amicon Centriplus YM-100 regenerated cellulose centrifugal devices (Millipore, Bedford, MA, USA) for 1 hr at 3000 rpm at 25ºC until the volume in the filter reach 1ml.

2.3.2 Fibrinogen dialysis

Dialysis tubing (Sigma-Aldrich, St Loius, MO, USA) was prepared by boiling the tubing in 1 M EDTA. Pooled fibrinogen was dialysed via dialysis tubing cellulose membrane, size 10x6 mm against 50 $mmol^{-1}$ Tris, 100 $mmol^{-1}$ NaClpH 7.4 at 4ºC for 2 consecutive hrs and overnight with stirring (50rpm). Subsequently, the concentration of the protein was adjusted using a ND-1000 spectrophotometer and a coefficient ofE = 15.1

2.3.3 Integrity of fibrinogen samples (SDS page)

The integrity of all samples was checked using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Each sample (21 µl) was mixed with 7 µl 4 x LDS sample buffer (Invitrogen, Carlshad, CA USA) and 3 µl 10x sample reducing agent (Invitrogen, Carlshad, CA USA). The mixture were then reduced at 95ºC for 10 minutes to break the disulphide bonds and ran on a 4-12% Bis-Tris gradient gel, 1.5 mm x 10 well (Invitrogen, Carlshad, CA USA) using 20x MES SDS running buffer (Invitrogen, Carlshad, CA USA).

Gels were then stained using GelCode blue stain reagent (Pierce, Rockport, IL, USA) for 1 hour and subsequently washed with distilled water three times for 2

consecutive hours. and overnight. The gel was visualised using Chemi-imager software (Alpha Innotech, San Leandro, CA, USA) using a trans-white light. Three bands were expected (for the α, β, and γ chains of fibrinogen), and correct sizes verified using an appropriate ladder (66, 65 and 48 KDa respectively) as shown in Figure 2.2. Once the integrity and purity of the protein had been confirmed, the samples were aliquoted in 50-100 µl aliquots and stored at -80°C for further analysis.

2.4 Fibrinogen acetylation analysis

2.4.1 Materials

Buffers and reagents were prepared as follows: 1×TBS buffer was composed of 25 mM Tris base, 150 mM NaCl, 2 mM KCl at pH 7.4. 1×TBS Tween 20 was prepared by mixing 100 ml TBS with 50µl Tween 20.Blocking bufferwas prepared by adding 5 g milk powder (Marvel, Republic of Ireland, Dublin). Transfer buffer was made by adding 6.06 g Tris, 28.84 g Glycine, 400 ml methanol (Sigma) to 2 L distilled H_2O and pH 8.1-8.4. The buffer was stored at 4°c until use. Gels used were 4-12% Bis Tris (Invitrogen), NuPAGE SDS running buffer (20x) (Invitrogen).

2.4.2 Sample preparation

In order to determine which chain of fibrinogen is acetylated, pooled protein from patients or controls were incubated *ex vivo* with different concentrations of aspirin 0, 10 mg/l and samples that blotted using an antibody against acetylated-lysine residues (Cell signaling technology).

2.4.3 Running the gel

25 µl samples + 8.3 µl NuPAGE SDS sample buffer (Invitrogen) + 3.4 µl NuPAGE sample reducing agent (Invitrogen) were mixed and then heated for 5 minutes at 95°C. 15 ul of sample was loaded per well to a 4-12% Bis Tris gel (Invitrogen) and run for 45 minutes at 200V. Ladder used was low range BioRad 161-0306.

After electrophoresis, the gel was removed from the plastic casing and placed in transfer buffer until use. An appropriate sized membrane (pore size 0.45uM immobilon-P) was cut and activated by soaking in methanol for 15 seconds, distilled water for 2 minutes then in transfer buffer until use. The membrane was placed directly over the gel inbetween2 pieces of filter paper (pre-soaked in transfer buffer) and transferred for 1 hour at 100V.

The membrane was then removed and blocked for 1 hour at RT on a shaker. The blocker was then removed and replaced with primary antibody (for PT 1:1000 dilution, 10 ml) and then left in thefridge overnight. The primary antibody was Acetylated-lysine Antibody (cell Signalling, USA) which detects proteins posttranslationally modified by acetylation on the epsilon-amine groups of lysine residues. The membrane was then washed in TBS-T, 1x15 minutes and 2x5 minutes. The secondary antibody was added (anti-mouse IgG 1:4000) and the membrane was placed on a shaker for 1hour at room temperature and then washed as above.

2.4.4 Radioactive aspirin and fibrinogen

This work was led by a member of the team (Dr. Alzahrani), and my main role was to help conducting the experiments. A total of 25 µl of acetyl-1-

[^{14}C]salicylic acid ([^{14}C]ASA or ([^{14}C]aspirin), specific activity 55 Mci/mmol) supplied in from the American Radiolabeled Chemicals, St Louis, USA) was added to 25 µl of human purified fibrinogen from diabetes subjects or controls (at 0.5mg/ml). Aspirin labelled with ^{14}C at the carboxyl group was used as a negative control. The mixture was then incubated overnight in a water bath at 37°C. Subsequently, radiolabelled fibrinogen was run on NuPage (4-12%) Bis-Tris Gel (Invitrogen) slowly over 210 minutes to allow enough time for separation of fibrinogen chains, which were subsequently cut out separately. Gel pieces were then mixed with 12.5 ml of aqueous counting scintillant which contains Xylene and methanol (Amersham Bioscience UK). Finally, radioactivity was counted in disintegrations per minute using a liquid scintillation analyser (Tri-Carb 2800TR).

2.5 Fibrin structure

2.5.1 Fibrin clot turbidity and lysis (Clot formation test)

Clot turbidity measurements were conducted as described by Carter (Carter et al., 2007). Briefly, for turbidity measurements, plasma samples were thawed in a water bath at a temperature of 37° for 30 minutes. Tubes were vortexed and spun down for 30 seconds and then 25 µl of each sample was added to a 96 well Greiner plate (in duplicate). A total of 75 µl of assay buffer (0.05 mol/lTris-HCl, 0.1 mol/ NaCl, pH 7.4) was added to each well using multi-channel pipette. 50 µl of activation mix containing assay buffer, 0.0075 U/ml thrombin (Calbiochem) and 7.5 mmol/l $CaCl_2$was added to each well using multi-channel pipette at an interval of 10 seconds. The plates were shaken and read at 340

nm every 12 sec for 1 hr using ELX-808 IU ultramicroplate reader (BIO-TEK Instruments).

2.5.2 Clot formation and lysis test (turbidity and lysis)

In clot formation and lysis, plasma samples were thawed in a water bath at a temperature of 37° for 30 minutes. Tubes were vortexed and spun down for 30 seconds. 25 µl of plasma was added in a 96 well clear polystyreneflate bottom microplate (Grenier Bio-one, Gloucester, UK). Each sample was tested in duplicates, to ensure reproducibility and any variability that exceeds 10% was repeated. A total of 75 µl lysis mix (LM) containing (0.05 M Tris and 0.1 M NaCl and 2.4 nM tissue plasminogen activator, tPA; Technoclone, Vienna, Austeria) was added to each well using multi-channel pipette (Finnpipette Thermo scientific) at an interval of 10 seconds. The time of adding the lysis mix was recorded to adjust plate reader times to the start of the clot formation. Three minutes after adding the lysis mix to the first column, polymerisation and lysis were initiated by the addition of 50 µl activation mix, composed of 0.05 M Tris, 0.1M NaCl, and 0.09U/mlthrombin (Calbiochem, Nottingham, UK) and 22.5 mM $CaCl_2$ was then added using multichannel pipette at an interval of 10 seconds between each column. The plates were shaken and read in 32°C condition at 340 nm every 12 sec for 1 hour for clot formation and then every 2 minsfor up to 9 hrs for lysis time using EL_x-808 IU ultramicroplate reader (BIO-TEK Instruments INC,Winooski, VT, USA). In analysing the *in vivo* effect of aspirin on plasma clot, a special software application was used that provides several clot structure parameters and the following variables that represent the average of two readings were recorded (Figure 2.3, 2.4). For purified fibrinogen

experiments, data were analysed manually as the software is not designed to handle these data.

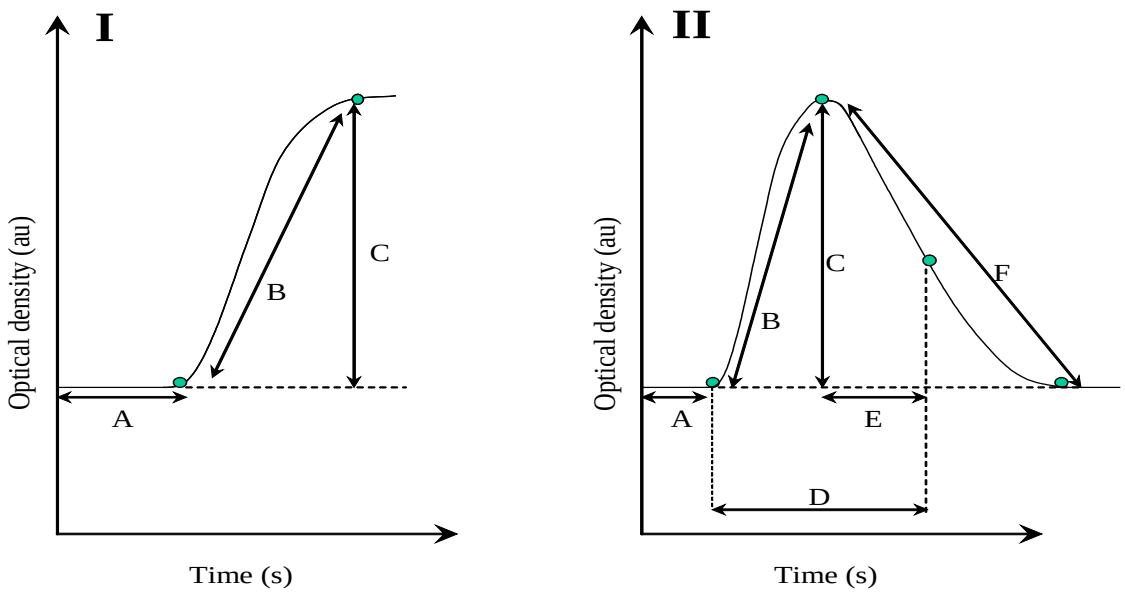


Figure 2.3 Different parameters used in the turbidimetric analysis are summarised (LTA technique). I represents turbidity analysis, whereas II demonstrates turbidity and lyses. A: lag phase. B: rate of clot formation. C: maximum turbidity (absorbance).D: time from clot formation to 50% lysis. E: time from full clot formation to 50% lysis. F: rate of clot lysis (Figure kindly provided by my supervisor).

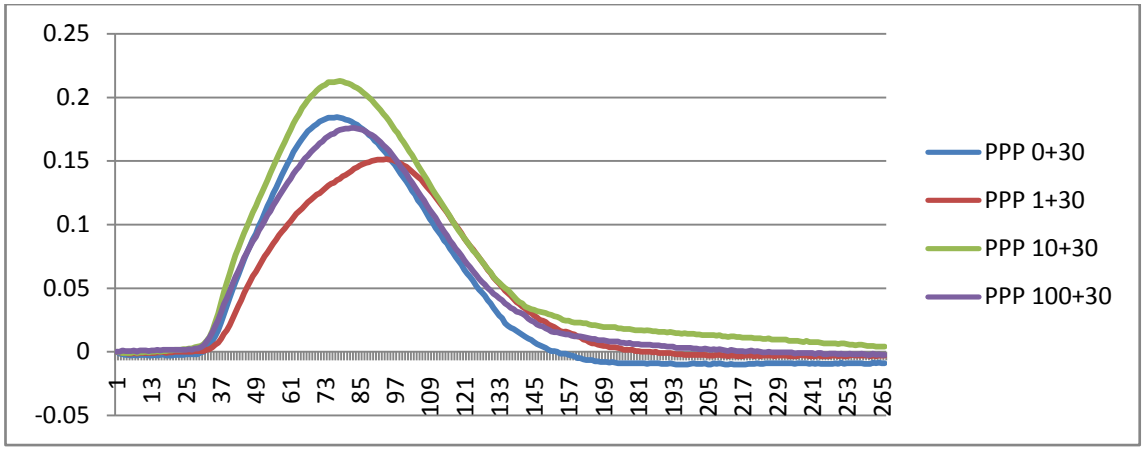


Figure 2.4 Example of the turbidimetric analysis of optimisation experiment (LTA technique). I demonstrate turbidity and lyses analysis of experiment that used to optimise the effective *ex vivo* concentration of aspirin 0,1,10,100 mg/l incubated for 30 minutes. X axis represents time (S), Y axis represents optical density.

2.5.3 Clot formation and lysis test using purified fibrinogen

In this assay, turbidity and lysis of fibrin clot developed from a pool of purified fibrinogen from subjects with diabetes and a pool of purified fibrinogen from control subjects were used. 50 µl of purified fibrinogen at 0.6 mg/ml was mixed with 50µl LM, containing 0.2 µM plasminogen (Enzyme Research Laboratories, Swansea, UK) in 0.1 M NaCl, 0.05 M Tris, pH 7.4 (permeation buffer [PB]), in a Greiner 96 well clear polysteren flat bottom microplate in duplicate. To ensure adequate crosslinking, 5µl of FXIII (20µg/ml, 1.56 nM) was added. Polymerisation and lysis were initiated by the addition of 50 µl AM containing 0.37 U/ml thrombin, 3.3 nM tPA and 7.5 mM $CaCl_2$ in 0.1 M NaCl, 0.05 M Tris, pH 7.4. Increase in turbidity at 340 nm was continuously monitored every 12 seconds on ELx-808 IU ultramicroplate reader over a period of 60 minutes.

2.6 Clot image analysis by microscopy (laser scanning confocal microscopy, LSCM)

Confocal microscopy was used to visualise the architecture of hydrated clots and to study lysis of mature fibrin networks. In contrast to scanning electron microscopy, samples do not need fixing and dehydration, and therefore they are viewed in a more physiological status.

2.6.1 Sample preparation for LSCM (plasma fibrin clots)

For confocal image of plasma fibrin clots, preparation was made as follows: 7.5 µl of plasma was mixed with 63.2 µl PB (50 mmol/l Tris and 100 $mmol^{-1}$ NaCl and pH 7.4) followed by the addition of 0.036 mg ml^{-1} fluorescent labelled fibrinogen (Alexa 488; Invitrogen, Manchester, UK). Activation mixture (AM), containing 0.035 M $CaCl_2$ (final concentration 5 mmol/l), human thrombin (Calbiochem, final concentration 0.35 u/ml)and 63.2 µl permeation buffer was prepared. 5 µl of this (AM) was added to 30 µl Alexa stained plasma. Then immediately, the resulting mixture was pipetted into ibidi slides (Applied Biophysics, New York, USA).

2.6.2 Sample preparations for LSCM (purified fibrinogen clots)

For confocal image of fibrin clot produced from purified fibrinogen, 19.5 µl of control fibrinogen was added to 9.5 µl permeation buffer followed by adding 0.036mg ml^{-1}fluorescent labelled fibrinogen. Activation mix (AM) was prepared by ading4.9µg/mlfactor XIII,5 mmol/l $CaCl_2$ and 0.05 U/ml thrombin to 61.2 µl permeation buffer. 5 µl of this (AM) was added to the Alexa stained fibrinogen. Then immediately, the fibrinogen pipetted into the Ibidi slides, which was

subsequently kept in a humidified environment to preserve the hydrated clot until viewing by confocal microscope (LSM 510 META microscope from Carl Zeiss; UK)

2.7 Imaging fluorescent samples

Images were viewed under 40×/1.3 oil ph3 objective lens. Z-stacks images were captured at 20 µm intervals for 10 images (2 µm intervals for each image). The scan format was 512×512 pixels and the pinhole set to 90 µm.

2.7.1 LSCM clot lysis

To monitor clot lysis, time series function was used with 400 scans with 1 second between scans. 20 µl of lysis mix, composed of 2 µl tissue plasminogen activator (10 µg/ml), 2 µl plasminogen (0.1mg/ml) and permeation buffer (50 mmol/l Tris and 100 $mmol^{-1}$ NaCl and pH 7.4), was added to the edge of the clot and the slide tapped gently for 30 seconds to encourage the lysis mix into the clot channel. The slide was then left for 3 minutes in a vertical position before loading onto the stage and clot visualisation. After 3minutes, lysis was monitored and recorded using LSM image browser (Zeiss).

2.7.2 Scanning electron microscopy

Although scanning electron microscopy requires clot manipulation (fixing and dehydration) it remains the best technique to investigate fibrin network ultrastructure.

Clot preparation

A lid from 0.5 ml Eppendorf tube was cut off and pierced with needle several times before covering the bottom of the lid with parafilm in order to cover the holes. For plasma clots, 50 µl of plasma was diluted 1:2 with PB and 5 µl AM (0.05 M $CaCl_2$, 11 U/ml thrombin in PB) added. Thiswas mixed with a 200 µl tip that had the end removed to ensure even distribution of the AM. For purified fibrinogen clots, 45 µl of fibrinogen (0.5 mg/ml) was mixed with 5 µl of AM (0.025 M $CaCl_2$, 5 U/ml thrombin in PB). The clots were left to form in the lid for 2 hours in a humidified chamber.

Fixing and dehydration

After removing the parafilm, the clots were washed by placing the lids containing the clots in a beaker of sodium cacodylate buffer (0.078 M Cacodylic acid (Sigma), pH 7.4) for 10 minutes, which was then replaced with fresh buffer and left for a further 10 minutes. The clots were then fixed by transferring the lids to 2% Glutaraldehyde (Sigma) in sodium cacodylate buffer for 30 minutes followed by washing steps as above. Once fixed, the clots were dehydrated by placing them in acetone at increasing concentrations, 30%, 50%, 70%, 80%, 90% and 95% for 10 minutes each, after which they were transferred to 100% acetone 3 times for 10 minutes.

Critical point drying

I was unable to perform critical point drying (CPD) which was carried out by Martin Fuller at the University of Leeds using a E3000 critical point dryer (Quorum Technologies Ltd, UK). This involves dehydrating the fibrin clot in

order for it to be coated with platinum and be detected bythe EM. It is crucial not to disturb the surface of the fibrin network during the drying process as EM relies on analysing the surface morphology of biological specimens. Air drying is, therefore, unsuitable as it may lead to deformation and collapse of protein structures as water has high surface tension to air. As liquid in the sample evaporates, the specimen is subjected to large forces at the boundary between the water in the sample and surrounding air. By replacing the water in the sample with liquid $CO_{2,}$ this surface tension is reduced, and the surface tension between the liquid in the sample and surrounding air is progressively lowered. Liquid CO_2 then passes from liquid to gas witoutan abrupt change in state and avoids damaging the surface of the sample.

Sputter coating

In order to prevent the clots from charging under the electron beam, the samples were covered with a thin layer of conducting metal. The clots were mountedonto aluminium specimen mounts that were covered in a carbon film. They were then coated with a 7 nm thick layer of platinum applied in 208 HR high resolution sputter coater (Cressington, UK).

Sample imaging

Samples were viewed and photographed using a Quanta 200F FEGESEM field-emission scanning electron microscope (FEI, Oregon, USA) in 10 different areas of each clot, at themagnification (x10000). Images were captured using FEI's own software, designed to run with the EM. Fiber diameters of all clots

were determined using image analysis software package ImageJ 1,23y (Ajjan et al., 2009).

2.8 Platelet-fibrinogen interaction studies

Platelet–fibrinogen interaction measurement was based on the methods for measurement of platelet adhesion in microtiter plates technique (Bellavite, 1993, Oberprieler et al., 2007).

2.8.1 Static platelet-fibrinogen interaction

100 µl of 1 mg/ml fibrinogen (Calbiochem) was added to each well of microtiter plate (Greiner) for at least 24 hrs at 4°C before performing the test. Ten ml blood tube with 10% ACD anticoagulant (113.8 mM D-Glucose, 29.9 mMTris-Na citrate, 72.6 mM NaCl, 2.9 citric acid pH 6.4) was centrifuged at 900 rpm at 20°C for 20 mins. PRP was then transferred into a falcon tube and 50 ng/ml final concentration of prostaglandin E1 “PG E1” (Sigma) was added followed by centrifugation at 1900 rpm 20°C for 12 minutes. The supernatant consisting of PPP was then removed and tube was left to drain. One ml of modified tyrode’s buffer MTB which consists of (15 mM NaCl, 5 mM HEPES, 0.55 mM NaH2PO4, 7 mM NaHCO3, 2.7 mM KCl, 0.5 mM MgCl2, 5.6 mM D-Glucose anhydrous, pH 7.4) was added to platelet pellet. Manual platelet count “according to manual platelet count protocol” was performed and the platelet pellet solution was titrated with MTB to reach 1×10^{8} platelet/ml final pellet concentration. Platelet suspension was left for 1 hr to be recovered from PGE1 followed by incubation with 200 µM RGDS (Tocris) for 20 minutes in 37°C. Meanwhile, fibrinogen was washed from the wells by PBS followed by blocking uncovered plastic surface with 5% human serum for 30 min at room temperature. After a washing step,

100 µl of platelet suspension was added to each well and incubated for 1 hr. Wells were washed and 150 µl of citrate buffer added [containing 29 mM citric acid, 68 mM sodium citrate dehydrate, 5 mM p-nitrophenyl phosphate (Sigma) and 0.1%Triton×100 (Sigma) pH 5.4]. After 1 hr incubation, 100 µl of 2M NaOH was added to stop the reaction and wells were read at 405 nm using ultramicroplate reader.

2.8.2 Flow cytometry

Flow cytometry experiments require fibrinogen to be fluorescently labelled and similar concentration used to test for binding to platelets.

Labelling of fibrinogen

A buffer free of ammonium ions or primary amines is essential in order to successfully undertake the labelling process and to avoid competition with the amine groups of the buffer with the reactive dye. Purified fibrinogen was therefore dialysed by slide-A-Lyzer MINI Dialysis Devise (Thermo Scientific, Loughborough, UK) in a beaker containing 1 L phosphate-buffered saline (PBS).

After dialysis of fibrinogen, the protein was labelled using Alexa Fluor 647 monoclonal antibody labelling kit (Invitrogen) as per manufacturer's instructions. Briefly, 1 M solution of sodium bicarbonate (pH 8-9) was prepared by adding 1 ml of deionised water (dH_2O) to the provided vial of sodium bicarbonate. To raise the pH of the reaction mixture, $1/10^{th}$ volume of 1 M sodium bicarbonate buffer was added to fibrinogen solution. Subsequently, 100 µl of the fibrinogen solution, with concentrations ranging between 0.5-0.6mg/ml was added to the

reactive dye then capped and gently inverted a few times to fully dissolve the dye. The solution was then incubated for 1 hour at room temperature. The vial was gently inverted every 10-15 minutes several times to mix the two reactants and increase the labelling efficiency. During the incubation period, the spin column for the purification was prepared by stirring the purification resin. One ml was addedonto the column and allowed to settle by gravity. More of the suspension was added until the resin bed volume reached ~1.5 ml. The column buffer was allowed to drain by gravity. To elute first drops of buffer, the spin column was put in one of the collection tubes and centrifuged for 3 minutes at 1100×*g*. The 100µl reaction solution (labelled fibrinogen after 1hr incubation) was loaded drop wise onto the centre of the spin column. The solution was allowed to be absorbed into the resin bed. The spin column was then placed into empty collection tube and centrifuged for 5 minutes at 1100×g. After centrifugation, the collection tube contained labelled fibrinogen in approximately 100 µl of PBS, pH 7.2 with 2 mM sodium azide. Free dye remained in the column bed and was not eluted.

Measuring fibrinogen concentration

Protein concentration was estimated three times using the NanoDrop. The labelled fibrinogen was then stored at -20° C and protected from light.

Human blood was taken from healthy volunteers by clean venepuncture into acid citrate dextrose (29.9 mM sodium citrate, 113.8 mM glucose, 72.6mM sodium chloride and 2.9mM citric acid, pH 6.4) as anticoagulant in a ratio of 1:5 (vol:vol).In 3 ml tubes, 5 µl of blood from the healthy donor was added to 50 µl modified tyrode's buffer (MTB) which consists of (15 mM NaCl, 5 mM HEPES,

0.55 mM NaH2PO4, 7 mM NaHCO3, 2.7 mM KCl, 0.5 mM MgCl2, 5.6 mM D-glucose anhydrous, pH 7.4) solution containing 50 µg/ml final concentration of labelled fibrinogen from each sample (it was prepared according to labelled fibrinogen concentration readings, as mentioned in Table 2-1). A total of 2µl of FITC mouse CD42b antibodies was added to platelet detection tubes. To stop $\alpha_{IIb}\beta 3$ receptors from binding to fibrinogen, 2 µl of ethylene glycol tetra-acetic acid (EGTA, 100 mM) was added to a tube to provide a negative control for fibrinogen binding (final concentration of EGTA was 4mM). The tubes were then flicked gently three times and fibrinogen negative control was left for two minutes. This was followed by the addition of 5 µl ADP to each tube at a final concentration of 4 uM in a timing step. After exactly 10 minutes, the reaction was fixed with 0.5 ml of 0.2% formal saline (See table 2-2). The samples were then kept at 4°c for 20 minutes before undertaking the analysis.

Sample (aspirin/glucose)	Fibrinogen concentration (mg/ml)	Sample volume to be added	Permeation buffer volume Ul
Control	0.26	9.6	40.4
Control+aspirin	0.22	11.4	38.6
Control+glucose	0.22	11.4	38.6
Control+glucose+aspirin	0.20	12.5	37.5
Patient+0mg	0.24	10.4	39.6
Patient+10mg	0.25	10	40
Patient+glucose	0.24	10.4	39.6
Patient+g+10	0.24	10.4	39.6
Control+75mg	0.45	5.6	44.4
Control+300mg	0.39	6.4	43.6
Patient+75	0.41	6.1	43.9
Patient+300	0.53	4.7	45.3

Table 2.1 Volume of labelled fibrinogen added to MTB solution to achieve a final fibrinogen concentration of 50 ug/ml.

	Fibrinogen negative	Platelet detection	Sample	
			Basal	With ADP
C/S + labelled fibrinogen	50 ul at 50 ug/ml final concentration of fibrinogen	50 ul of c/s only	50 ul	50 ul
EGTA (100mM)	2 ul	×××	×××	×××
FITC CD42b	×××	2 ul	×××	
Blood	5 ul	5 ul	5 ul	5 ul
ADP (100uM) (in timing process)	×××	×××	×××	5ul
F/Saline (after 10 min)	0.5 ml	0.5 ml	0.5 ml	0.5 ml

Table 2.2 Steps undertaken for conducting flow cytometry experiments of labelled fibrinogen.

Running flowcytometry test

Measurement of the binding of labelled fibrinogen with platelets was carried out by single-colour analysis. Platelets were identified on the basis of their forward scatter (FS) and side scatter (SS) and then analysed for binding with APC labelled fibrinogen. To exclude readings of platelet-fibrinogen binding using receptors other than $\alpha_{IIb}\beta 3$, tubes containing blood, fibrinogen and EGTA inhibitor $\alpha_{IIb}\beta_3$ were run and the population was determined. To exclude false

autofluorescence coming from platelets and to determine platelet populations, tubes containing blood and CD42b were run and the fluorescence was determined. On the plot of FS and SS, a gate was drawn around the platelet population. Ten thousand platelet events were collected and a histogram of fluorescence gated on the platelet region was drawn. Results are presented as percentage of fluorescent platelets as well as median fluorescence for each sample.

Chapter 3

Effects of glycation and blood glucose levels on platelet response to aspirin therapy

3.1 Introduction

The efficacy of aspirin in diabetes is compromised but the exact mechanisms responsible remain largely unclear. The reasons for reduced efficacy of aspirin, often termed aspirin variable response, can perhaps be broadly categorised into pharmacodynamic and pharmacokinetic factors (Weber et al., 2002). Pharmacokinetic causes explore reasons for insufficient blood concentration of aspirin whereas, pharmacodynamic factors investigate incomplete suppression of platelet activity despite sufficient plasma drug levels (Benedek et al., 1995, Cerletti et al., 2003). Benedek et al 1995 have assessed the concentration of aspirin after 80 mg/day oral dose of aspirin within 0, 4 and 24 hrs of drug administration in healthy controls. The investigators found that plasma aspirin, or acetyl salicylic acid (ASA), concentration reached 1ug/ml after 30 minutes of drug administration and then started to decline rapidly withthe terminal half-life of 0.4 hrs. Salicylic acid (SA), the main ASA metabolite, was rapidly formed to reach ~4ug/ml within approximately 1hr and had a terminal half-life of 2.1 hrs. An interesting observation in this study was that pharmacokinetic and platelet aggregation sensitivity to aspirin showed noticeable intra- and inter-subject variability. This heterogeneity in aspirin concentration between different individuals, add to the complexity in unravelling the causes for the reduced efficacy of aspirin in high-risk vascular patients.

Concentrating more specifically on patients with diabetes, previous work by Watala et al 2005 has shown a potential interaction between glycation and acetylation as a cause for the reduced clinical efficacy of aspirin in diabetes (Watala et al., 2005). However, this study was conducted in older individuals with type 2 diabetes, with various confounding factors, and therefore the

observed changes could not be attributed to glycaemia alone. For example, some patients were on insulin therapy, whereas others were on oralhypoglycaemiatherapy with metformin and/or sulfonylureas. Also, their cardiovascular history was unclear and the presence of microvascular complications was not documented.

Therefore, studies that control for confounding factors are needed in order to understand the main mechanistic pathways responsible for the reduced efficacy of aspirin in diabetes. In order to address pharmacokinetic variability between individuals, potential effects of co-morbidities and various treatments, my aims in this chapter were to: i) develop an *ex vivo* system to enable the analysis of similar aspirin concentrations on platelet reactivity in young individuals with type 1 diabetes, not on any treatment other than replacement doses of insulin, ii) analyse the effects of high glucose concentration on platelet response to aspirin therapy in both groups, to differentiate between the effects of short and long-term glycaemia, iii) complement the work with *in vivo* studies to understand the clinical validity of the *ex vivo* techniques.

3.2 Materials and Methods

3.2.1 Patients and controls

For platelet aggregation experiment, a total of 25 T1DM patients and 26 controls were collected. I was planning to collect 29 samples from patients and 29 samples from controls but during the collection period and doing the experiment (which extended for two and half years). A total of 4 patients and 3 controls were lost to follow up due to reasons related to inconvenient timing of the samples or pulling out of the study for personal reasons. Therefore, the

attrition rate was 13.7% and 10.3% for patients and controls, respectively. Patients were recruited from the local diabetes clinic whereas healthy controls who were friends of the patients, medical students or University of Leeds employees acted as controls. The criteria of patients and controls selection was detailed in section 2.1.1

3.2.2 Sample collection

Blood samples were taken in the morning after a light breakfast, without a tourniquet. The first 5 ml of blood sample was used for clinical tests and the rest was collected for platelet experiments or to separate plasma samples. Samples were collected in tubes contain hirudin anticoagulant (Canyon, USA) for platelet aggregation test using platelet rich plasma light transmission aggregometry, which used in optimisation experiments and hirudin (Multiplate™, Germany) for platelet aggregation test using whole blood.

3.2.3 Sample preparation

To investigate the *ex vivo* effects of glucose and aspirin, tubes were prepared with 0 mg aspirin, 1 mg/l aspirin and 10 mg/l aspirin in the presence and absence of additional 20 mmole/L glucose. Samples were then incubated at 37°C for 10 minutes and then at RT for 20 minutes before analysis**.** Analysis of platelet aggregation was done within 90 minutes as described previously.

To investigate the *in vivo* effect of aspirin, blood samples were collected from both patients and healthy controls before giving aspirin (baseline). Then they were given 75mg aspirin daily dose for two weeks followed by collection of blood as detailed above. After a wash out period of three weeks, patients and

controls were given 300mg aspirin daily dose for two weeks followed by sample collection (Figure 3.1).

3.2.4 Assessment of platelet reactivity

After 30 minutes of incubation with aspirin, platelet aggregation in response to three agonists were analysed, which included arachidonic acid (AA), collagen and adenosine diphosphate (ADP). Details of the methodology are described in Chapter 2.

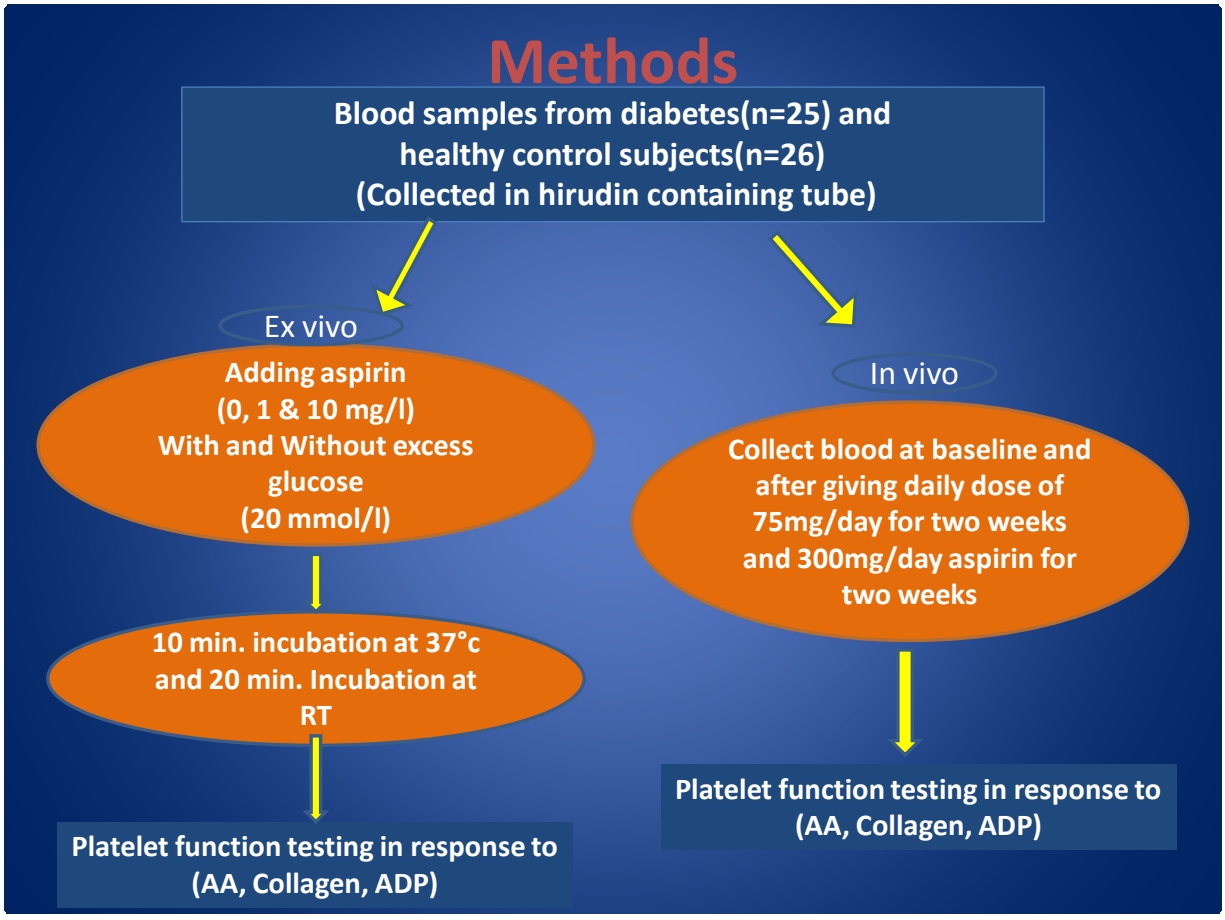


Figure 3.1 Sample selection and preparation. For *ex vivo* experiment, blood samples from diabetes and controls were collected at baseline. Different concentrations of aspirin were then added to whole blood samples (0,1,10 mg/l), with and without additional glucose, and incubated for 30 minutes. This was followed by testing platelet aggregation in response to arachidonic acid (AA),collagen and ADP. For *in vivo* experiments, blood samples from diabetes and controls were collected before and after treatment with aspirin 75mg/day and 300mg/day (two weeks each with three weeks wash out period). This was followed by analysing platelet aggregation to AA, collagen and ADP was tested.

3.3 Results

3.3.1 Patients and controls characteristics

Analysis of all patients (n=29) and controls (29) profiles has been done as shown in table 3.1

	Patients (n=29)	Controls (n=29)
Age	24±1.2	25 ± 1.3
Gender male/female	16/13	15/14
HbA1c	76 ± 3.6	37.4 ± 3
systolic blood pressure(SBP)	113 ± 2.4	114 ± 2
Diastolic blood pressure(DBP)	78 ± 2	76.8 ± 1.8
Body mass index (BMI)	24.9 ± 0.7	22.5 ± 1.3
Weight	73.6 ± 1.6	66.8 ± 3.5
Smokers	2	0

3.1 patients and controls charectristics

3.3.1 Optimisation of the *ex vivo* dose response and incubation time of aspirin using PRP (LTA technique)

In order to optimise the effective concentration of aspirin following 2 hrs incubation, aspirin had no significant effect on ADP or collagen-induced platelet aggregation, even at high concentrations. In contrast, aspirin resulted in

complete inhibition of aggregation to AA at 0.1 mg/l, starting with significant inhibition at 0.01 mg/l; p=0.02 (Figure3.2). The readings show the average of three repeats of experiment±SEM.

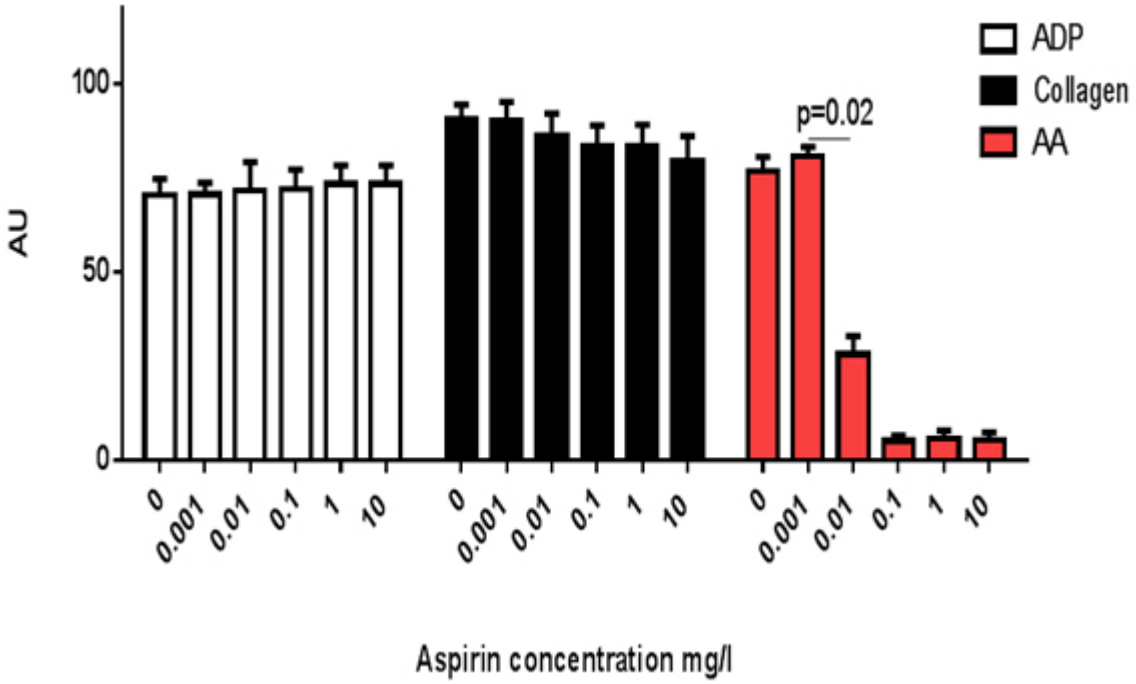


Figure 3.2 Aggregation of healthy control platelet in response to different agonists after incubation with aspirin using LTA technique. Platelet response to ADP (10 µmol/l; white columns), collagen (16 ug/ml; black columns) and AA (1 mM, red columns) after incubation with a range of aspirin concentrations (0-10 mg/l for 2 hrs. The readings show the mean±SEM of three repeat experiments. AU: arbitrary units.

3.3.2 Optimizing *ex vivo* time response of aspirin using PRP

As shorter incubation time is important to avoid platelet damage, time course experiments were conducted. Using 0.5 mg/l aspirin, partial inhibition occurred after 5 minutes p>0.05 and full inhibition to AA occurred after 30 minutes incubation p=0.02 in platelets from healthy volunteers, with no difference

observed in ADP or collagen response (Figure 3.3). The readings show the average of three repeats of experiment±SEM.

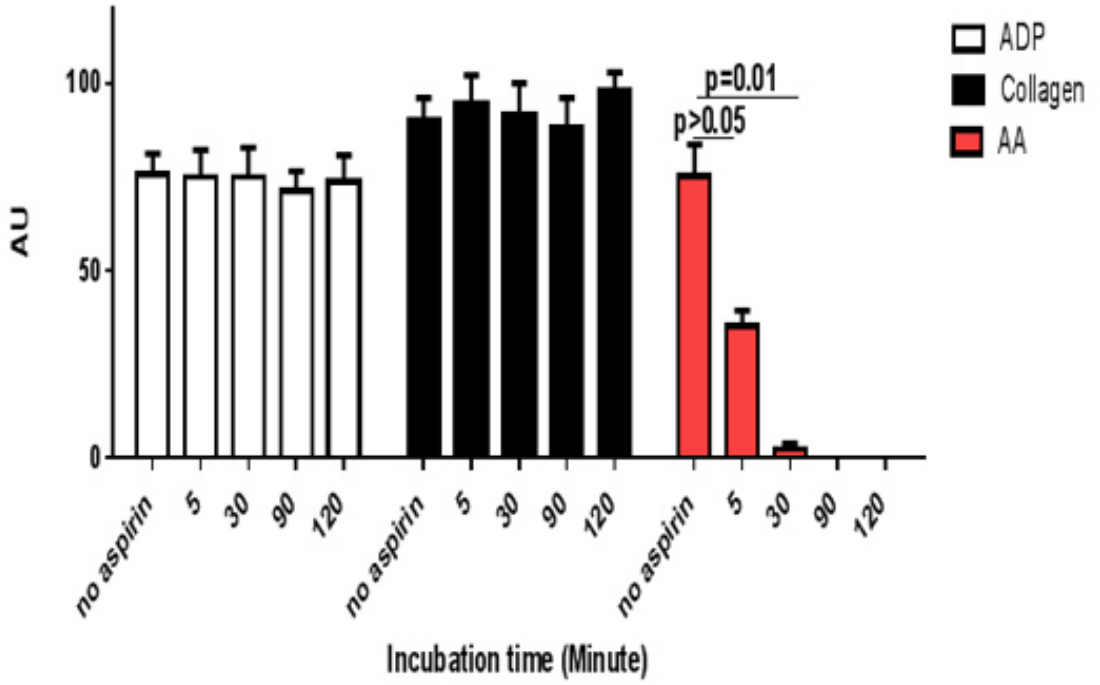


Figure 3.3 Aggregation of healthy control platelets in response to different agonists after incubation with aspirin using LTA technique. Platelet response to ADP (10 µmol/l; white columns), collagen (16 ug/ml; black columns) and AA (1 mM, red columns) after incubation with 0.5 mg/l aspirin for 5, 30, 90, 120 minutes. The readings show the mean±SEM of three independent experiments each conducted in duplicate. AU: arbitrary units.

3.3.3 Optimizing *ex vivo* aspirin concentration using whole blood (Multiplate)

When compared with LTA test, different concentrations of aspirin were necessary when assessing platelet inhibition using whole blood samples. Aspirin at 10 mg/l was needed to cause full suppression, which occurred at 30 mins. Marginal inhibition to aspirin was noted with 1 mg/l. Platelet reactivity to ADP was not affected, whereas partial inhibition of platelet respnse to collagen was noted at 10 mg/l after 30 mins incubation as shown in Figure 3.4.

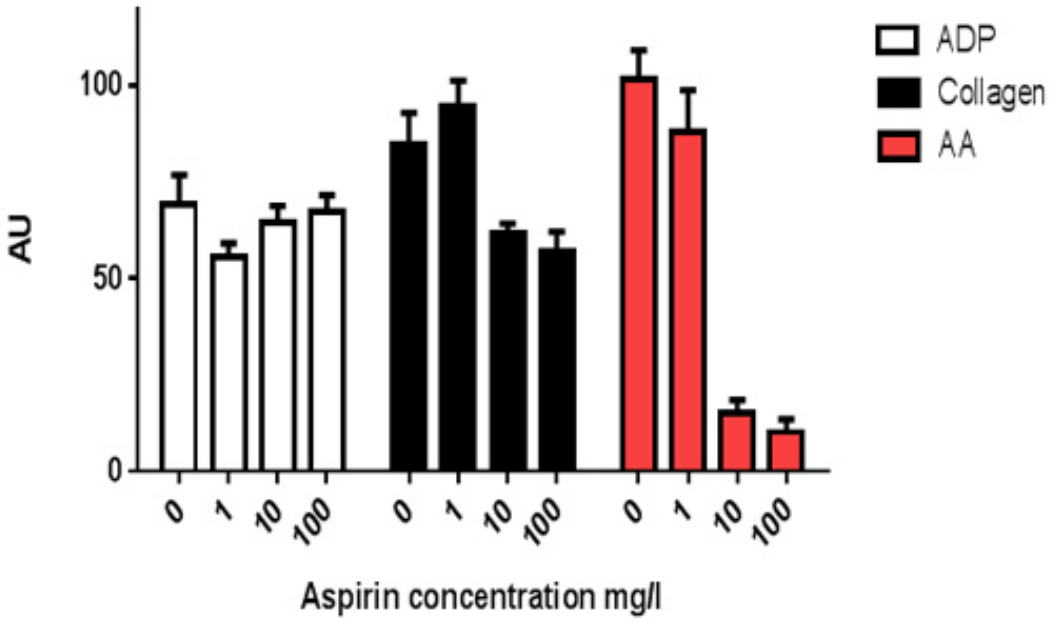


Figure 3.4 Platelet aggregation using whole blood from of healthy controls in response to different agonists after incubation with aspirin using Multiplate technique. Platelets response to ADP (white), collagen (black) and AA (red) after incubation with different aspirin concentrations 0, 1, 10, 100 mg/l aspirin for 30 minutes. The readings show the mean±SEM of three independentexperiments,each conducted in duplicate.AU:aggregation unit.

3.3.4 Effects of *ex vivo* aspirin on platelet reactivity in diabetes and control subjects

Given that whole blood represents a more physiological environment to test platelet aggregation, further experiments were conducted by adding aspirin, with and without additional glucose, to whole blood with platelet function tested after stimulation with arachidonic acid (AA), collagen and ADP.

Arachidonic acid (AA)

Given that smoking may affects platelet activity and clot structure, I analysed the data of the whole group as well as the group excluding the two smokers.Low aspirin concentration at 1 mg/l inhibited AA-stimulated platelet aggregation by (12.7%, p=0.01) in patients with diabetes (Figure 3.5A)and by (13.1%, p=0.03) in controls (Figure 3.5B). Data were similar when smokers were excluded (11.9% inhibition, $p<0.05$ compared with controls). Interestingly, individuals with poor diabetes control (HbA1c≥8.5% or >75 mmol/mol) had no response to the inhibitory effects of low concentration of aspirin, suggesting interaction between acetylation and glycation (Figure 3.5C). High aspirin concentration exerted a more profound inhibitory effect on AA-mediated stimulation of platelet aggregation (48%, or 50% after excluding the two smokers) and 48% reduction in diabetes and control subjects, respectively, $p\leq0.01$ for both) (Figure 3.5 A,B). High aspirin concentration was effective at inhibiting AA-mediated stimulation of platelets in subjects with poor diabetes control, suggesting that the potential interaction between acetylation and glycation can be overcome by increasing aspirin concentrations (Figure 3.5C).

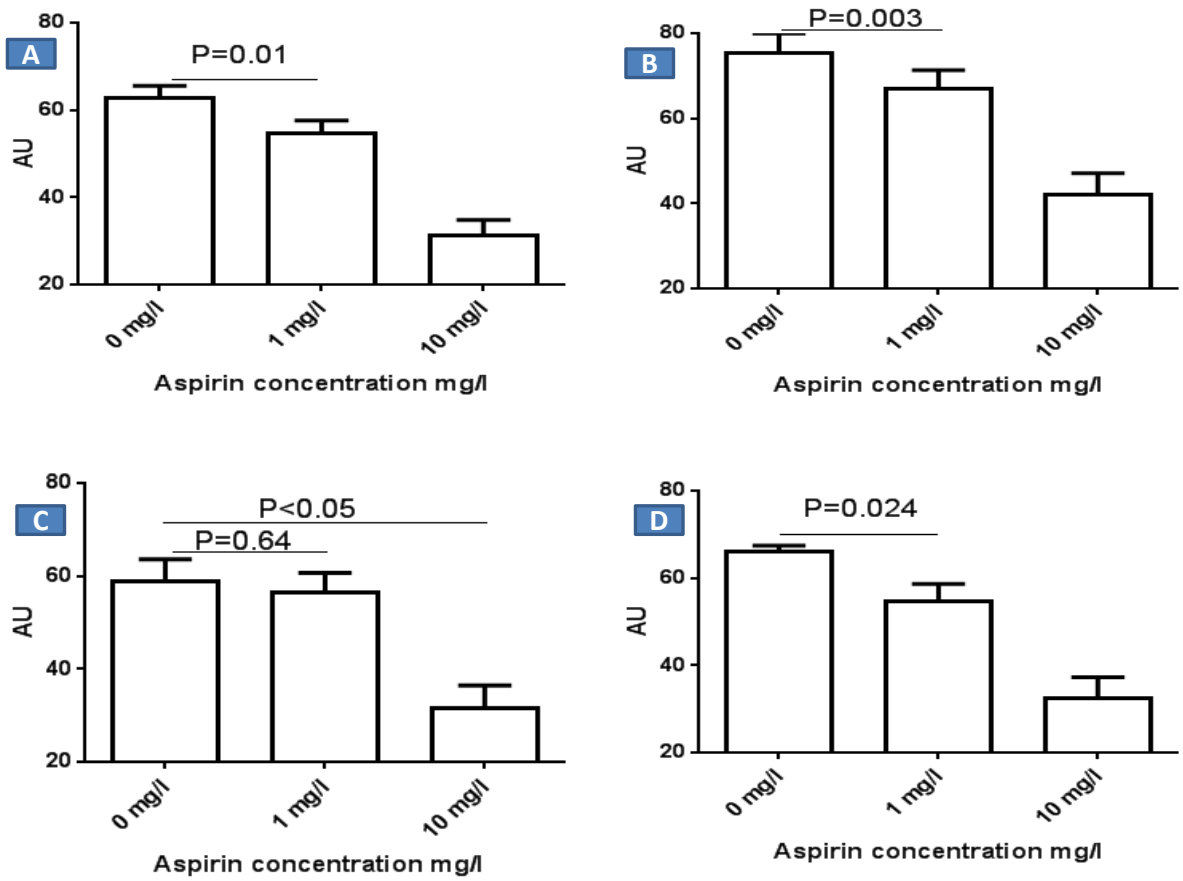


Figure 3.5 *Ex vivo* effect of different concentrations of aspirin on platelet aggregation in response to AA using Multiplate. Platelets were stimulated with arachidonic acid (AA) in subjects with diabetes (n=25; A), healthy controls (n=26; B), subjects with poor diabetes control (n=14; C) and subjects with better diabetes control (n=11; D).

Collagen

Low aspirin concentration at 1 mg/l inhibited collagen-stimulated platelet aggregation by 7.4% (8.3% after excluding the two smokers) in diabetes patients (p=0.037) and by 12.4% in controls (p<0.01; Figure 3.6 A, B). Interestingly, individuals with poor diabetes control (HbA1c ≥ 8.5%) had no response to the inhibitory effects of aspirin, suggesting again interaction between acetylation and glycation (Figure 3.6C). High aspirin concentration exerted a more profound inhibitory effect on collagen-mediated stimulation of

platelet aggregation (30% or 28.6% after excluding the two smokers) and 32.3%, reduction in diabetes and control subjects, respectively; p<0.01 for both). High aspirin concentration was effective at inhibiting collagen mediated stimulation of platelets in subjects with poor diabetes control, suggesting that the interaction between acetylation and glycation can be overcome by increasing aspirin concentrations (Figure 3.6C).

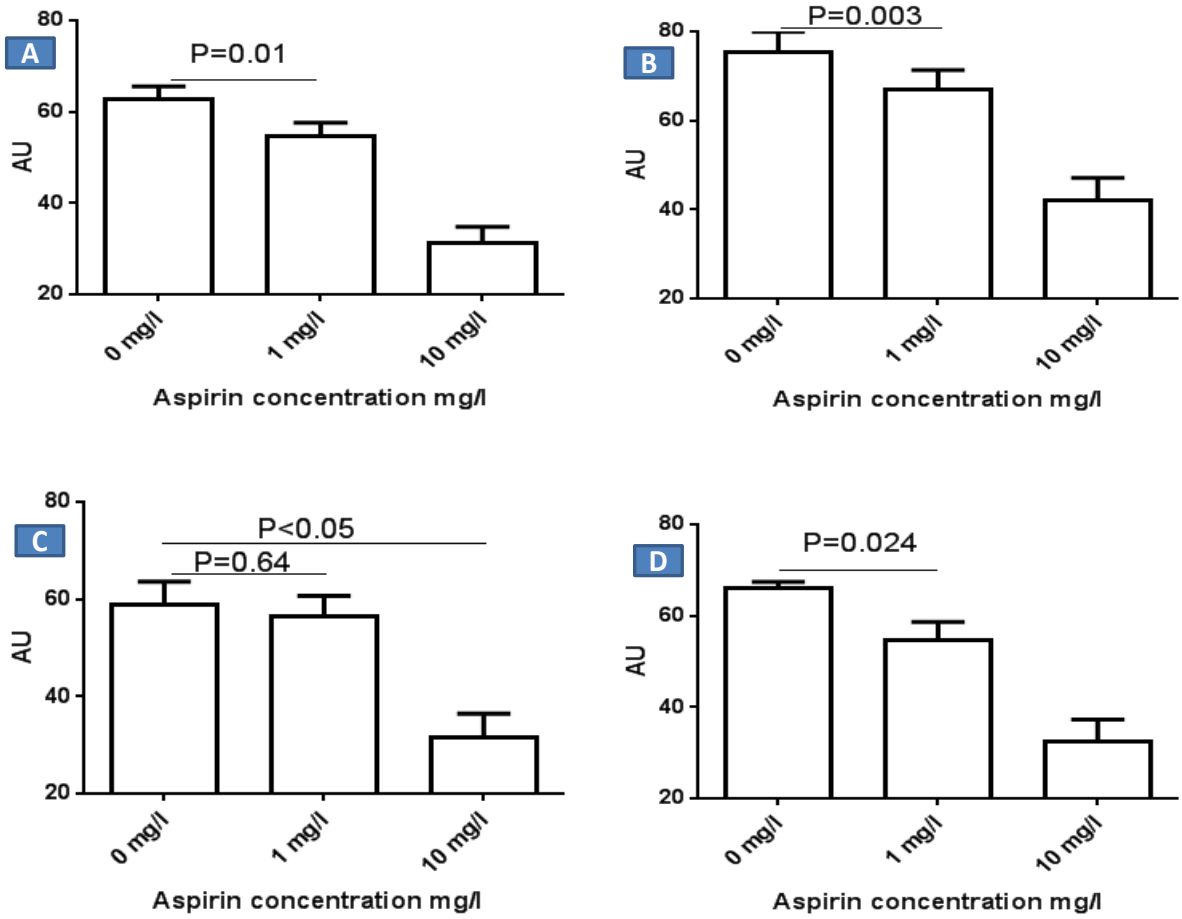


Figure 3.6 *Ex vivo* effect of different concentrations of aspirin on platelet aggregation in response to collagen Multiplate. Platelets were stimulated with collagen in subjects with diabetes (n=25; A), healthy controls (n=26; B), subjects with poor diabetes control (n=14; C) and subjects with better diabetes control (n=11; D).

Adenosine diphosphate (ADP)

Neither low nor high aspirin concentrations had significant inhibitory effects on ADP-stimulated platelet aggregation in both patients and controls (figure 3.7A, B). There was no difference in ADP response to aspirin treatment comparing diabetes patients with poor and good glycaemic control (Figure 3.7C, D).

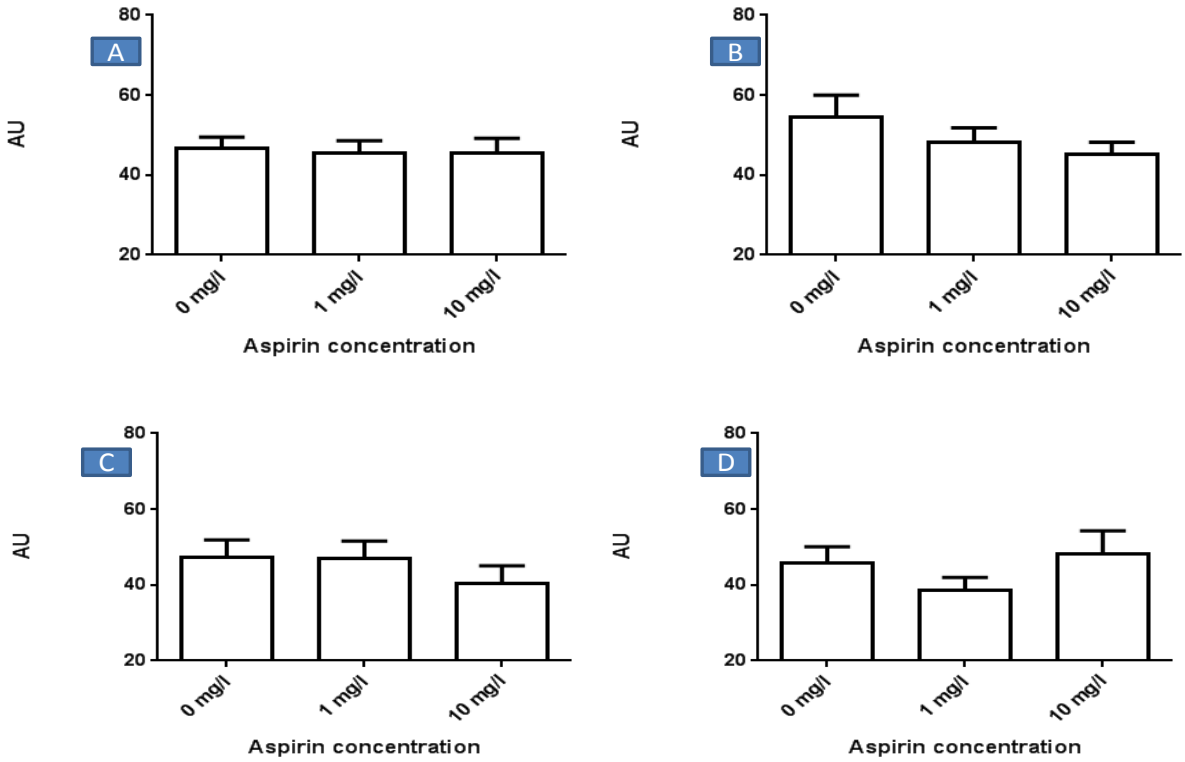


Figure 3.7 *Ex vivo* effect of different concentrations of aspirin on platelet aggregation in response to ADP using Multiplate. Platelets were stimulated with ADP in subjects with diabetes (n=25; A), healthy controls (n=26; B), subjects with poor diabetes control (n=14; C) and subjects with better diabetes control (n=11; D).

3.3.5 Effects of *ex vivo* aspirin on platelet reactivity in diabetes and control subjects in the presence of excess glucose

Arachidonic acid (AA)

In individuals with diabetes, the presence of additional glucose (20 mmol/l), resulted in reductionin the platelet inhibitory effect of low aspirin concentration after AA stimulation of platelet aggregation.

Low aspirin concentration at 1 mg/l, inhibited AA-stimulated platelet aggregation by (7.5% p=0.063, 6.9%, p=0.1after excluding the two smokers) in patients with diabetes (Figure 3.8A) and by (7.0%, p=0.074) in controls (Figure 3.8B). Individuals with poor diabetes control (HbA1c≥8.5%) had no response to the inhibitory effects of aspirin with a trend seen in those with better glycaemic control (Figure 3.8C,D). High aspirin concentration was effective in inhibiting AA-mediated stimulation of platelets by (56.4%) in subjects with diabetes (56.4% in patients after excluding the two smokers) and by (54%) in healthy controls. In subjects with poor diabetes control, high aspirin concentration was effective in inhibiting AA mediated stimulation of platelet by (59%; Figure 3.8C).

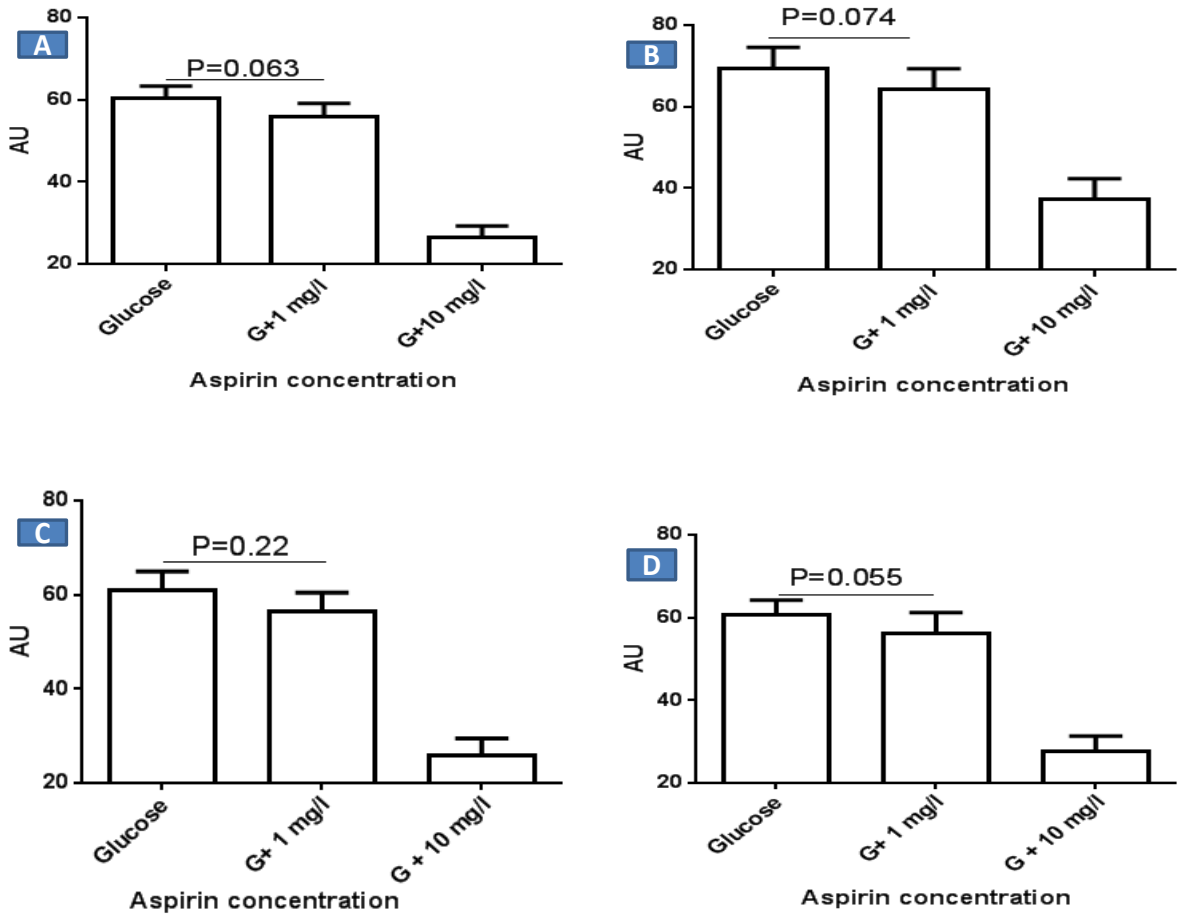


Figure 3.8 *Ex vivo* effect of different concentrations of aspirin, in the presence of excess glucose, on platelet aggregation in response to AA using Multiplate. Platelets were stimulated with arachidonic acid (AA) in subjects with diabetes (n=25; A), healthy controls(n=26; B), subjects with poor diabetes control (n=14; C) and subjects with better diabetes control (n=11; D).

Collagen

In the presence of excess glucose, low aspirin concentration at 1 mg/l had no effect on inhibiting collagen-stimulated platelet aggregation in patients or controls (Figure 3.9A,B). Glycaemic control was not a factor in determining platelet responseto collagen stimulation (Figure 3.9C,D). In contrast, high aspirin concentration was effective at inhibiting collagen-mediated stimulation of platelets by 27.3% ($p<0.0001$; 23% after excluding the two smokers) in subjects with diabetes and by 31.1% ($p<0.0001$) in healthy controls. Moreover, in subjects with poor and better diabetes control, high aspirin concentration showed a significant inhibition of collagen mediated stimulation of platelet activation by 33.3% (32% after excluding the two smokers) and 22.3%, respectively ($p<0.002$ for both; Figure 3.9C).

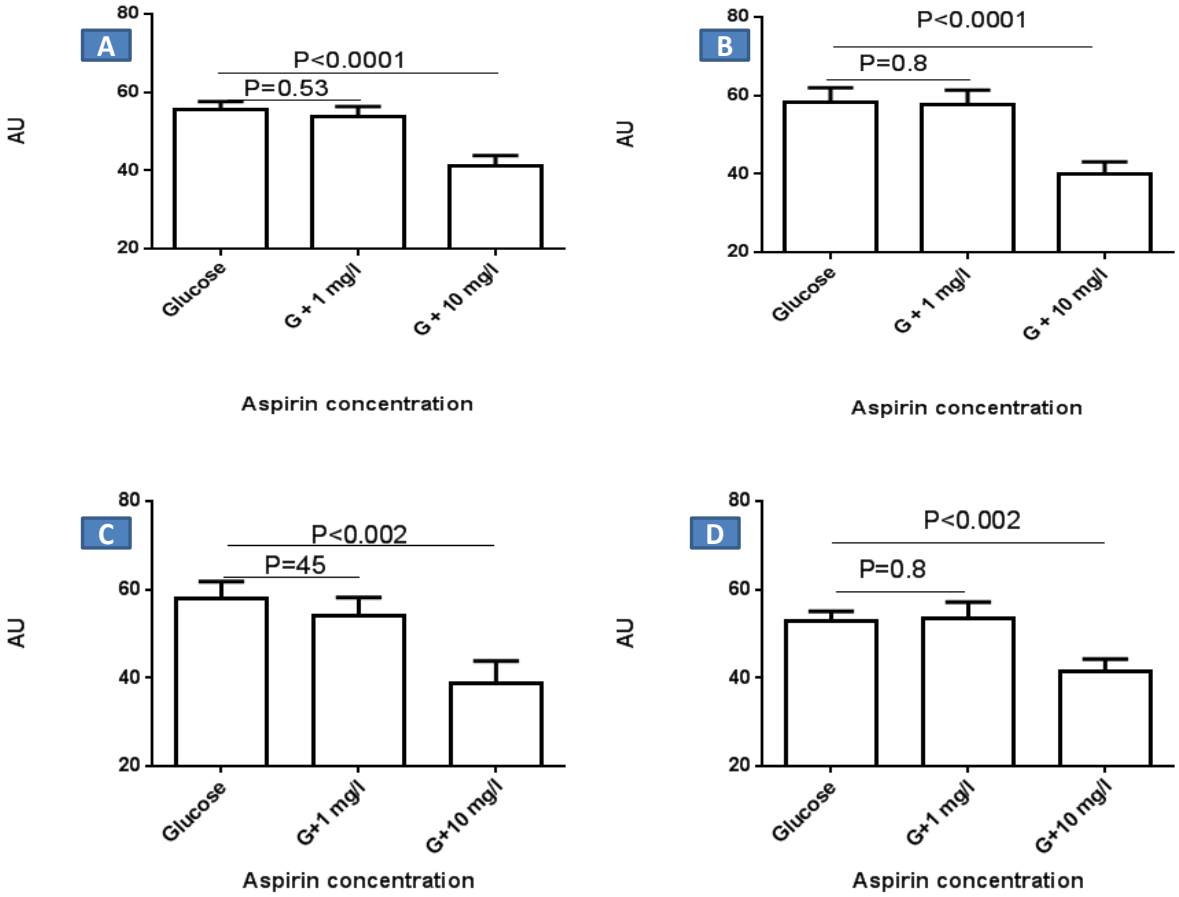


Figure 3.9 *Ex vivo* effect of different concentrations of aspirin, in the presence of excess glucose, on platelet aggregation in response to collagen using Multiplate. Platelets were stimulated with collagen in subjects with diabetes (n=25; A), healthy controls (n=26; B), subjects with poor diabetes control (n=14; C) and subjects with better diabetes control (n=11; D).

Adenosine diphosphate (ADP)

Neither low nor high aspirin concentration had any effect on ADP-mediated platelet aggregation in the presence of additional glucose. Results were similar in those with poor and better glycaemic control (figure 3.10 A-D).

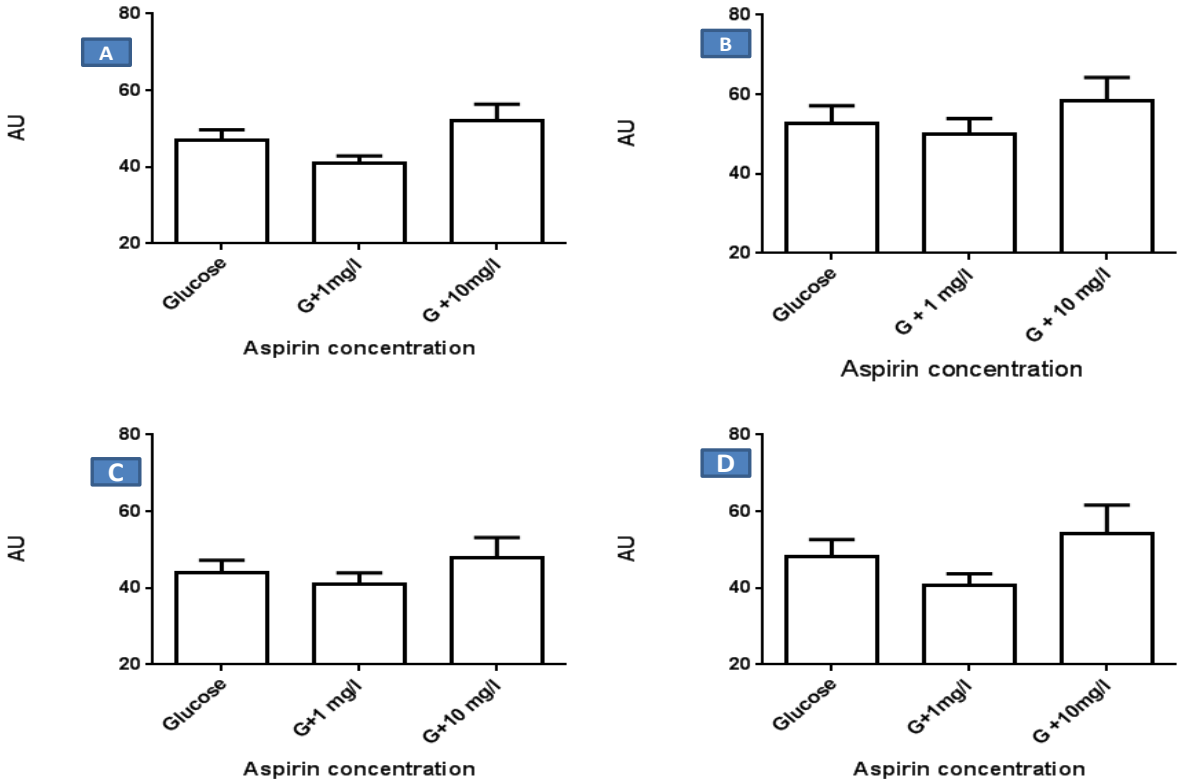


Figure 3.10 *Ex vivo* effect of different concentrations of aspirin, in the presence of excess glucose, on platelet aggregation in response to ADP using Multiplate. Platelets were stimulated with ADP in subjects with diabetes (n=25; A), healthy controls (n=26; B), subjects with poor diabetes control (n=14; C) and subjects with better diabetes control (n=11; D).

3.3.6 Effects of *in vivo* aspirin on platelet reactivity in diabetes and control subjects

All samples were taken approximately 2 hours after low (75 mg/day) or high (300 mg/day) aspirin ingestion in patients with diabetes and healthy controls.

Arachidonic acid (AA)

Low daily dose of aspirin (75 mg/day) inhibited AA-stimulated platelet aggregation by 72% in patients with diabetes (70% after excluding the two smokers) ($p<0.0001$; Figure 3.11A) and by 72% in controls ($p<0.0007$; Figure

3.11B). Nearly the same observation was found in individuals with poor and good diabetes control with reductions of 69% (68% after excluding the two smokers smoker) and 79%, respectively (p<0.0001 for both). High aspirin daily dose (300mg/day) exerted a more profound inhibitory effect on AA-mediated stimulation of platelet aggregation at 75% (76%after excluding the two smokers) (p<0.0001) and 82% p<0.0008 in patients with diabetes and controls. In those with poor glycaemic control, inhibition was 67% (65%after excluding the two smokers) (p<0.003) whereas in those with good diabetes control the inhibition was 83% (p<0.0002) (Figure 3.11 A-D).

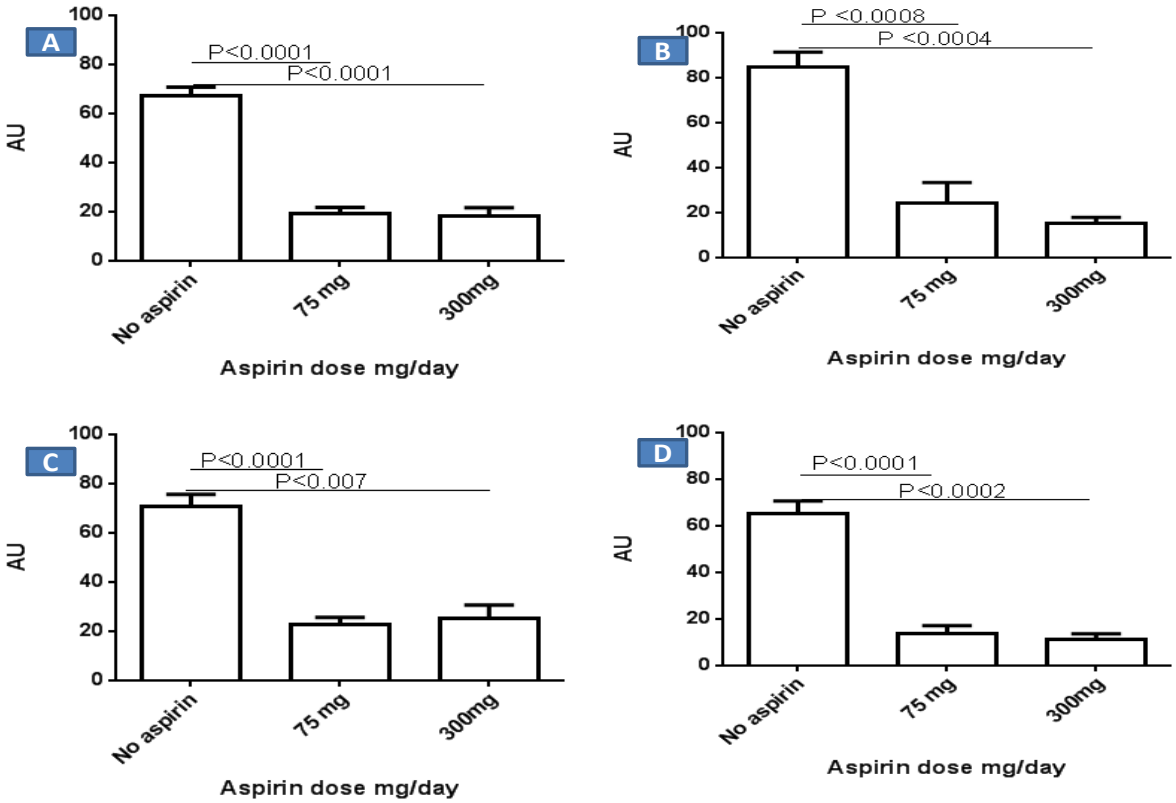


Figure 3.11 *In vivo* effect of different daily doses of aspirin on platelet aggregation in response to AA using Multiplate. Platelet response to arachidonic acid (AA) in subjects with diabetes (n=25A), healthy subjects (n=26; B), subjects with poor diabetes control (n=14; C) and subjects with better diabetes control (n=11; D).

Collagen

Low daily dose of aspirin (75 mg/day) inhibited collagen-stimulated platelet aggregation by 26% (p=0.014) in patients with diabetes while it failed to show a significant effect in healthy controls (Figure 3.12A,B). Higher aspirin dose inhibited collagen-mediated platelet aggregation in both diabetes patients and controls by 25.2% (p=0.03) and 34.6% (p=0.001), respectively. When patients were split into poor and better glycaemic control, the trend was similar but the inhibitory effect of aspirin on collagen-mediated platelet aggregation were not significant (Figure 3.12C, D).

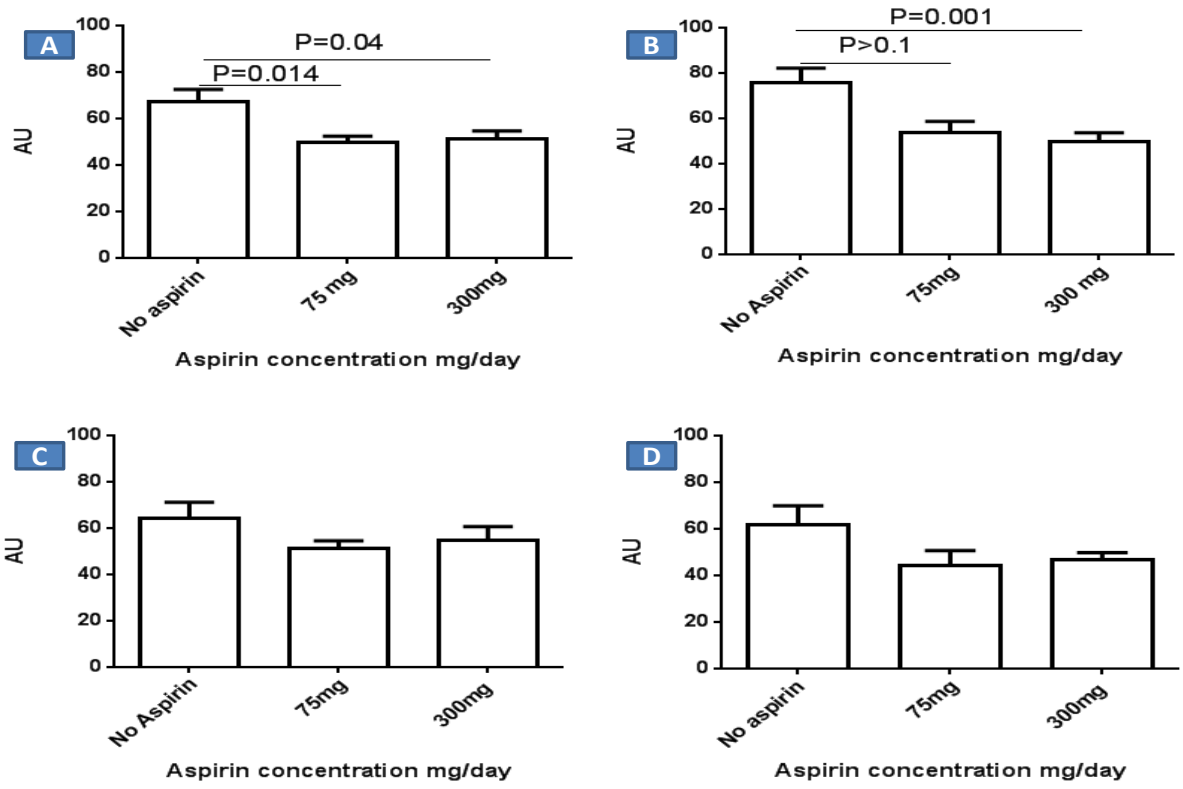


Figure 3.12 *In vivo* effects of different daily doses of aspirin on platelet aggregation in response to collagen using Multiplate. Platelet response to collagen in subjects with diabetes (n=25; A), healthy controls (n=26; B), subjects with poor diabetes control (n=14, C) and subjects with better diabetes control (n=11; D).

Adenosine diphosphate (ADP)

Aspirin had no significant inhibitory effect on ADP-stimulated platelet aggregation in either patients or controls, regardless of the dose of aspirin used (Figure 3.13 A,B).

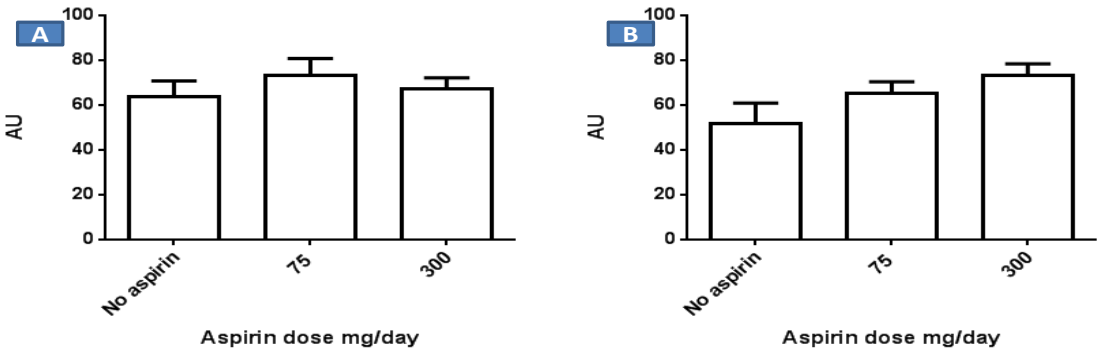


Figure 3.13 *In vivo* effects of different daily doses of aspirin on platelet aggregation in response to ADP. Platelet response to ADP in subjects with diabetes (n=25; A) and healthy controls (n=26; B).

3.4 Discussion

In this ChapterI describe optimisation experiments to test for the *ex vivo* platelet inhibitory effects of aspirin in response to AA, collagen and ADP.Following this, I assessed the *ex vivo* inhibitory effect of low and high aspirin concentration on platelet aggregation in both patients with diabetes and healthy controls. Also, I investigated the *ex vivo* effect of high glucose concentration on aspirin inhibitory capacity of platelet aggregation in response to these agonists.These

experiments were complemented by analysing the *in vivo* effects of low and high dose aspirin on platelet aggregation following AA, collagen and ADP stimulation in both patients with diabetes and healthy controls.

There are a number of novel observations emerging from my studies, i) Addition of *ex vivo* aspirin incubated to whole blood for 30 minutes is able to assess platelet response to this agent, ii) Although aspirin suppresses AA and collagen-stimulated platelet reactivity in both diabetes subjects and controls, the efficacy of low aspirin concentration appears to be compromised in individuals with diabetes and poor glycaemic control, iii) The platelet inhibitory activities of low aspirin concentrations are abolished in the presence of excess glucose in both diabetes subjects and controls, iv) Low aspirin concentrations fail to inhibit collagen-stimulated platelet reactivity in the presence of excess glucose and v) in vivo aspirin administration at 75 or 300 mg/day causes suppression of AA-induced platelet aggregation in samples collected 2 hours following aspirin ingestion. As expected, aspirin had no *ex vivo* or *in vivo*effect on ADP-mediated platelet stimulation.

The prostanoid thromboxane A2 (TXA2) is a key platelet activator that can also cause vasoconstriction thus stopping bleeding following vessel injury. Activated platelets release AA, which is modified by cyclooxygenase (COX)-1, yielding cyclic endoperoxides (prostaglandin G2 “PGG2” and “PGH2”), representing the main TXA2 substrate. PGH2 and TXA2 bind to thromboxane/prostaglandin (TP) platelet receptor (Yan and Phillips, 2005), resulting in platelet activation as detailed in Section 1.7.1.

Aspirin acts by acetylation of platelet cyclooxygenase-1(COX-1) at position serine 529. This chemical modification inactivates the enzyme and as a result,

cyclic endoperoxides (prostaglandin G2 “PGG2” and “PGH2”) production from arachidonic acid is reduced, consequently decreasing TXA2 synthesis, (Roth and Calverley, 1994). Hyperglycaemia has been implicated in reduced efficacy of aspirin in diabetes (Ajjan et al., 2008). Several mechanisms have been proposed for increased platelet reactivity with high plasma glucose, including increased expression of platelet $\alpha_{IIb}\beta 3$, upregulation of P-selectin expression (Keating et al., 2003), increased platelet intracellular calcium secondary to glycation of circulating low-density lipoprotein (LDL) (Ferretti et al., 2002). An additional key mechanism for hyperglycaemia increasing platelet reactivity is non-enzymatic glycation of platelet membrane, resulting in loss of membrane fluidity and enhancement of platelet adhesion capacity (Winocour et al., 1992, Watala et al., 1998). Therefore, the general increase in platelet reactivity secondary to diabetes may be one mechanism for relative aspirin treatment failure or, alternatively, direct interaction between acetylation and glycation may be the main mechanism responsible. Both mechanisms have been proposed for the reduced clinical efficacy of aspirin, but no conclusive evidence is provided to fully back either (Rendell et al., 1986, Swamy-Mruthinti and Carter, 1999, Watala et al., 2005).

Even if we accept that hyperglycaemia reduces response to aspirin treatment, it remains unclear whether this is due to protein glycation, or it is simply the result of high glucose levels interfering with the acetylation process. Understanding the exact mechanisms may have important therapeutic implications. For example, if high glucose, rather than glycation, is the cause of reduced aspirin efficacy, then normalising glucose levels within hours of cardiac ischaemia becomes a priority. In contrast, if glycation is more important, then acutely

normalising glucose levels following vascular ischaemia becomes less of a priority.

My data show, for the first time, that both glycation and high glucose levels interfere with the action of aspirin. Although low aspirin concentration inhibited platelet response to AA stimulation, this effect was compromised in patients with poor glycaemia control. In contrast, higher aspirin concentrations inhibited AA-induced stimulation of platelet aggregation in both diabetes patients and controls and were not affected by glycaemic control. Previous work has shown that plasma aspirin levels drop relatively quickly following drug administration and therefore very low levels are expected 8-12 hours post ingestion (Benedek et al., 1995). Given this fact and increased turnover of platelets in diabetes (Ajjan and Grant, 2006a, Guthikonda et al., 2007), my results indicate that individuals with poor diabetes control may not be covered by aspirin for the whole 24 hours. The counter-argument is that *in vivo* administration of aspirin showed good platelet inhibition to AA-stimulation regardless of glycaemic control in the patient group. It should be remembered, however, that blood samples were taken approximately 2 hours after aspirin administration, when plasma levels of this agent are still high. In retrospect, the study design should have included an additional blood sampling before aspirin administration.

Previous work has shown incomplete inhibition of platelet aggregation in response to AA in patients with poor glycaemic control when receiving 30mg daily dose of aspirin. However, higher dose of aspirin, resulted in improved platelet inhibition, further supporting my data(Lemkes et al., 2012). In addition to glycation, it appears that high glucose levels can directly compromise the efficacy of aspirin. The *ex vivo* addition of excess glucose (20mmol/l) abolished

the inhibitory effect of low concentration of aspirin on platelet aggregation in both patients and controls. This indicates that high glucose levels have a direct effect on aspirin efficacy, the mechanism of which remains an area for future research.

Collagen is one of the most important componentsthat triggers platelet activation following vessel injury (Roberts et al., 2004). It is proposed that there are two main receptors involved in platelet-collagen interaction: collagen first adheres to α2β1 on platelet surface then reacts with glycoprotein VI (Poole and Watson, 1995). Collagen activates platelet aggregation and stimulates the release of α granular protein, β-thromboglobulin and production of TXA2 (Valles et al., 1991). At high concentration of collagen, platelet activation occurs through a process ending with the release of calcium either from the dense tubular system (Brass and Joseph, 1985) or by influx from the extracellular milieu (Roberts and Bose, 2002). However, at lower collagen concentration, its ability to activate platelet is enhanced by the production of TXA2 (Nakano et al., 1989). Therefore, the addition of aspirin will partially inhibit the ability of collagen to enhance platelet aggregation. This explains the inhibition of platelet activation by collagen following aspirin treatment *ex vivo* or *in vivo*. Moreover, the failure of aspirin to exert an effect in those with poorly controlled diabetes indicates that suppression of TXA2 production in these patients is compromised. Moreover, it is not only long-term glycaemia that is an issue, but high glucose levels also play a role. Nearly the sameobservation was found when assessing the *in vivo* effect of aspirin on collagen-mediated platelet response. Although low dose aspirin (75 mg/day) showed an effect in both subjects with diabetes

and healthy controls, this effect was reduced in those with poor glycaemic control.

A large body of evidence is available in the literature in relation to the effect of diabetes on platelet reactivity and decreased response to aspirin treatment (Watala et al 2004). However, most of these studies have assessed the *in vivo* effect of diabetes on the effectiveness of aspirin on platelet response to agonists. To my knowledge, there is only one study that assessed both the *in vivo* and *ex vivo* platelet response to aspirin (Watala et al 2004). Although there are some similarities compared with my work, there are a number of differences that should be highlighted.First, the cohort of patients analysed was different in the two pieces of work: I focused on young type1 diabetes patients devoid of complications and not on any treatment other than insulin. This allowed me to investigate the effect of glycaemia away from confounding factors frequently encountered in older type 2 diabetes patients in the study by Watala et al. Second, my study investigated the effects of glycation as well as high glucose levels on the platelet inhibitory effects of aspirin. Thirdly, I analysed platelet response to three agonists to provide a more complete picture of platelet reactivity in patients and controls.

Keating et al 2003 have studied the *ex vivo* effect of increased concentration of glucose or mannitol on platelet reactivity in 14 healthy volunteers and in hospitalized patients with (n=14) or without (n=7) type 2 diabetes mellitus.They documented increased activation of platelet $\alpha_{IIb}\beta 3$ and P-selectin expression after 1hour incubation with high concentration of glucose or mannitol. In my study, glucose per se had no effect on platelet activation but it reduced the platelet inhibitory effects of aspirin. Also, it should be noted that the study by

Keating analysed a limited number of samples and again used type 2 diabetes patients who have a more variable profile than type 1 diabetes, as discussed earlier.

Although the idea of aspirin resistance was floated more than two decades ago, the exact mechanisms for this phenomenon remain elusive (Ajjan et al., 2008). There are a large number of factors that may play a role in aspirin resistance in patients with diabetes, including decreased endothelial nitric oxide production and the general increase in platelet activation. My study further established that both long-term glycaemia and high glucose levels can interfere with the action of aspirin. Interestingly, however, minor elevation in HbA1c was not associated with decreased response to aspirin. This suggests that only individuals with very poor diabetes control (HbA1c≥8.5%) have reduced response to aspirin thus explaining some of the contradictory data on aspirin resistance. In reality, HbA1c cut off that is not associated with impaired response to aspirin is likely to be different from patient to patient, but what this study shows is that very tight control of blood glucose is not necessary to improve response to aspirin therapy. Naturally, future clinical studies are necessary to test this hypothesis, which may have important long-term clinical implications.

A clear strength of my study is the use of type 1 diabetes patients who have no other pathology, enabling me to dissect the role of glycaemia in response to aspirin treatment using a combination of *ex vivo* and *in vivo* experiments. However, there are a number of drawbacks including: i) number of patients studied remains relatively small and therefore full characterisation of the subgroup with reduced response to aspirin was not possible, ii) for the *in vivo* work, an additional blood sample should have collected before aspirin

administration, which would have given us a better understanding of the time course of aspirin action *in vivo,* and iii) the exact pathways responsible for the interaction between glycation/high glucose and acetylation are not clarified in this study and remain an area for future work. iv) No measurements of thromboxane levels have been done in this study which could have further helped to assess the effects of aspirin on this pathway, and v) This study is limited to type 1 diabetes patients and the results do not necessarily reflect what takes place in type 2 diabetes. However, the reason for choosing type 1 diabetes was to ensure that the effects of glycaemia was studied away from the large number of confounding factors often present in patients with type 2 diabetes.

In conclusion, my study demonstrates that *ex vivo* work is a useful tool to address specific aspects of reduced efficacy of aspirin in diabetes. Both long-term and short-term hyperglycaemia are associated with impairment in platelet inhibition by aspirin in this condition. It should be noted, however, that aspirin is not only an inhibitor of platelet function but can also affect fibrinolytic potential. Therefore, to fully understand the mechanisms of aspirin treatment failure in diabetes, studies are required to analyse both the cellular and protein arms of coagulation.

Chapter 4

Diabetes, blood glucose and modulation of the fibrin clot by aspirin

4.1 Introduction

Fibrin networks with compact structure, containing thin fibrin fibres with small pores, are associated with increased risk of thrombosis and cardiovascular disease (Fatah et al., 1996b, Collet et al., 2006, Undas et al., 2010b) . Fibrinolysis is compromised in tighter clots, offering one mechanism for higher thrombosis risk and poor outcome in individuals with this kind of clot structure (Collet et al., 2000).

Ex vivo clots from individuals with diabetes display a compact structure with impaired fibrinolysis (Dunn et al., 2006). Improving clot structure and fibrinolytic efficiency in diabetes represent one strategy to reduce thrombotic risk in this population.

As discussed before, aspirin is effective at altering fibrin clot structure, related, at least in part, to acetylation of fibrinogen (Ajjan et al., 2009, Undas et al., 2003). However, in patients with diabetes, the acetylation process by aspirin may be compromised. One mechanism implicated is related to an interaction between glycation and acetylation, secondary to high plasma glucose levels (Rendell et al., 1986, Swamy-Mruthinti and Carter, 1999).

Researchers have investigated the effect of high plasma glucose on fibrin clot structure using *ex vivo* glycated fibrinogen (Ney et al., 1985, Mirshahi et al., 1987, Brownlee et al., 1983) or studying samples from patients with diabetes (Dunn et al., 2005, Nair et al., 1991b, Lutjens et al., 1988, Jorneskog et al., 1996, Pieters et al., 2006). The advantage of *ex vivo* glycation is the ease of obtaining samples with different degrees of specific glucose-related post-translational modification. The drawback, however, is related to the non-physiological nature of the process, and therefore not representing the *in*

*vivo*environment. Obtaining samples from diabetes patients is more physiological but has the inconvenience of being affected by inter-individual variability (genetic and environmental), making data interpretation problematic.

To further complicate matters, some researchers used plasma samples to investigate fibrin networks, a methodology that takes into account the effects of various plasma proteins on the clotting process (Nair et al., 1991a, Nair et al., 1991b, Jorneskog et al., 1996, Pieters et al., 2006).Others employed purified fibrinogen in order to dissect out the effect of diabetes on the fibrinogen molecule (Dunn et al., 2005, Lutjens et al., 1988, Dunn et al., 2006).

Although studies have shown potential interaction between glycation and acetylation (Rendell et al., 1986, Swamy-Mruthinti and Carter, 1999), this has not been appropriately studied using samples from diabetes patients and matched controls. Also, a general criticism of studies to date is the failure to investigate the role of instantaneous high glucose on clot structure and response to aspirin treatment. Therefore, to obtain a comprehensive assessment of glucose and glycation on clot structure and response to aspirin therapy, I conducted *ex vivo* studies to analyse the effect of aspirin on the fibrin network with and without high glucose level in individuals with diabetes and in healthy controls.

4.2 Materials and methods

4.2.1 Patients and controls

Plasma samples were stored from the same patients and controls who gave blood samples for the platelet test (section 2.1). Clot structure studies were

conducted in this group using plasma samples or plasma-purified fibrinogen. A total of 29 patients and 29 healthy controls were studied.

4.2.2 Sample preparation

To investigate the *ex vivo* effects of glucose and aspirin, samples were prepared in two ways, using whole blood or plasma-purified fibrinogen (Figure 4.1).

Whole blood preparations

Samples of whole blood were collected in sodium citrate followed by incubation with aspirin at 0mg/l, 1mg/l or 10mg/l in the presence and absence of additional 20µM glucose. Samples were then incubated at 37°C for 10 minutes and then on ice for 50 minutes**.** Blood samples were then centrifuged at 3000 rpm for 20 minutes and platelet poor plasma was aliquoted (0.5 ml plasma/tube) and stored at -80°C for further analysis (Figure 4.1). After collection of all samples, turbidimetric assays were conducted on all samples as described in section 2.5.

Purified fibrinogen

Samples collected in lithium heparin were centrifuged for 20 minutes at 3000 rpm and fibrinogen was purified from plasma samples as detailed in section 2.3. Pools of plasma purified fibrinogen were then constructed using 12 samples from healthy controls, 12 samples from poor diabetes control (HbA1c≥8.5%). To these pools of purified fibrinogen, different concentrations of aspirin were added at 0mg/l, 1mg/l and 10mg/ in the presence and absence of additional 20 µM glucose. Samples were then incubated for 24 hours at 4°C.

4.2.3 Assessment of clot structure

Clot structure and fibrinolysis were assessed using turbidimetric assays, confocal microscopy and, electron scanning microscopy as described in Chapter 2 (section 2.5 and 2.6).

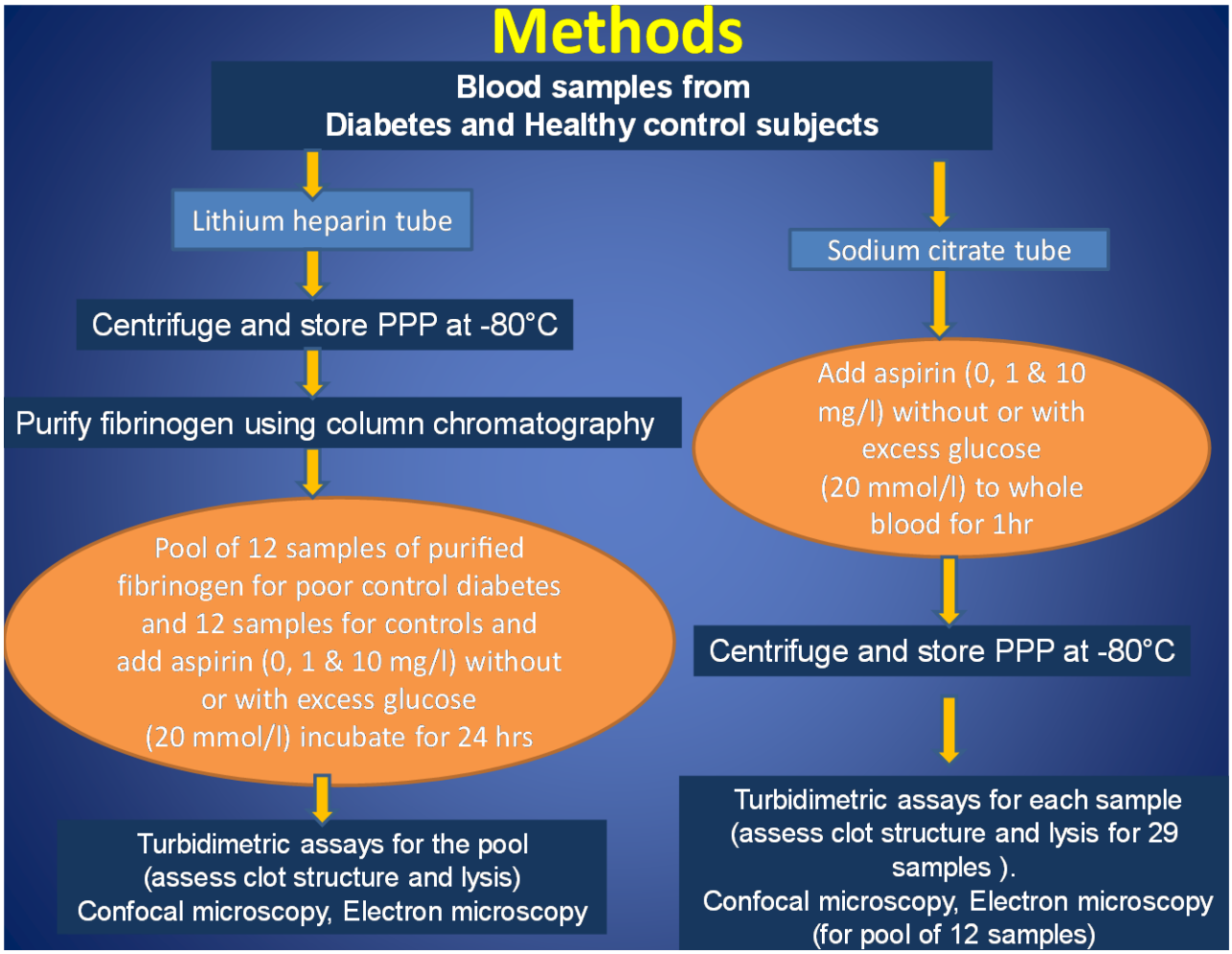


Figure 4.1 Summary of sample selection and preparation. For plasma fibrin clot analysis, blood samples from diabetes (n=29) and controls (n=29) were collected at baseline in sodium citrate. Different concentrations of aspirin were then added to whole blood samples (0, 1, 10 mg/l), with and without additional glucose, and incubated for 60 minutes. Platelet poor plasma (PPP) was aliquotedand fibrin clot structure was analysed using turbidimetric assay in all 29 samples. Confocal microscopy and electron microscopy was used to analyse clot structure in pooled control and diabetes samples n=12 each. For clots made from purified fibrinogen, blood samples from diabetes and controls were collected in lithium heparin. Fibrinogen was then purifiedfrom individual samples followed by pooling healthy control and diabetes samples (n=12 each). Pooled fibrinogen was incubated for 24 hrs with different concentrations of aspirin (0, 1, 10 mg/l), with and without additional glucose (20 mmol/l). Fibrin clot properties were analysed using turbidimetric assay, confocal and electron microscopy.

4.3 Results

4.3.1 Turbidimetric analysis of plasma clots

Lag phase

Low aspirin concentration at 1 mg/l and high concentration at 10 mg/l had no effect on lag phase in patients (Figure 4.2A) or controls (Figure 4.2B). The same observation was found in individual with poor diabetes control, HbA1c≥8.5% and individuals with better diabetes control, HbA1c<8.5 (Figure 4.2 C, D).

In the absence of aspirin, lag phase did not show a significant difference comparing controls (357±56 sec.) with diabetes individuals (254±49 sec.), but a trend was observed with low concentration of aspirin (413±54 sec., 278±56 sec; p=0.09) that disappeared with high aspirin concentration (322±61, 241±48 p=0.3; Figure 4.3).

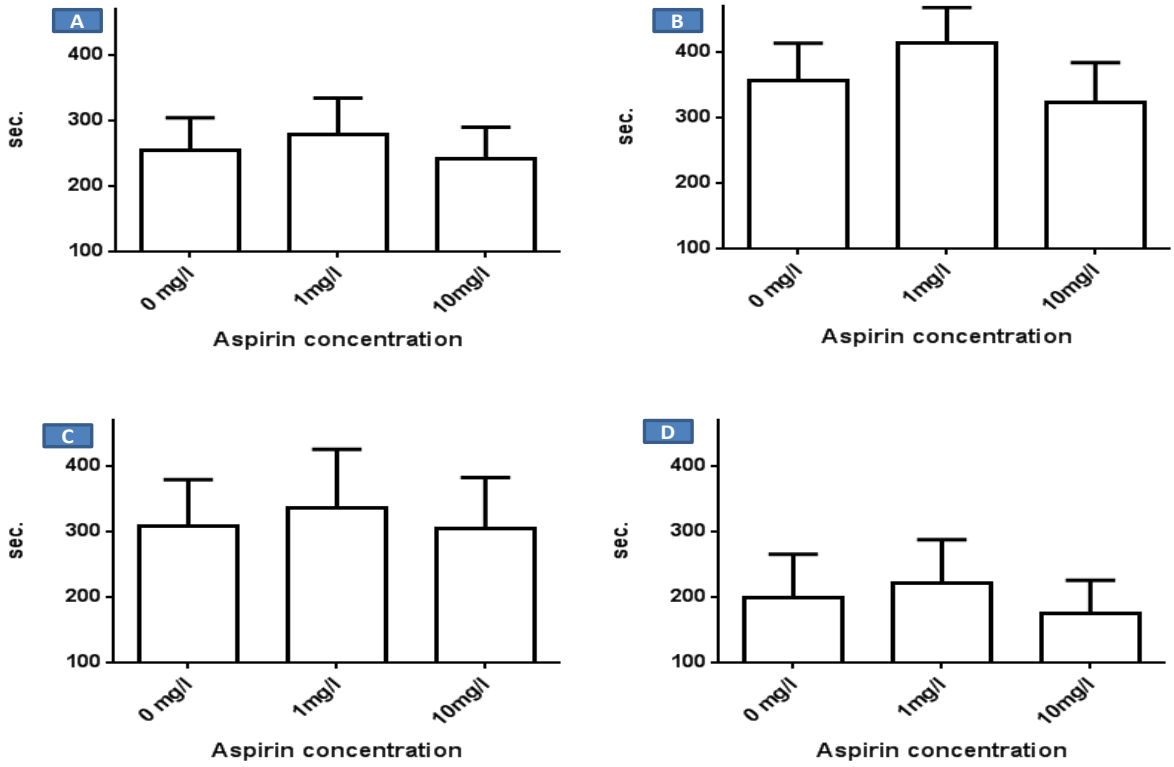


Figure 4.2 *Ex vivo* effect of different concentration of aspirin on lag phase (measured by seconds) of clot formation using turbidity and lysis technique. After addition of 0, 1, and 10 mg/l of aspirin into plasma of subjects with diabetes (A; n=29), healthy subjects (B; n=29), subjects with poor diabetes control (C; n=15) and subjects with better diabetes control (D; n=14), lag phase was measured.The results represent the mean±SEM.

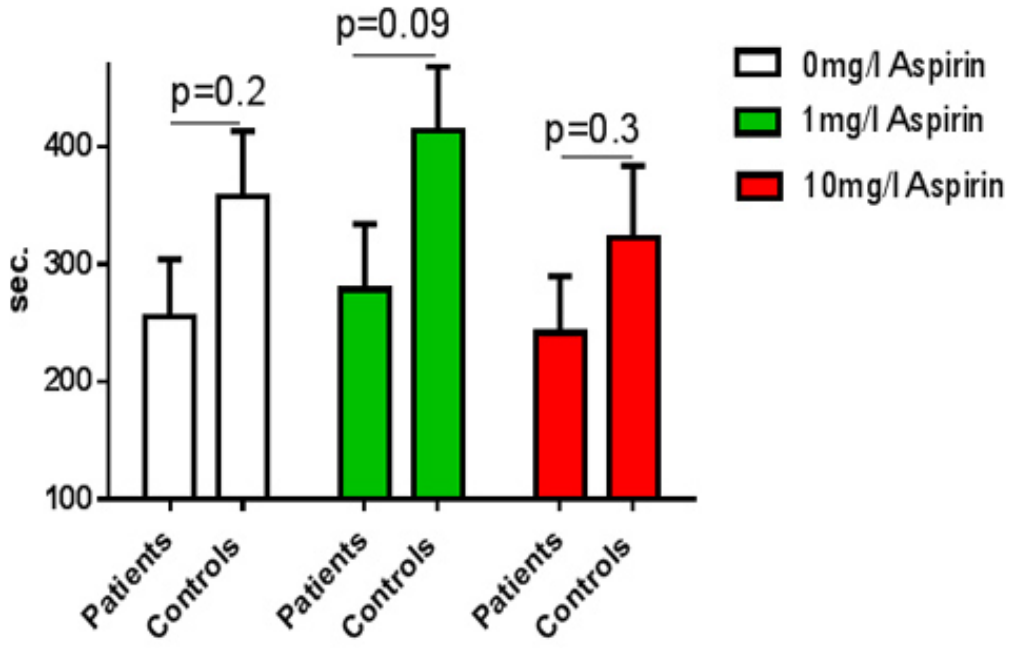


Figure 4.3 Analysis of the e*x vivo* effect of different concentration of aspirin on lag phase (measured by seconds) of clot formation in patients and controls using turbidity and lysis technique. After theaddition of 0, 1, and 10 mg/l of aspirin into plasma of subjects with diabetes (n=29) and healthy controls (n=29), lag phase was measured. The results represent the mean±SEM.

Maximum absorbance

Low aspirin concentration at 1 mg/l and high concentration at 10 mg/l had no effect on fibrin clot maximum absorbance in patients (Figure 4.4A) or controls (Figure 4.4B). The same observation was found in both individual with poor diabetes control (HbA1c≥8.5%) and individuals with better diabetes control (HbA1c<8.5; Figure 4.4 C, D).

Comparing individuals with poor and better diabetes control, maximum absorbance was consistently higher in the former group regardless of the concentration of aspirin used (Figure 4.5).

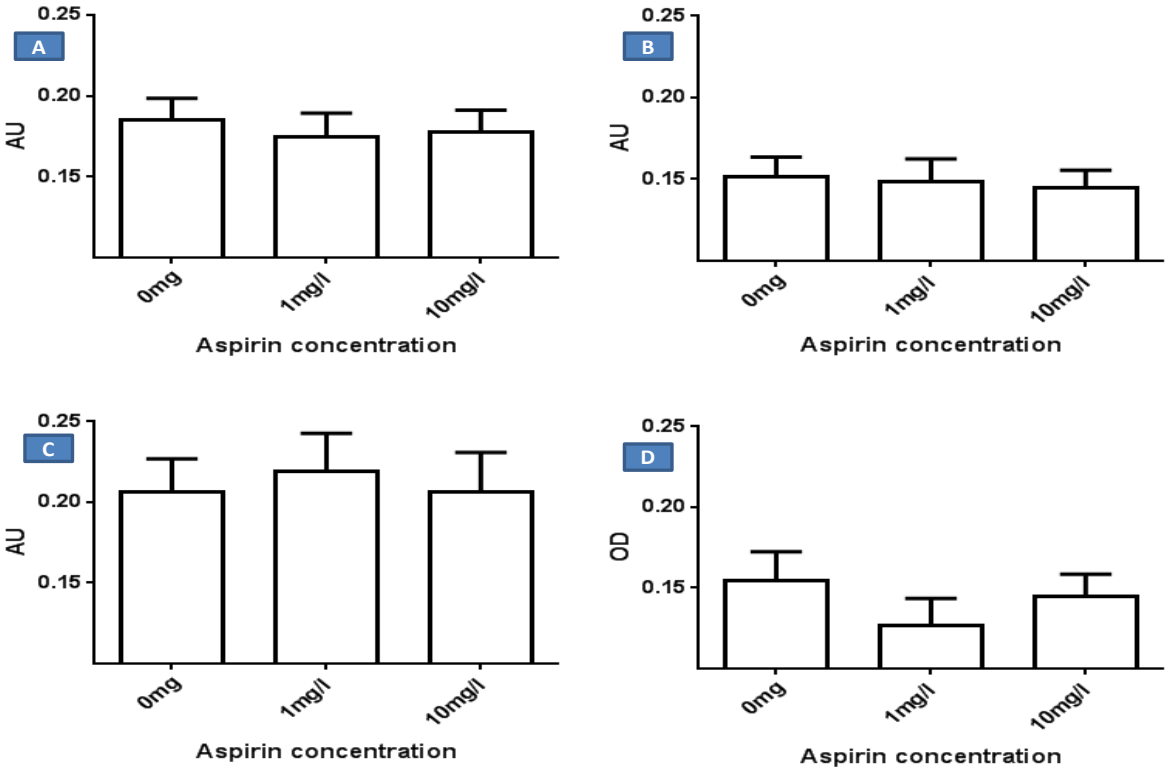


Figure 4.4 *Ex vivo* effect of different concentration of aspirin on clot maximum absorbance using turbidity and lysis technique. After addition of 0, 1, and 10 mg/l of aspirin into blood samples of subjects with diabetes (A; n=29), healthy subjects (B; n=29), subjects with poor diabetes control (C; n=15) and subjects with good diabetes control (D; n=14), maximum absorbance was measured. The results represent the mean±SEM.AU: arbitrary units.

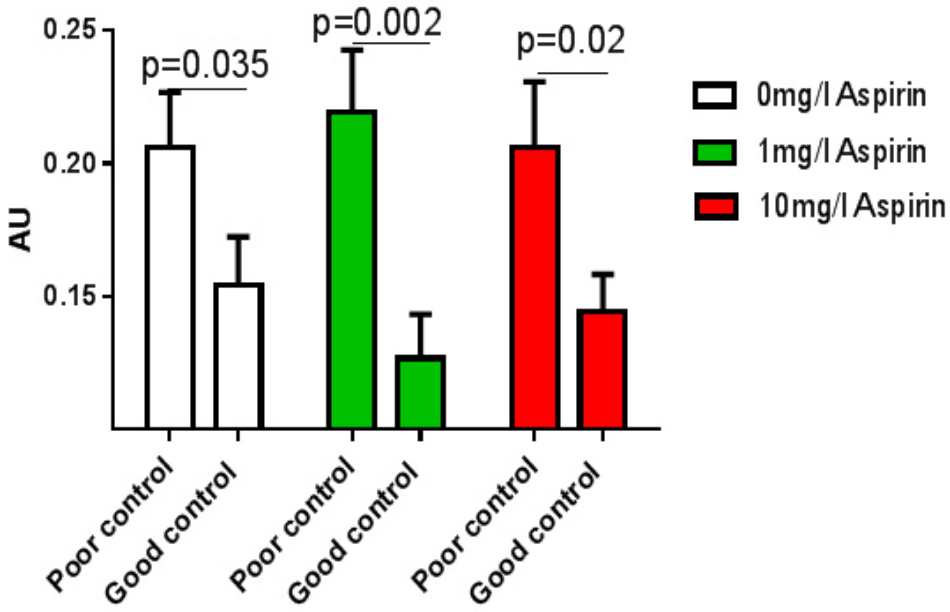


Figure 4.5 Analysis the e*x vivo* effect of different concentration of aspirin on maximum absorbance(measured by arbitrary unit) of clots formation in poor and good diabetes using turbidity and lysis technique. After addition of 0, 1, and 10 mg/l of aspirin into plasma of subjects with poor controlled diabetes (n=15) and subject with good controlled diabetes (n=14), maximum absorbance was measured. The results represent the mean±SEM. AU: arbitrary units.

Lysis time

There was no effect of low or high concentration of aspirin in clot lysis time in individuals with diabetes (Figure 4.6 A). However, in healthy controls there was reduction in lysis time from 600±63.5sec. before aspirin to 475±26.7sec. after 1mg/l aspirin, although only a trend was evident (p=0.06). Higher concentration of aspirin showed a similar pattern with a significantly shorter lysis time (489±28.6sec; p=0.04). Both Individuals with poor diabetes control (HbA1c≥8.5%) and individuals withgood diabetes control had no fibrinolytic response to aspirin (Figure 4.6C, D).

Comparing individuals with diabetes and healthy controls, there were significant differences in clot lysis time that were exaggerated in the presence of aspirin ($p<0.05$) (Figure 4.7).

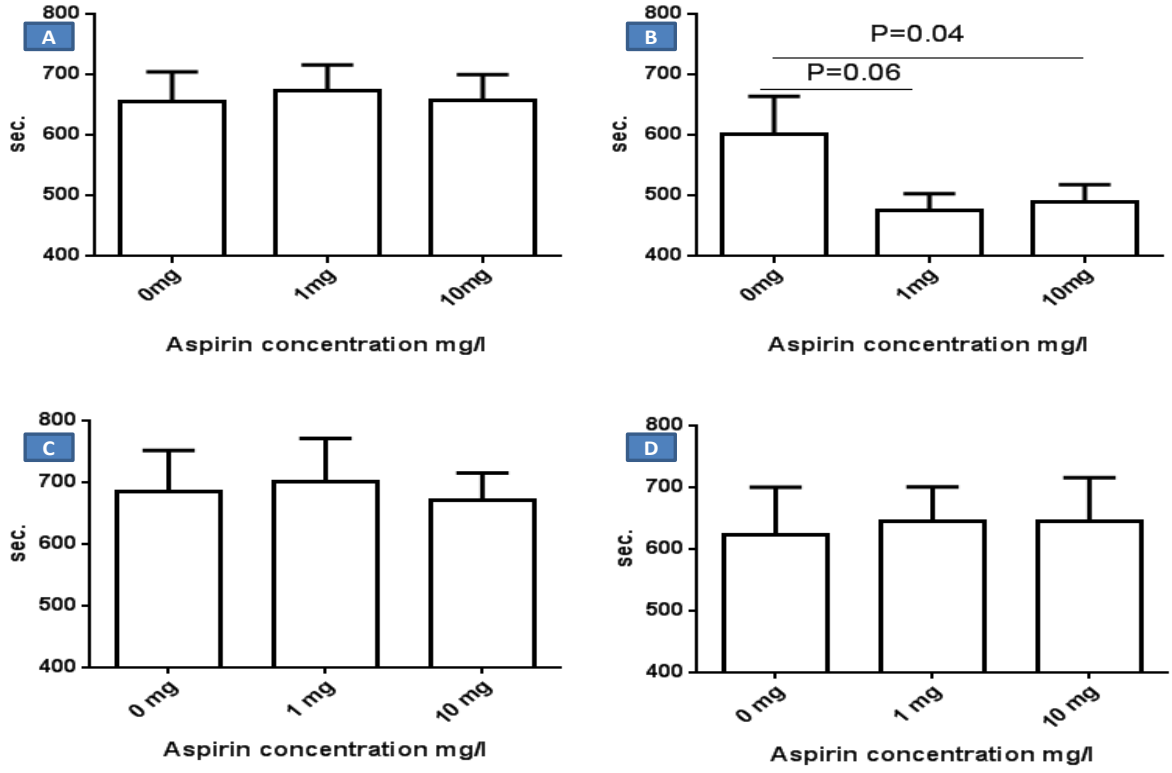


Figure 4.6 *Ex vivo* effect of different concentration of aspirin on plasma clots lysis time (measured by seconds) using turbidity and lysis technique. After addition of 0, 1, and 10 mg/l of aspirin into plasma of subjects with diabetes (A; n=29), healthy subjects (B; n=29), subjects with poor diabetes control (C; n=15) and subjects with better diabetes control (D; n=14), clot lysis was measured.The results represent the mean±SEM.

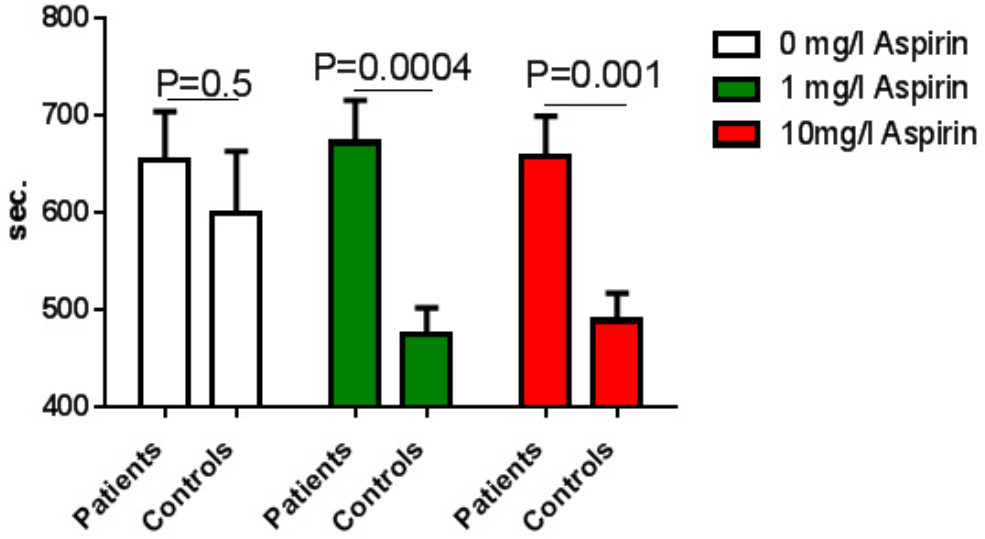


Figure 4.7 Analysis of e*x vivo* effect of different concentration of aspirin on plasma clots lysis time (measured by seconds) in patients and controls using turbidity and lysis technique. After addition of 0, 1, and 10 mg/l of aspirin into plasma of subjects with diabetes (n=29) and healthy subjects (n=29), clot lysis time was measured. The results represent the mean±SEM.

4.3.2 Scanning confocal microscopy of plasma clots (clot structure and clot lysis)

Fibrin structure before and after aspirin therapy of plasma clots from a pool of healthy controls is shown in Figure 4.8 A, B, respectively. Aspirin made the fibrin clots more porous and a similar finding, although to a lesser extent, was observed in patients with diabetes (Figure 4.9 C, D). As expected, there were differences in patients and controls in relation to plasma clot density (Figure 4.8 A and 4.9 C, respectively). Fibrinolysis of mature clots was observed in real time using laser scanning confocal microscopy. I recorded lysis time in at least four independent experiments for each condition. There was a reduction in lysis time of pooled plasma clots of healthy controls after incubation with 10mg/l

aspirin from 469±35 to 309±71 sec., although this failed to reach statistical significant (p=0.2, Figure 4.10). *Ex vivo* incubation of 10 mg aspirin paradoxically increased clot lysis time but the difference did not reach statistical significance (428±56 sec. and 526±143 sec. (p=0.7; Figure 4.10).

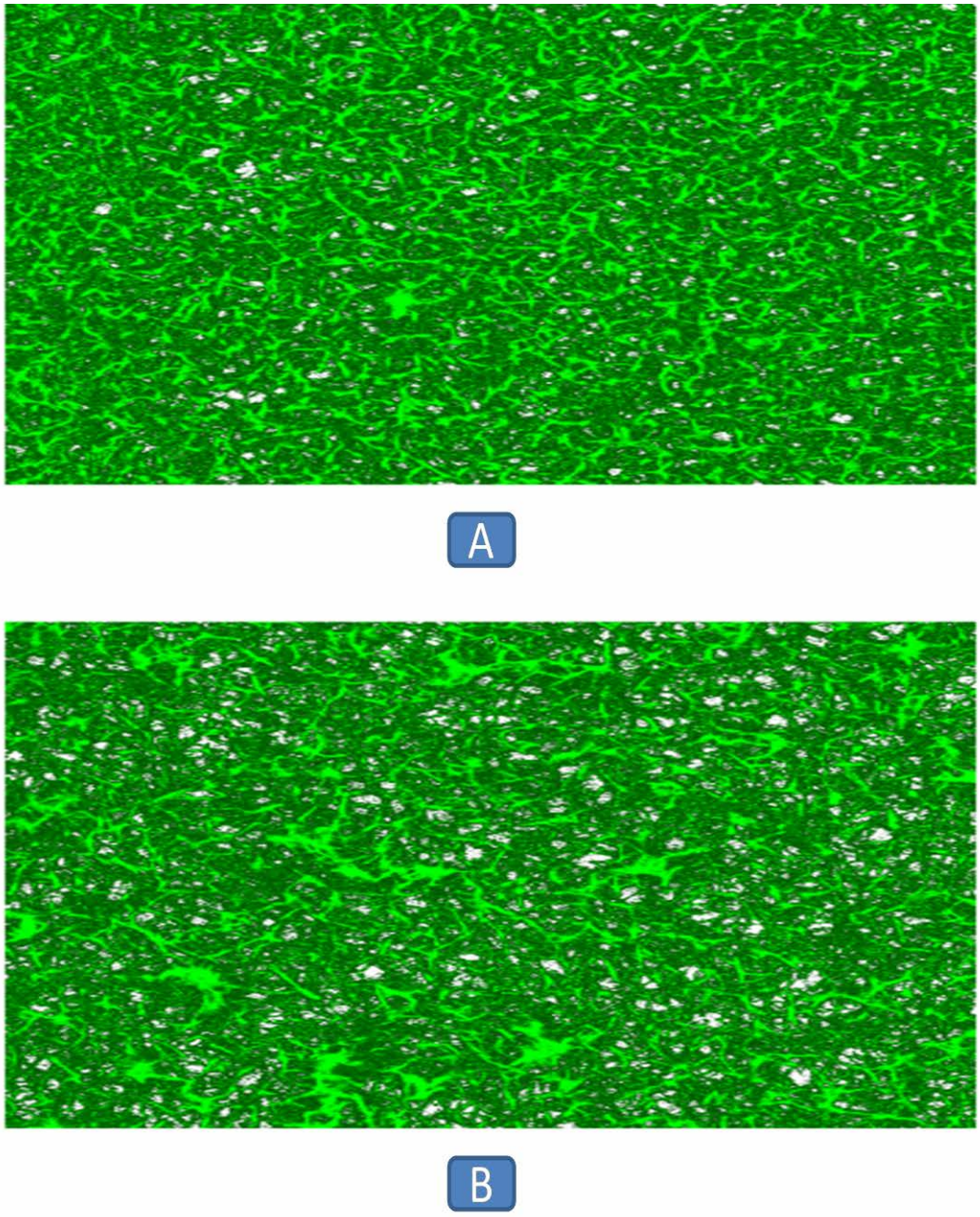


Figure 4.8 Confocal microscopy of 20 µm Z-stack (magnification of 400x) of mature fibrinclot of controls. Fibrin clots made from pooled plasma from healthy controls (n=12) incubated *ex vivo* with 0 mg/l (A), 10mg/l aspirin (B)

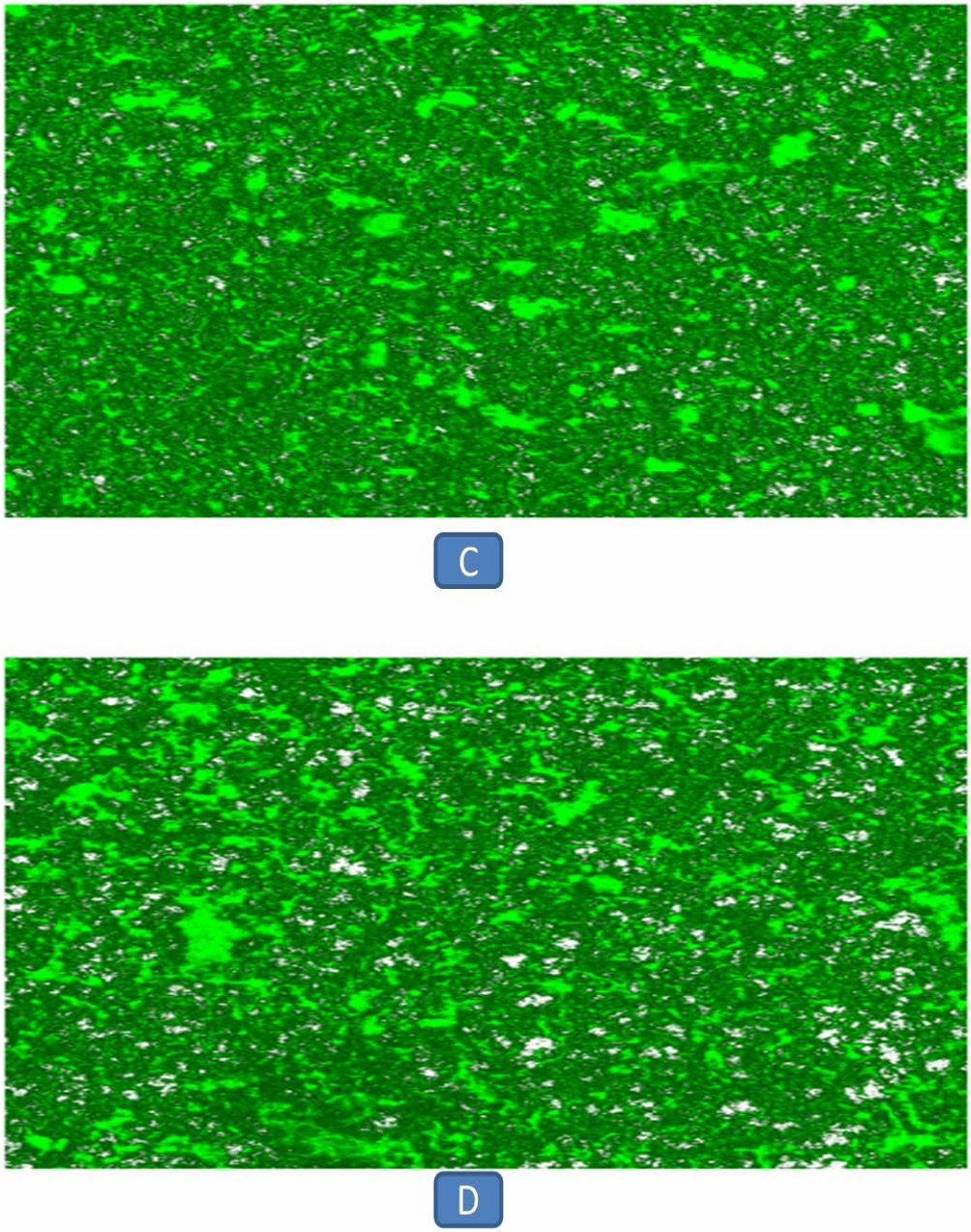


Figure 4.9 Confocal microscopy of 20 µm Z-stack (magnification of 400x) of mature fibrin clot of poor diabetes control. Fibrin clots made from pooled plasma ofpoor diabetes control (n=12) incubated *ex vivo* with 0 mg/l (C), 10mg/l aspirin (D).

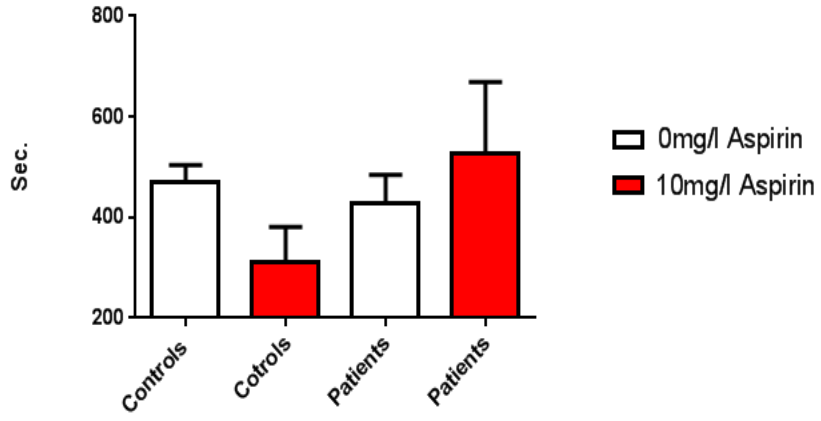


Figure 4.10 Fibrin clot fibrinolysis of mature clots measured usingconfocal microscopy of (magnification of 400x). The results represent the 3 repeats mean±SEM of 4 independent lysis experiments of clots made from plasma pool of healthy controls (n=12) or subjects from poor diabetes control.

4.3.3 Scanning electron microscopy of plasma clot

Scanning electron microscopy failed to show a significant difference in fibrin fibre thickness of clots made from pooled plasma of healthy controls before and after 10 mg/l aspirin therapy (72±2.5 and 76±2 nm, respectively; p=0.9). The same was observed in clots made from pooled diabetes plasma (59±1.3 and 63±1.8, respectively; p=0.07). Fibres of healthy control clots were significantly thicker than those of patients with diabetes (72±2.5 and 59±1.3, respectively; p=0.0001). The same significant difference was observedin the presence of aspirin (p=0.0001; Figure 4.11A). The pore size of clots from the control group before and after aspirin treatment was bigger than the pore size in clots made from diabetes patients (Figure 4.11 B-D).

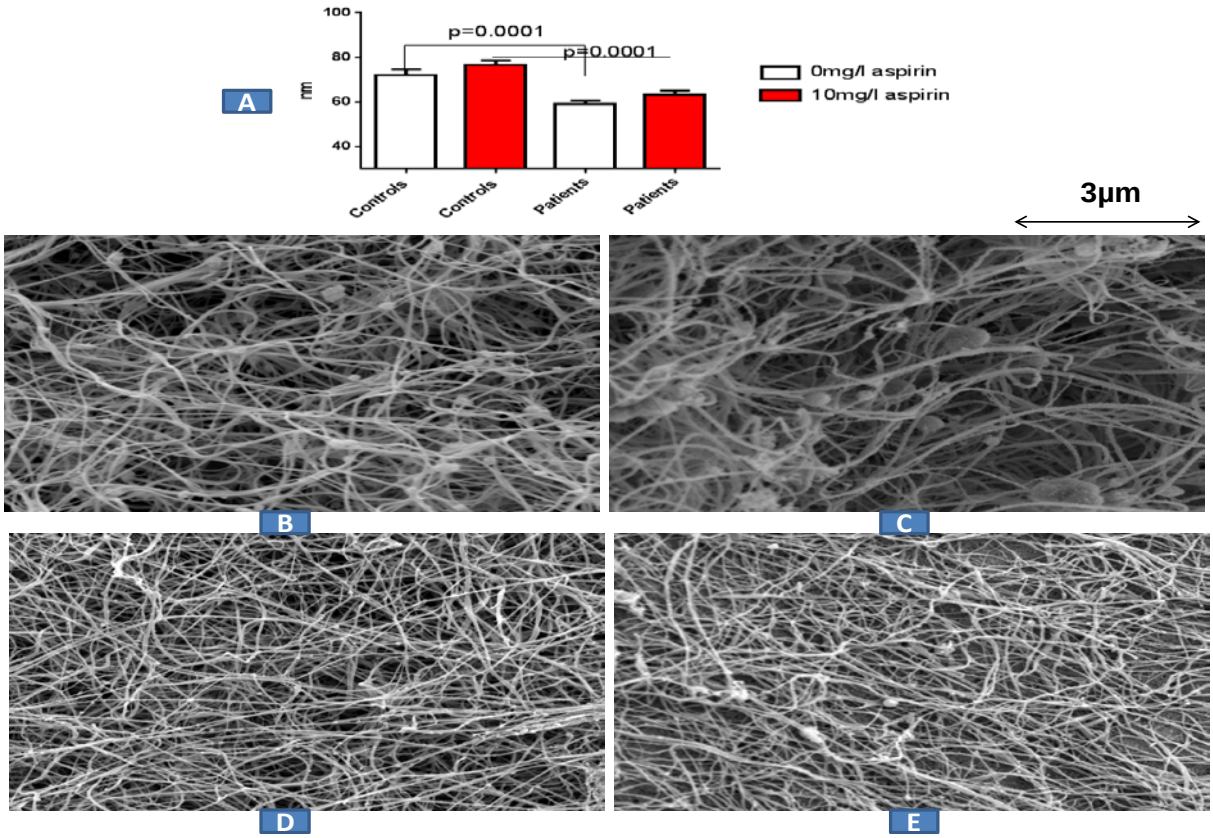


Figure 4.11 Fibrin clot analysis using electron microscopy (magnification of 10000x). Fibrin fibre thickness of clots made of pooled plasma of healthy controls or poor diabetes patients (n=12 in each pool) before and after 10mg/l aspirin treatment. Results represent the mean±SEM of 200 readings from 10different images captured from two fibrin clots(A). Clot Images of healthy controls before (B), and after treatment with 10mg/l aspirin (C). Clot image of diabetes patients before (D) and after treatment with 10mg/l aspirin (E).

4.3.4 Turbidimetric analysis (maximum absorbance and clot lysis) of clots made from purified fibrinogen

Aspirin addition, at 10 mg/l numerically increased fibrin clot maximum absorbance in controls from 0.033±0.002 to 0.037±0.006, but this failed to reach statistical significance (p=0.5). As similar pattern was observed in patients with poor diabetes controls with final turbidity at 0.05±0.01 and 0.06±0.01 before and after aspirin, respectively (p=0.2) (Figure 4.12 A). There was no significant difference in clot lysis time before and after incubation of purified

fibrinogen with aspirin in controls (151±27sec. and 146±17sec, respectively; p>0.1) with similar findings in individuals with poor diabetes control (197±31sec and 170±15 sec, respectively; p>0.1) (Figure 4.12 B). Although numerically longer, there was no significant difference between patients and controls in lysis time after the addition of either 0 or 10 mg/l aspirin (p>0.1 for both).

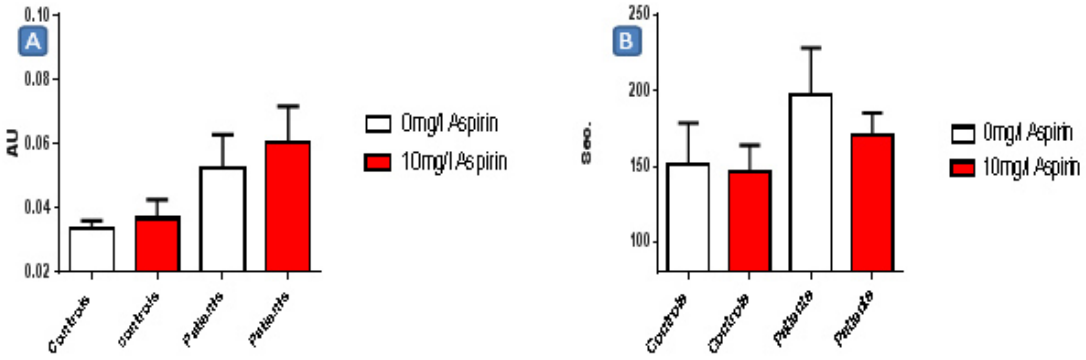
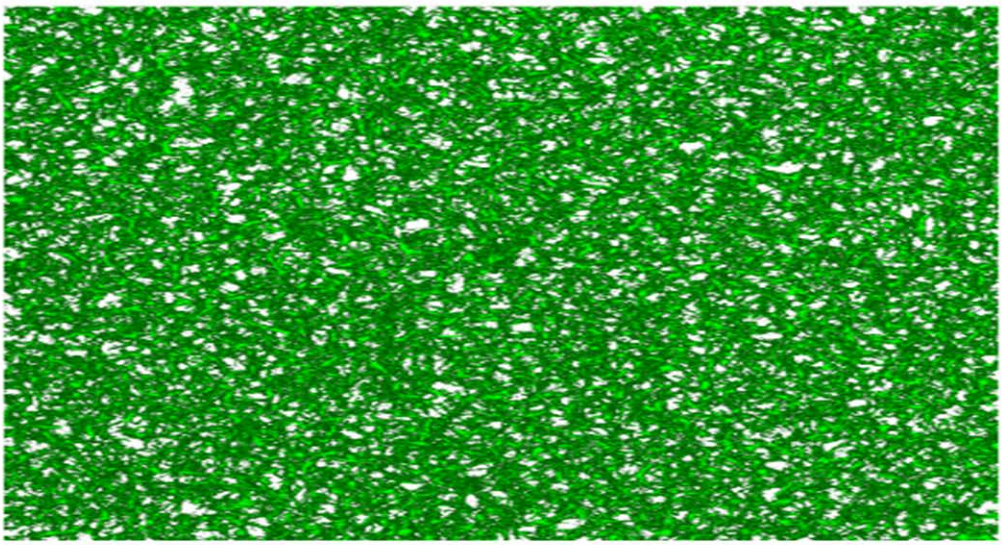


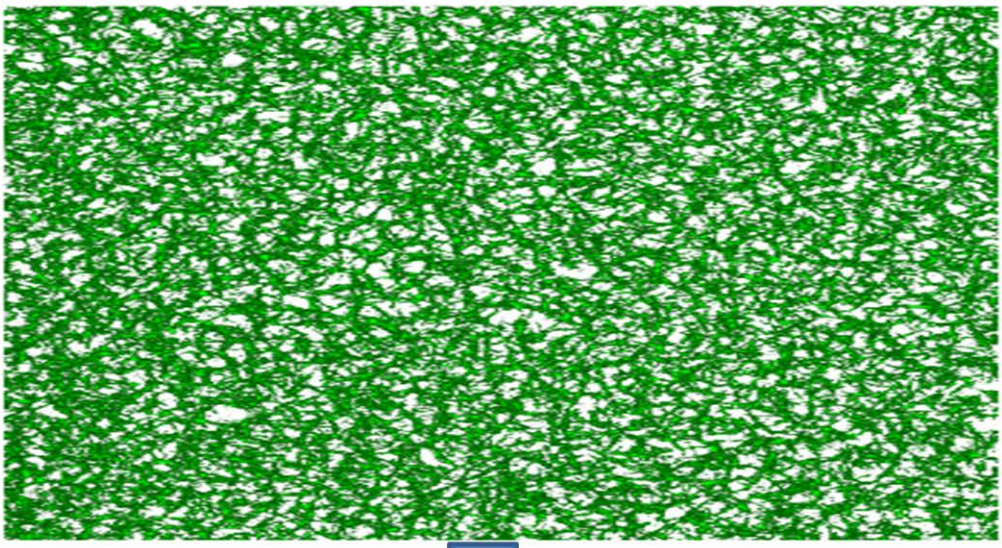
Figure 4.12 *Ex vivo* effect of different concentration of aspirin on maximum absorbance of purified fibrinogen fibrin clot formation and clot lysis time using turbidity and lysis. After addition of 0 and 10 mg/l of aspirin intopool of purified fibrinogen of subjects with poor diabetes control (n=12), healthy subjects (n=12), maximum absorbance was measured.(A). After addition of 0 and 10 mg/l of aspirin intopool of purified fibrinogen of subjects with poor diabetes control (n=12), healthy subjects (n=12), lysis time was measured (B). The results represent 3 repeats mean±SEM.

4.3.5 Scanning confocal microscopy of purified fibrinogen clots (fibrin clot structure and clot lysis)

The fibrin network appeared more porous after aspirin therapy using clots made from pooled purified fibrinogen from healthy controls (Figure 4.13 A,B), with largely similar findings, observed in clots made from pooled purified fibrinogen from poor diabetescontrol (Figure 4.14 C, D). The fibrin network was less compact comparing controls with diabetes clots (Figure 4.13, 4.14). There was no significant difference in lysis time of clots made from purified fibrinogen of pooled control samples before and after incubation with aspirin (405±13 and 466±42 sec., respectively; $p>0.1$; Figure 4.15 E). Also, there was no difference in lysis time of clots made from pooled purified fibrinogen of poor diabetes control before and after incubation with aspirin (525±111 and 467±53 sec, respectively; $p>0.1$). The numerical difference in clot lysis time comparing healthy control with patients with poor diabetes control samples did not reach statistical significance ($p>0.2$; Figure 4.15).



A



B

Figure 4.13 Confocal microscopy of 20 µm Z-stack (magnification of 400x) of mature fibrin clot of healthy control. Fibrin clot made from pooled fibrinogen from subjects from healthy control (n=12) incubated *ex vivo* with 0 mg/l (A), 10mg/l aspirin (B).

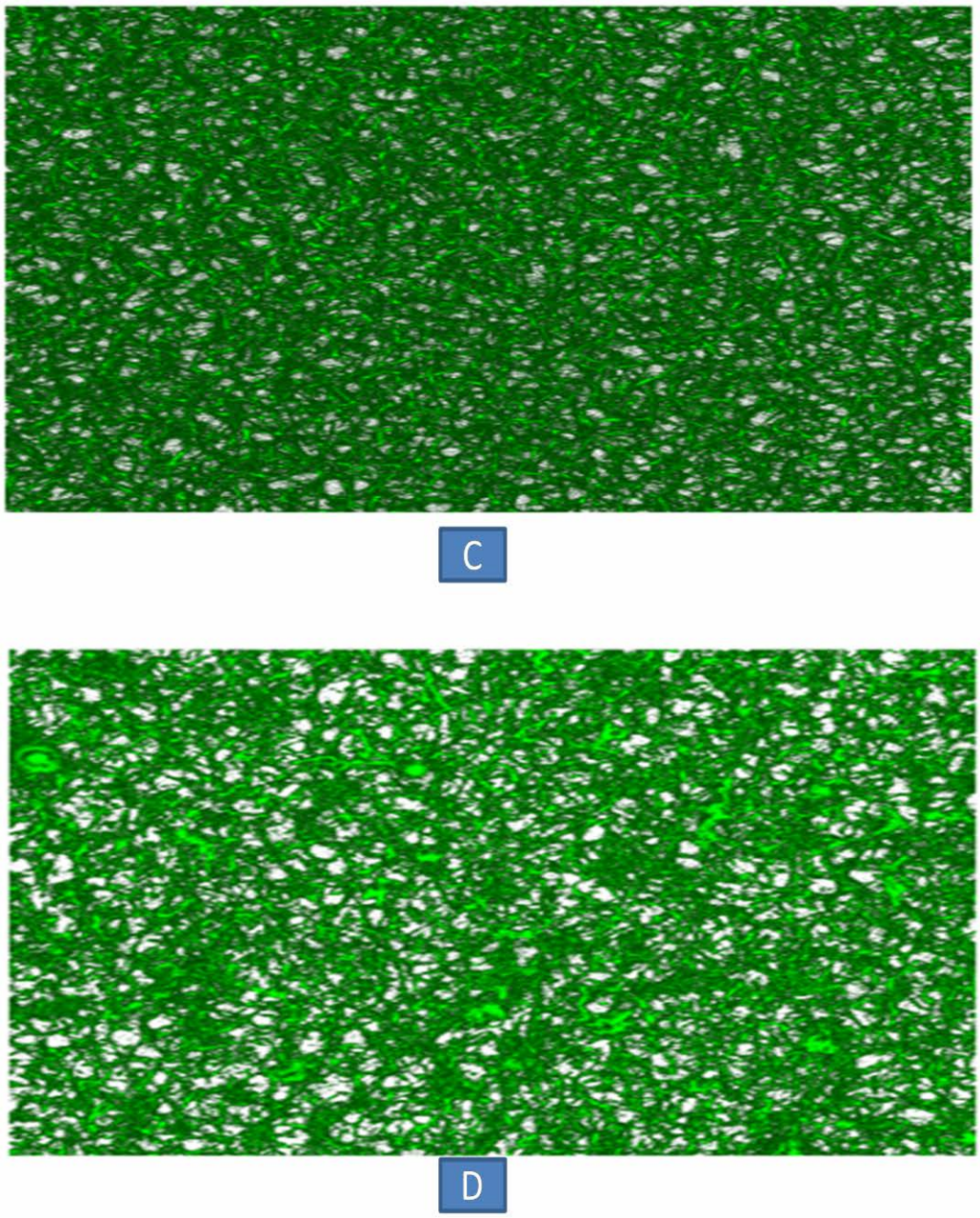


Figure 4.14 Confocal microscopy of 20 µm Z-stack (magnification of 400x) of mature fibrin clot of poor diabetes control. Fibrin clot made from pooled fibrinogen from subjects from poor diabetes control (n=12) incubated *ex vivo* with 0 mg/l (C), 10mg/l aspirin (D).

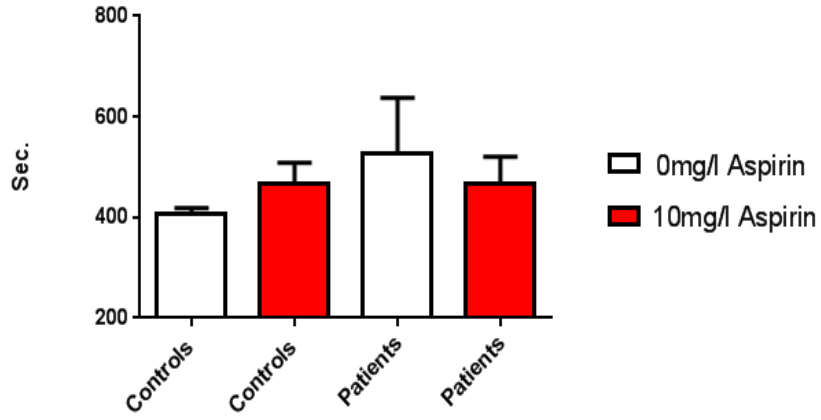


Figure 4.15 Fibrin clot fibrinolysis of mature clots measured using confocal microscopy of (magnification of 400x). The results represent the 3 repeats mean±SEM of 4 independent lysis experiments of clots made from fibrinogen pool of healthy controls (n=12) or subjects from poor diabetes control (n=12) incubated ex vivo with 0mg/l, 10mg/l aspirin.

4.3.6 Scanning electron microscopy of purified fibrinogen clot

Scanning electron microscopy showed that fibrin fibre thickness of clots made from purified fibrinogen of pooled healthy controls increased from 71.6±1.7 to 79.6±2.1nm after aspirin therapy ($p<0.05$). In contrast, aspirin had the opposite effect on fibre thickness of patients with diabetes with a reduction from 66±1.9 to 61±2.3 after aspirin therapy ($p<0.05$). Fibres of healthy control clots were thicker than those of patients with poor diabetes control (71±1.7 and 66±1.9 nm, respectively; $p<0.05$; Figure 4.16 A).

The pore size of clot from controls before and after aspirin treatment was bigger than the pore size in patients with the same condition (Figure 4.16 B-D).

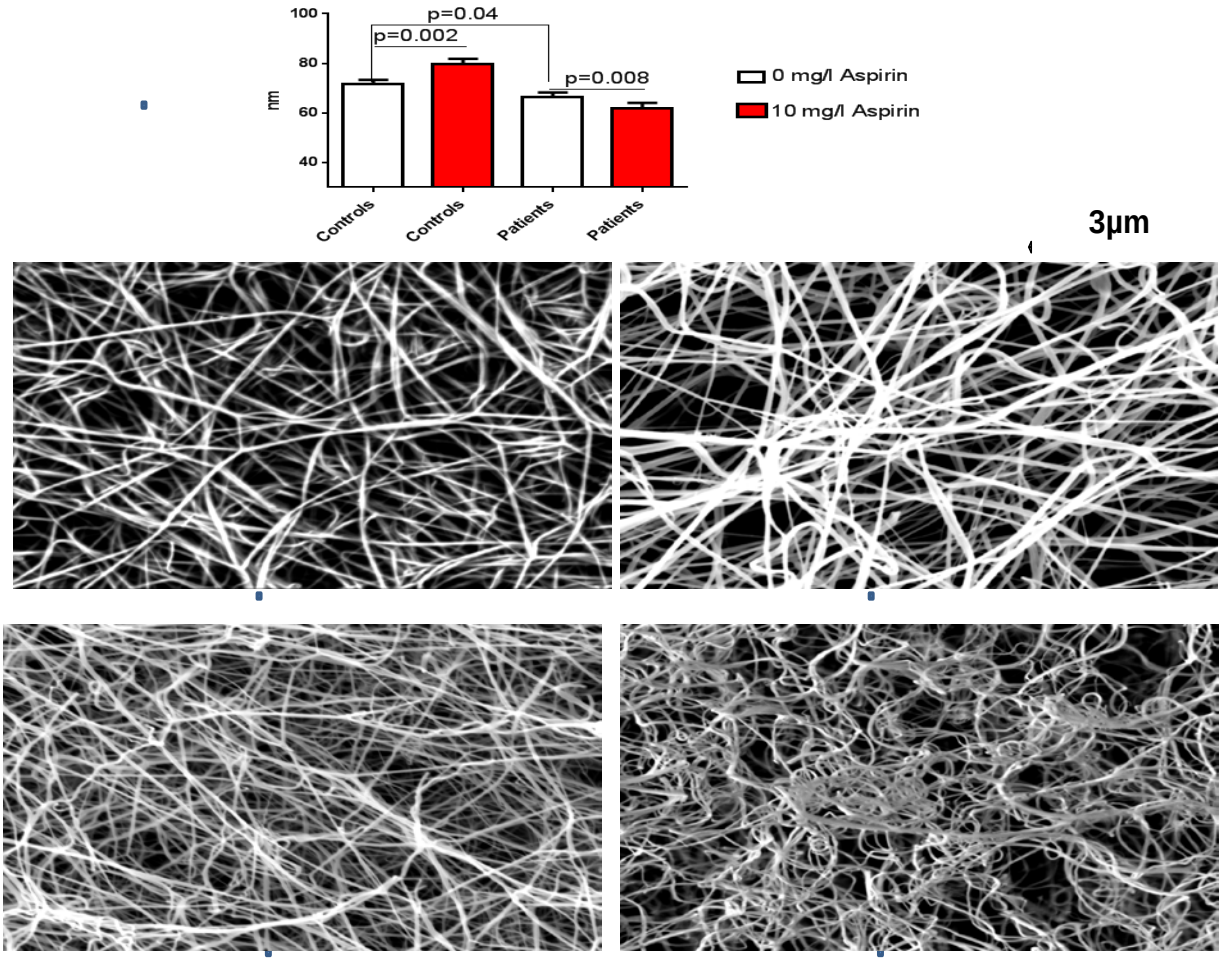


Figure 4.16 Fibrin clot analysis using electron microscopy (magnification of 10000x). Fibrin fibre thickness of clots made of purified fibrinogen from pooled healthy controls (n=12) or patients with diabetes (n=12) before and after *ex vivo* aspirin treatment. Results represent the mean±SEM of 200 readings from 10 imagescaptured from two fibrin clots. Aspirin resulted in increased fibrin thickness in healthy control with the opposite effect observed in clots made from patients with poor diabetes control. Fibrin fibres were thicker in healthy control compared with diabetes patients (A). Fibrin clots before and after treatment with 10mg/l aspirin in controls (B, C) or patients with diabetes D, E).

4.3.7 Turbidimetric analysis of plasma clots in the presence of excess glucose

Lag phase

Lag phase of plasma samples was not affected by aspirin in the presence of excess glucose in controls or patients with diabetes (Figure 4.17 A-D).

Lag phase in controls (398±56sec) was marginally longer than patients (263±47 sec; p=0.07) in the absence of aspirin. This difference became non-significant after adding 1 mg/l of aspirin to plasma samples (300±58 and 205±48 Sec, respectively; p=0.2). However, with higher aspirin concentration of 10 mg/l, the difference was significant (368±62 and 186±38, respectively; p=0.02, Figure 4.18).

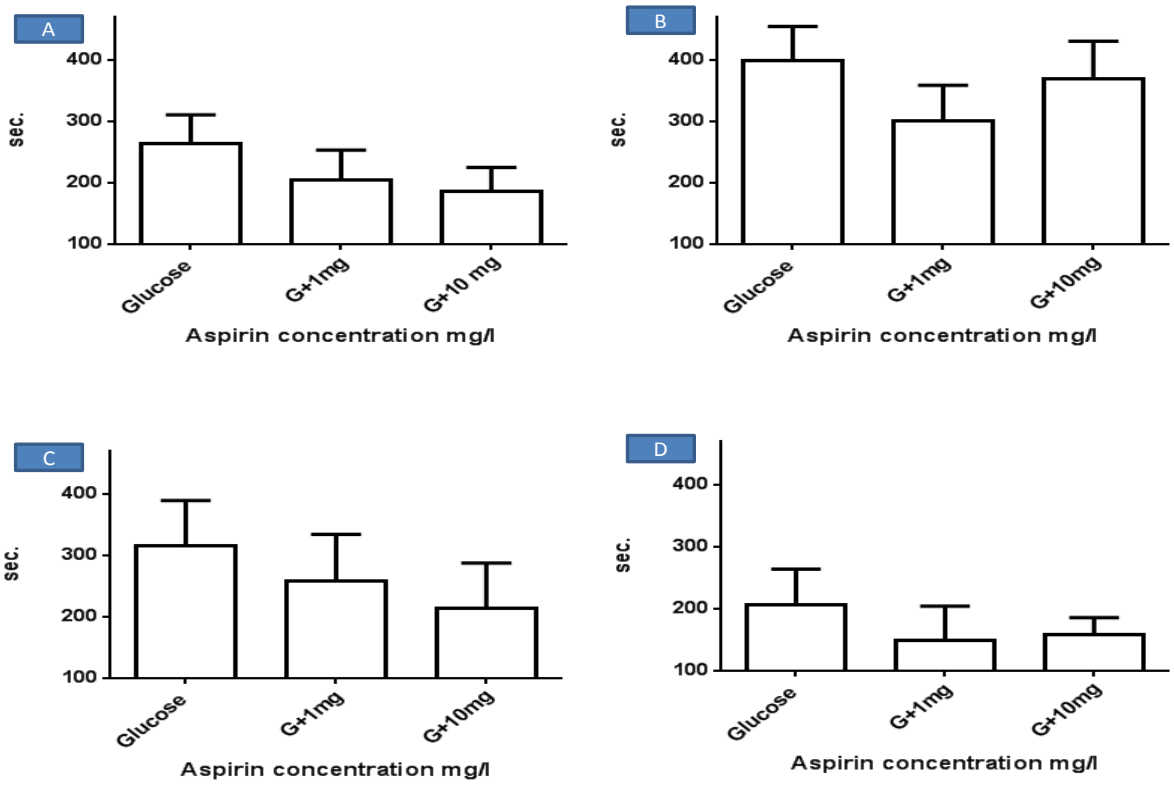


Figure 4.17 *Ex vivo* effect of different concentration of aspirin and 20 mmol/l glucose on lag phase (measured by seconds) of clot formation using turbidity and lysis technique. After addition of glucose, glucose+1mg/l, and glucose+10 mg/l of aspirin into plasma of subjects with diabetes (A; n=29), healthy subjects (B; n=29), subjects with poor diabetes control (C; n=15) and subjects with better diabetes control (D; n=14), lag time was measured.The results represent the mean±SEM.

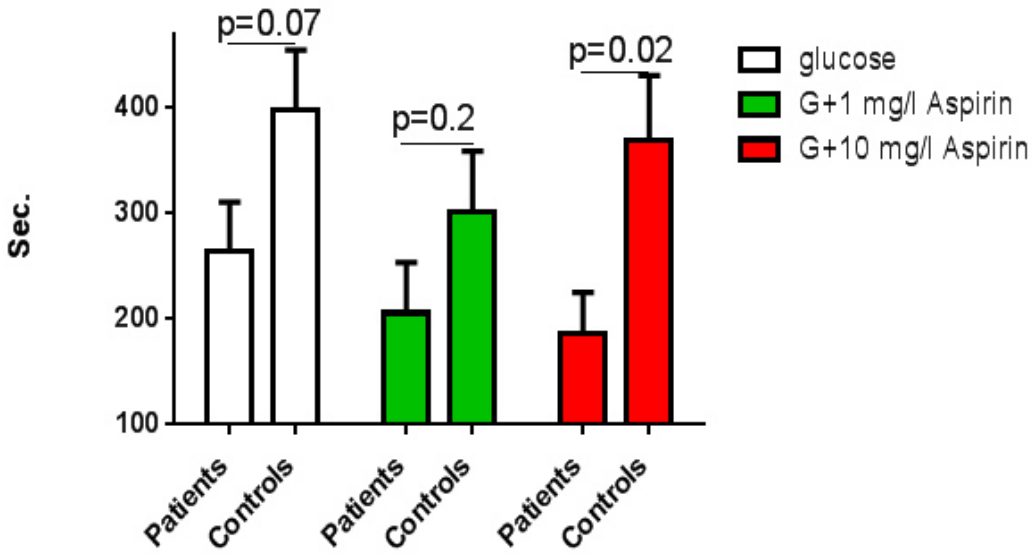


Figure 4.18 Analysis of the e*x vivo* effect of different concentration of aspirin and 20 mmol/l on lag phase (measured by seconds) of clot formation for patients and conrols using turbidity and lysis. After addition of glucose, glucose+1mg/l, and glucose+10 mg/l of aspirin into plasma of subjects with diabetes (n=29) and healthy controls (n=29), lag phase was measured. The results represent the mean±SEM.

Maximum absorbance

Both low and high aspirin concentrations had no effect on clot maximum absorbance in the presence of excess glucose in either patients (Figure 4.19A) or controls (Figure 4.19B). Glycaemic control appears to have affected response to aspirin therapy in the presence of additional glucose with a marginal difference detected between poor and good glycaemic control patients with 1 mg/l aspirin at 0.19±0.02 and 0.14±0.02, respectively; p=0.09). A clear difference was evident with 10 mg/l aspirin concentration at 0.2±0.01.and 0.13±0.01, respectively; p=0.01; Figure 4.19C, D and Fig 4.20).

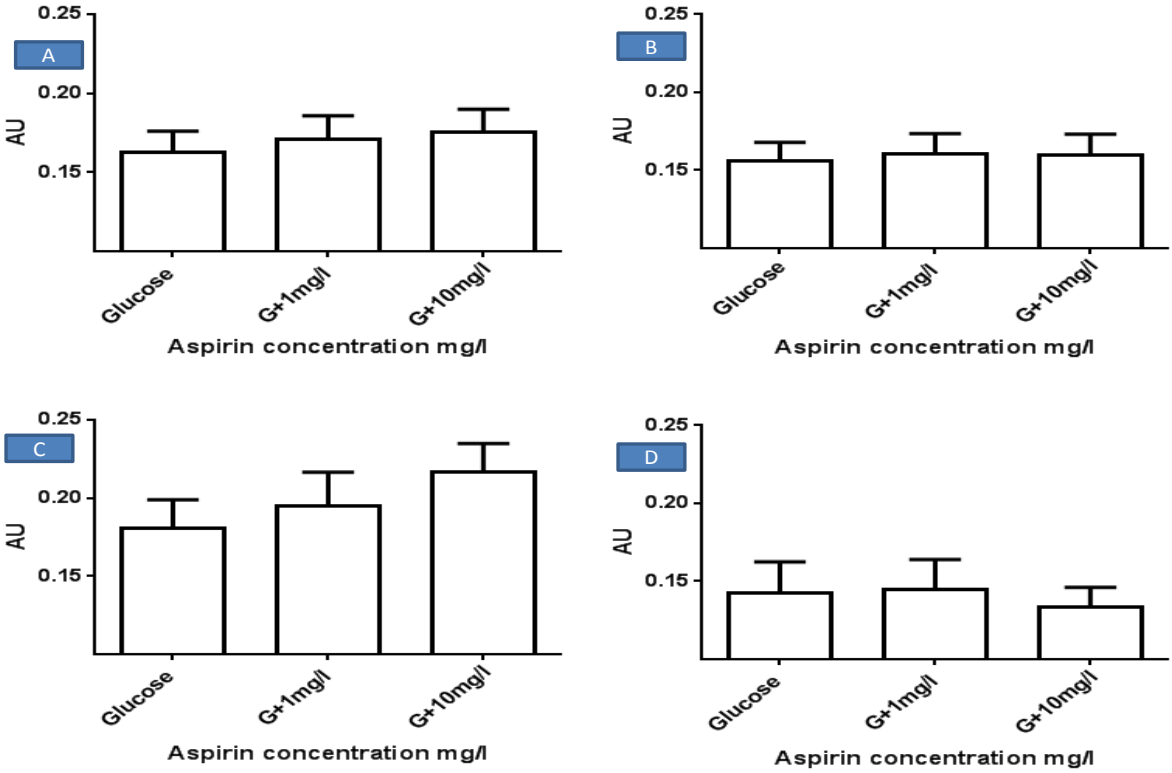


Figure 4.19 *Ex vivo* effect of different concentrations of aspirin in the presence of additional glucose on plasma clot maximum absorbance using turbidity and lysis technique. After addition of glucose (g), g+1mg/l, and g+10 mg/l of aspirin to blood samples of subjects with diabetes (A; n=29), healthy subjects (B; n=29), subjects with poor diabetes control (C; n=15) and subjects with better diabetes control (D; n=14), maximum absorbance was measured.The results represent the mean±SEM.AU: arbitrary units.

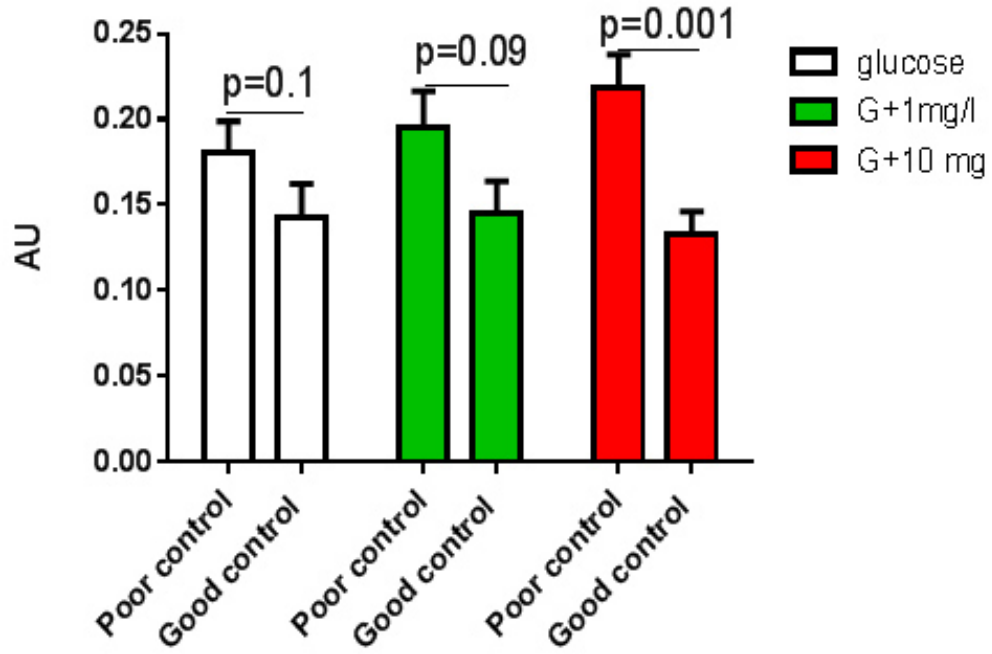


Figure 4.20 *Ex vivo* effect of different concentration of aspirin with 20mmol/l glucose on clot maximum absorbance using turbidity and lysis technique. After addition of glucose, glucose+1, and glucose+10 mg/l of aspirin into plasma of subjects with poor diabetes control (n=15) and better diabetes control (n=14), clot lysis time was measured. The results represent the mean±SEM.AU: arbitrary units.

Lysis time

There was no effect of low or high concentrations of aspirin with additional glucose on clot lysis time in individuals with diabetes or in healthy controls (Figure 4.21 A, B). Glycaemic control did not affect these results as shown in (Figure 4.21 C, D).

Comparing individuals with diabetes and healthy controls, lysis time was shorter in controls regardless of the presence or absence of aspirin (Figure 4.22).

Addition of glucose had no effect on clot lysis time in controls in the presence or absence of aspirin in controls (Figure 4.23).

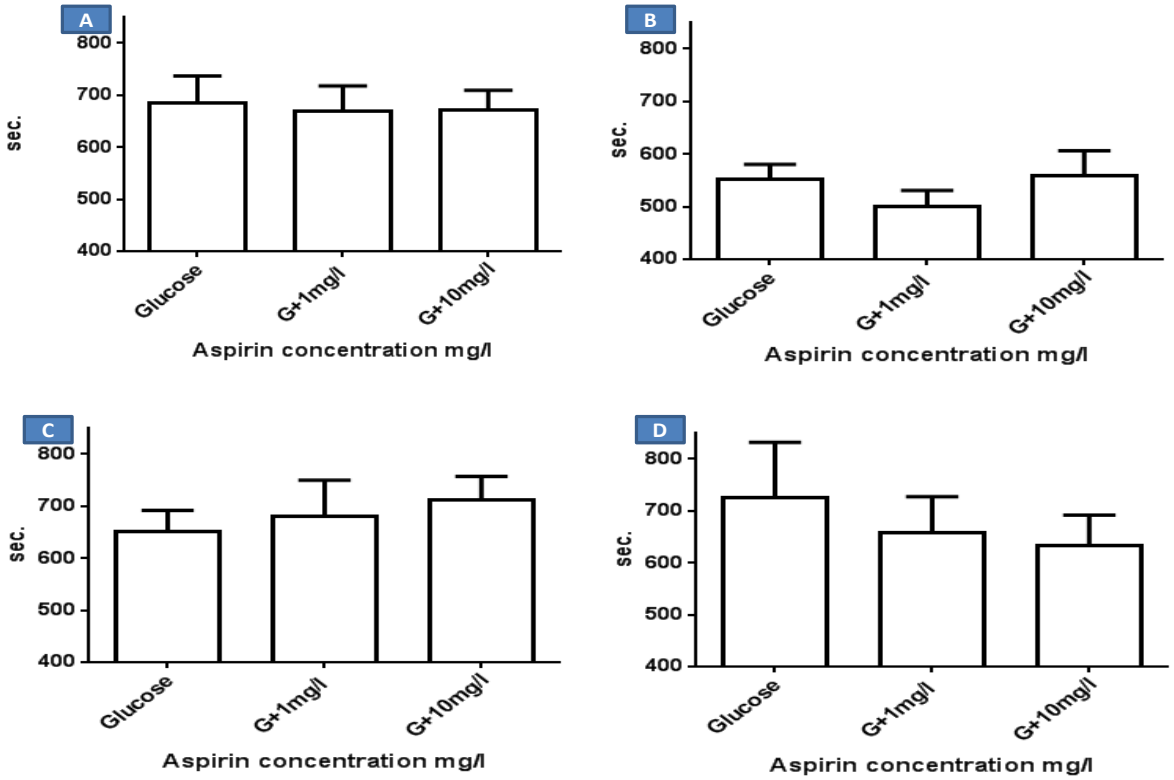


Figure 4.21 *Ex vivo* effect of different concentration of aspirin and 20 mmol/l glucose on plasma clots lysis time (measured by seconds) using turbidity and lysis technique. After addition of 0, 1, and 10 mg/l of aspirin into plasma of subjects with diabetes (A; n=29), healthy subjects (B; n=29), subjects with poor diabetes control (C; n=15) and subjects with better diabetes control (D; n=14), clot lysis time was measured.The results represent the mean±SEM.

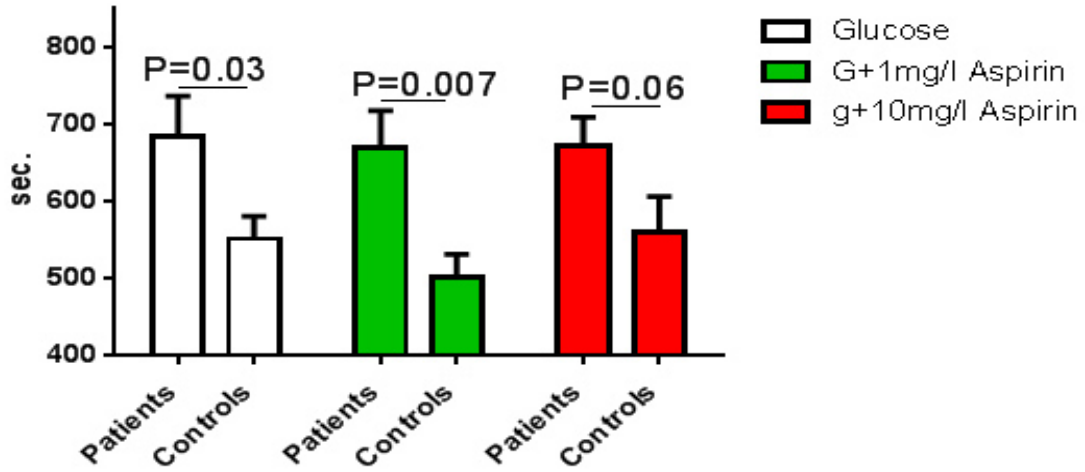


Figure 4.22 *Ex vivo* effect of different concentrations of aspirin with 20mmol/l glucose on clot lysis time (measured by seconds) using turbidity and lysis technique. After addition of glucose, glucose+1, and glucose+10 mg/l of aspirin into plasma of subjects with diabetes (n=29) and healthy controls (n=29), clot lysis time was measured. The results represent the mean±SEM.

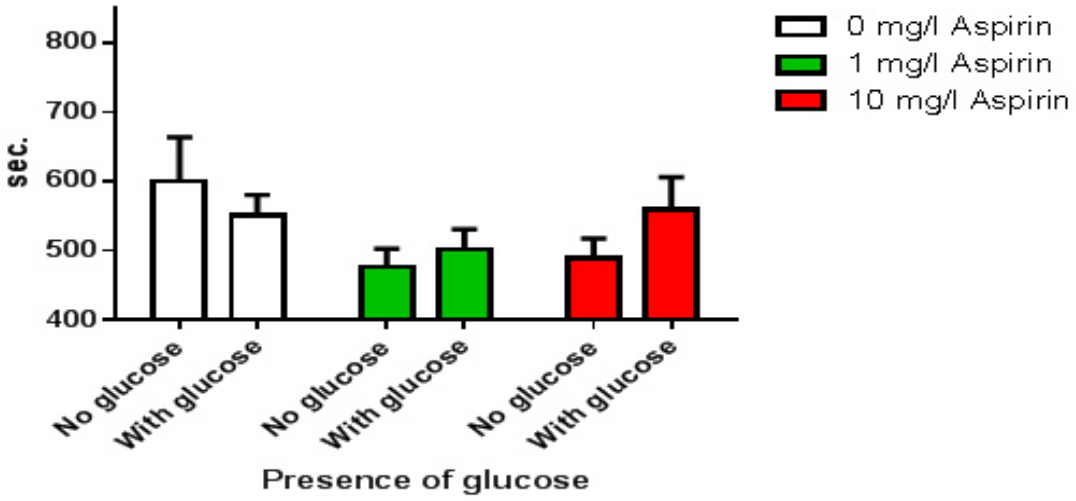


Figure 4.23 *Ex vivo* effect of different concentration of aspirin with 20mmol/l glucose on clot lysis time of controls (measured by seconds) using turbidity andlysis technique. After addition of glucose, glucose+1, and glucose+10 mg/l of aspirin to plasma of subjects with diabetes (n=29), clot lysis time was measured. The results represent the mean±SEM.

4.3.8 Scanning confocal microscopyplasma (clot image and lysis time) in the presence of extra glucoe

Fibrin structure before and after aspirin therapy of plasma clots from a pool of healthy controls is shown in Figure 4.24 A and Figure 4.24 B, respectively. There was no effect of aspirin on fibrin clot structure in healthy controls. A similar finding was observed in patients with diabetes (Figure 4.25 C and 4.25 D, before and after aspirin treatment respectively). Fibrinolysis of mature clots was assessed using laser scanning confocal microscopy. Lysis time in four independent experiments was recorded (Figure 4.26). There was no difference in lysis time of plasma pool clot of healthy control before and after incubation with 10mg/l aspirin in the presence of additional glucose (448±27, 502±130 sec, respectively; p=0.1). Aspirin had no significant effect on clot lysis time of pooled plasma frompatients with poor diabetes control subjects (657±30.sec to 514±106 sec, before and after aspirin; p>0.1). Lysis time in controls was shorter than in patients in the presence of additional glucose. However, no difference was detected in the presence of both additional glucose and aspirin (Figure 4.26).

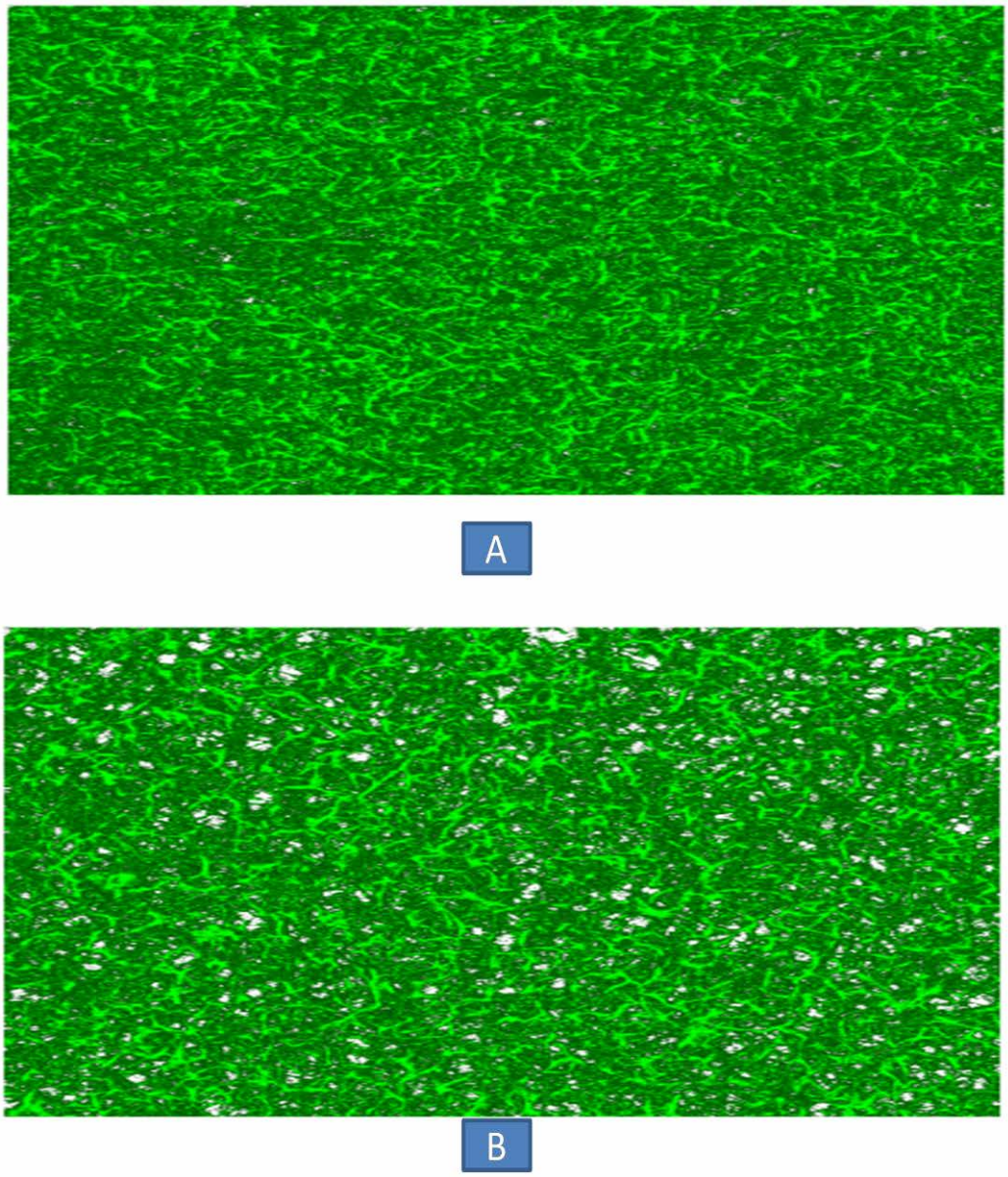
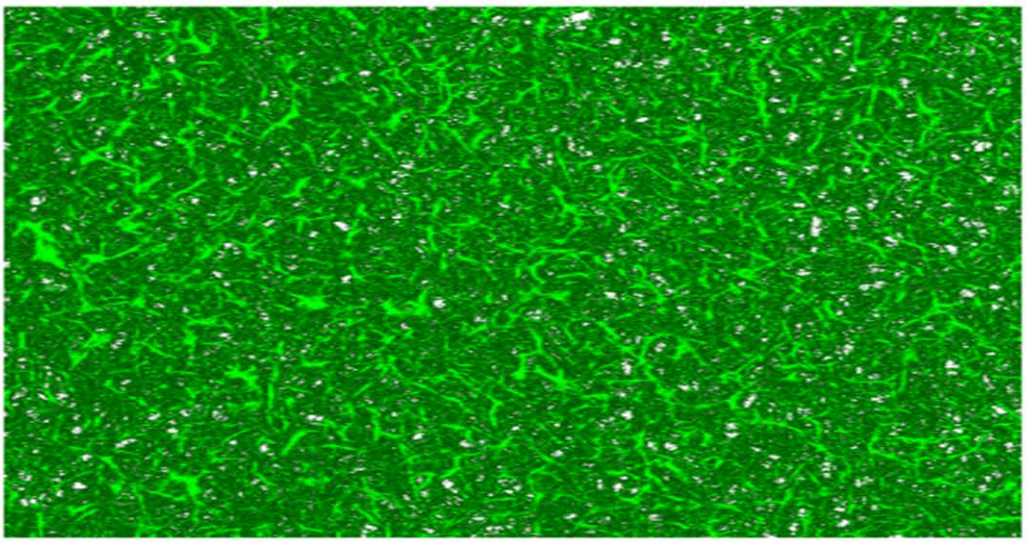
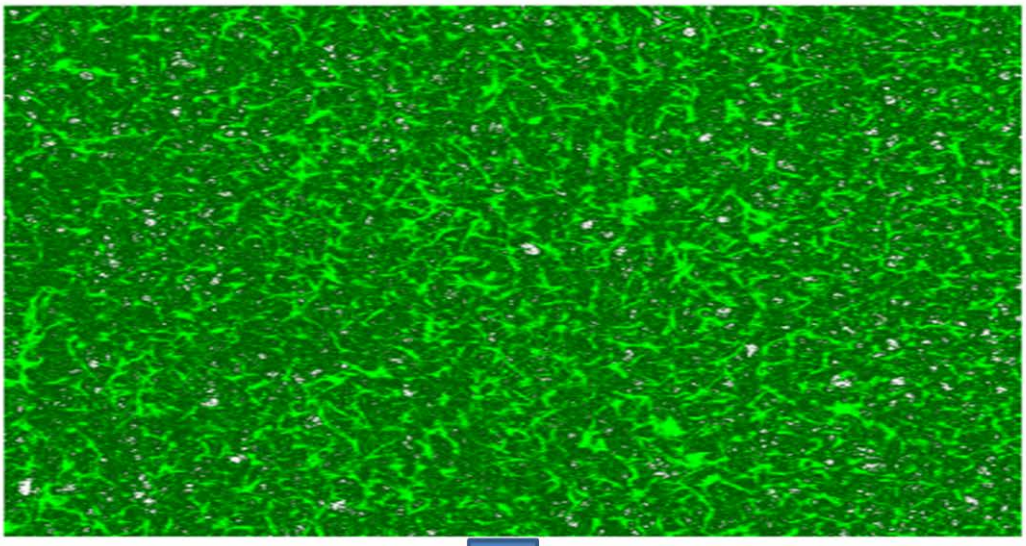


Figure 4.24 Confocal microscopy of 20 µm Z-stack (magnification of 400x) of mature fibrin clot of healthy control. Fibrin clot made from pooled plasmaof healthy control (n=12) incubated *ex vivo* with 20 mmol/l glucose (A), glucose+10mg/l aspirin (B).



C



D

Figure 4.25 Confocal microscopy of 20 µm Z-stack (magnification of 400x) of mature fibrin clot of poor diabetes control. Fibrin clot made from pooled plasmaof poor diabetes control (n=12) incubated *ex vivo* with 20 mmol/l glucose (C), glucose+10mg/l aspirin (D).

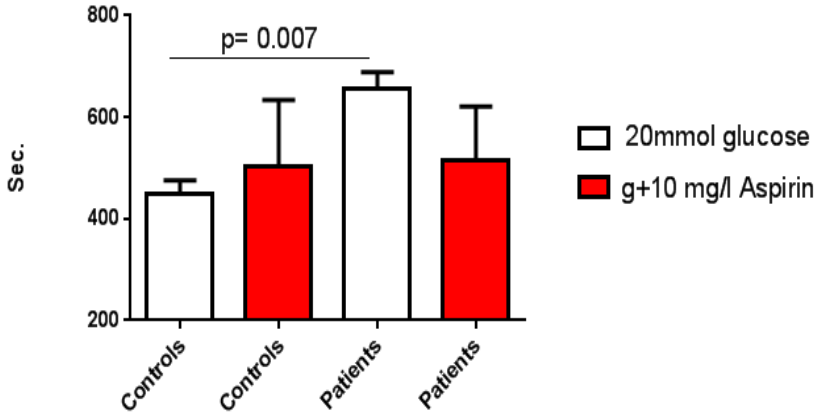


Figure 4.26 Fibrin clot fibrinolysis of mature clots measured using confocal microscopy of (magnification of 400x). The results represent the 3 repeats mean±SEM of 4 independent lysis experiments of clots made from pooled plasma of healthy controls (n=12) or subjects from poor diabetes control (n=12) incubated ex vivo with 20 mmol/l glucose, glucose+10mg/l aspirin.

4.3.9 Scanning electron microscopy of plasma clot after incubation with extra glucose

Scanning electron microscopy showed, both in controls and patients with poor diabete control, that aspirin had no effect on fibre thickness in the presence of additional glucose (Figure 4.27).

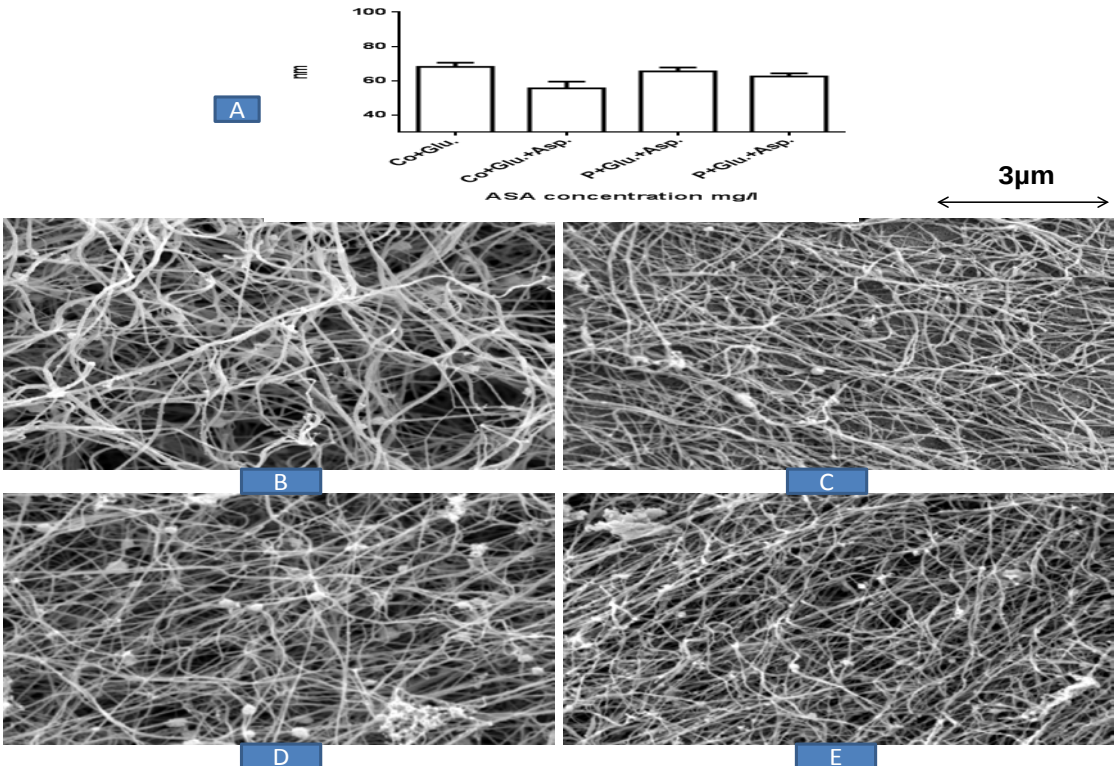


Figure 4.27 Fibrin clot analysis using electron microscopy (magnificatin of 10000x). Fibrin fibre thickness of clots made of pooled plasma of healthy controls or patients with poor diabetes control (n=12 in each pool) after incubation with 20mmol/l glucose or glucose+10mg/l aspirin treatment. Results represent the mean±SEM of 200 readings from 12 images (A). Clot Images of healthy controls after treatment with glucose(B)or glucose+10mg/l aspirin (C). D, E, representative images of clots from patients with additional glucose or glucose+10 mg/l aspirin.

4.3.10 Turbidimetric analysis of purified fibrinogen clots in the presence of excess glucose (Maximum absorbance and lysis time)

Aspirin failed to exert an effect on maximum absorbance of clots made from purified fibrinogen before and after aspirin treatment in the presence of additional glucose. This applied to both controls and patients with diabetes (Figure 4.28 A). In the presence of extra glucose, there was no difference in lysis time after aspirin treatment of clots made from pooled purified fibrinogen of control or subjects with diabetes control (Figure 4.28 B).

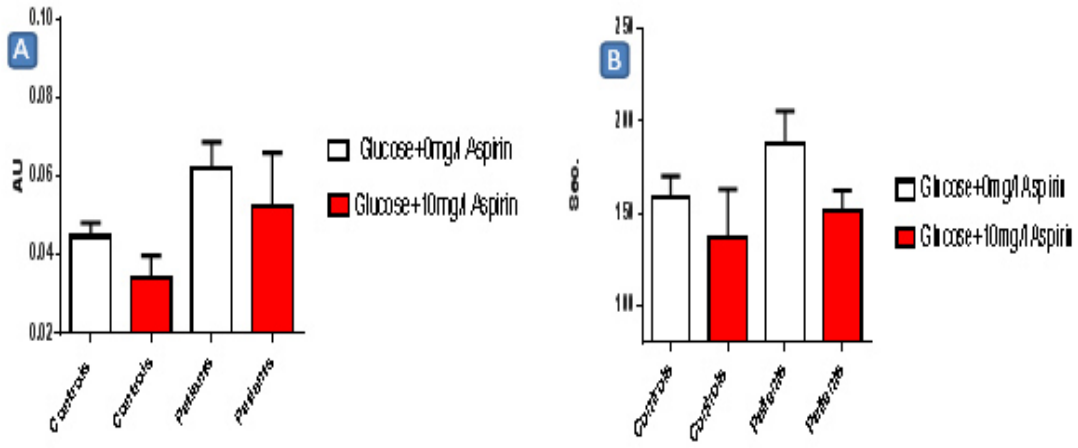


Figure 4.28 *Ex vivo* effect of different concentration of aspirin with 20mmol/l glucose on clot maximum absorbanceand on clot lysis time using turbidity and lysis. After addition of glucose and glucose+10 mg/l of aspirin into purified fibrinogen of patients with poor diabetes control (n=12) and good diabetes control (n=12), clot maximum absorbance was measured (A). After addition of glucose and glucose+10 mg/l of aspirin into pooled of purified fibrinogen of healthy subjects (n=12) and subjects with poor diabetes control (n=12), clot lysis time was measured (B).The readings represent the mean ±SEM of 4 repeat experiments.

4.3.11 Confocal scanning microscopy (clot image and lysis time) of purified fibrinogen clot after incubation with extra glucose

Fibrin networks appeared more porous in clots made from pooled purified fibrinogen of healthy controls after aspirin treatment in the presence of additional glucose. No such an effect was observed in individuals with diabetes (Figure 4.29, 4.30). Fibrinolysis was observed in real time using laser scanning confocal microscopy and the mean of 4 experiments were recorded (Figure 4.31). In the presence of extra glucose, aspirin had no effect onlysis time of clots made form pooled purified fibrinogen of healthy control. Aspirin appeared to increase clot lysis time in patients with diabetes, although the difference did not reach statistical significance (p=0.3). Clot lysis time of healthy controls was significantly shorter than diabetes in the presence of additional glucose and aspirin (Figure 4.31).

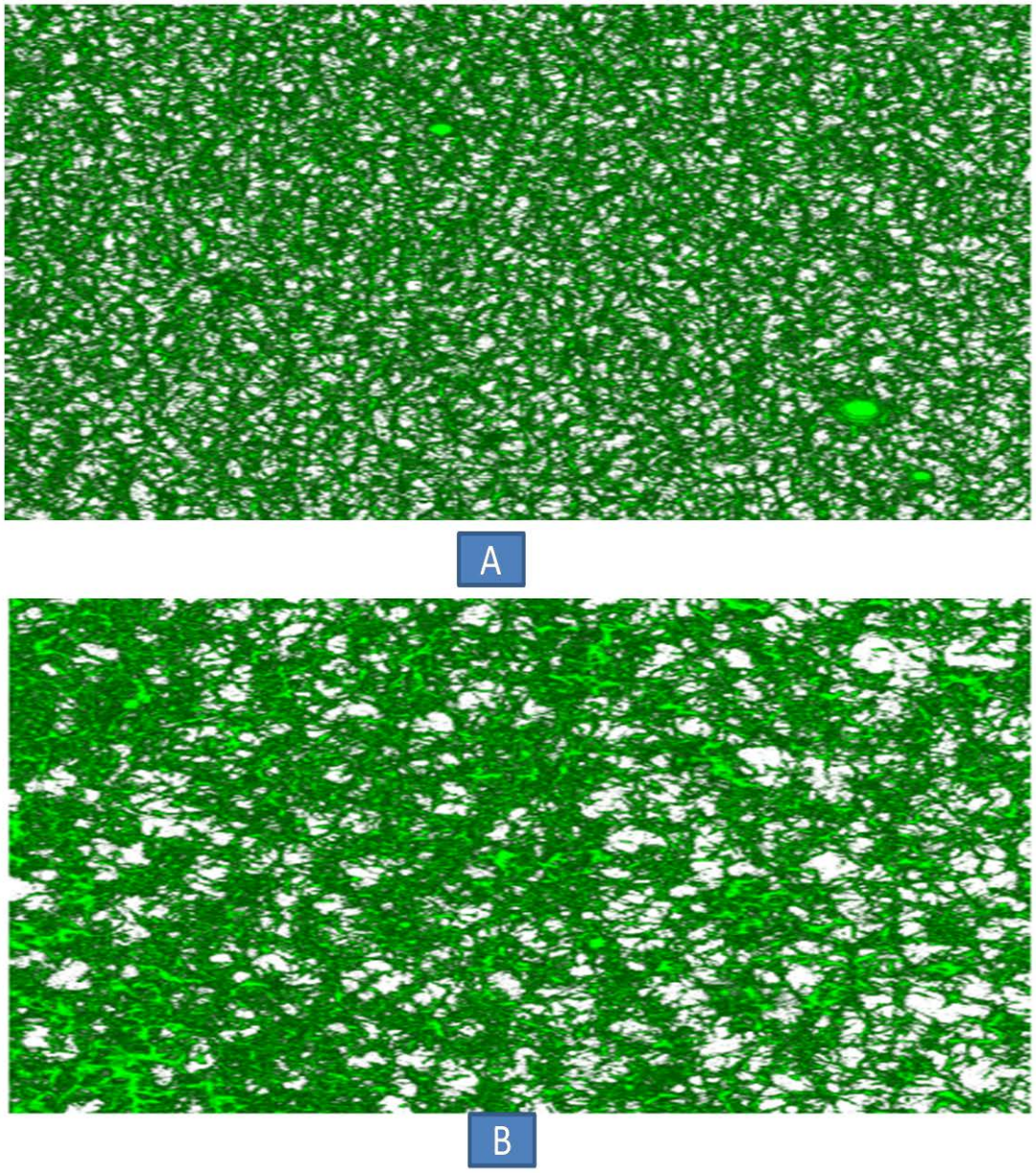


Figure 4.29 Confocal microscopy of 20 µm Z-stack (magnification of 400x) of mature fibrin clot of healthy control. Fibrin clot made from pooled fibrinogenof healthy control (n=12) incubated *ex vivo* with 20 mmol/l glucose (A), glucose+10mg/l aspirin (B).

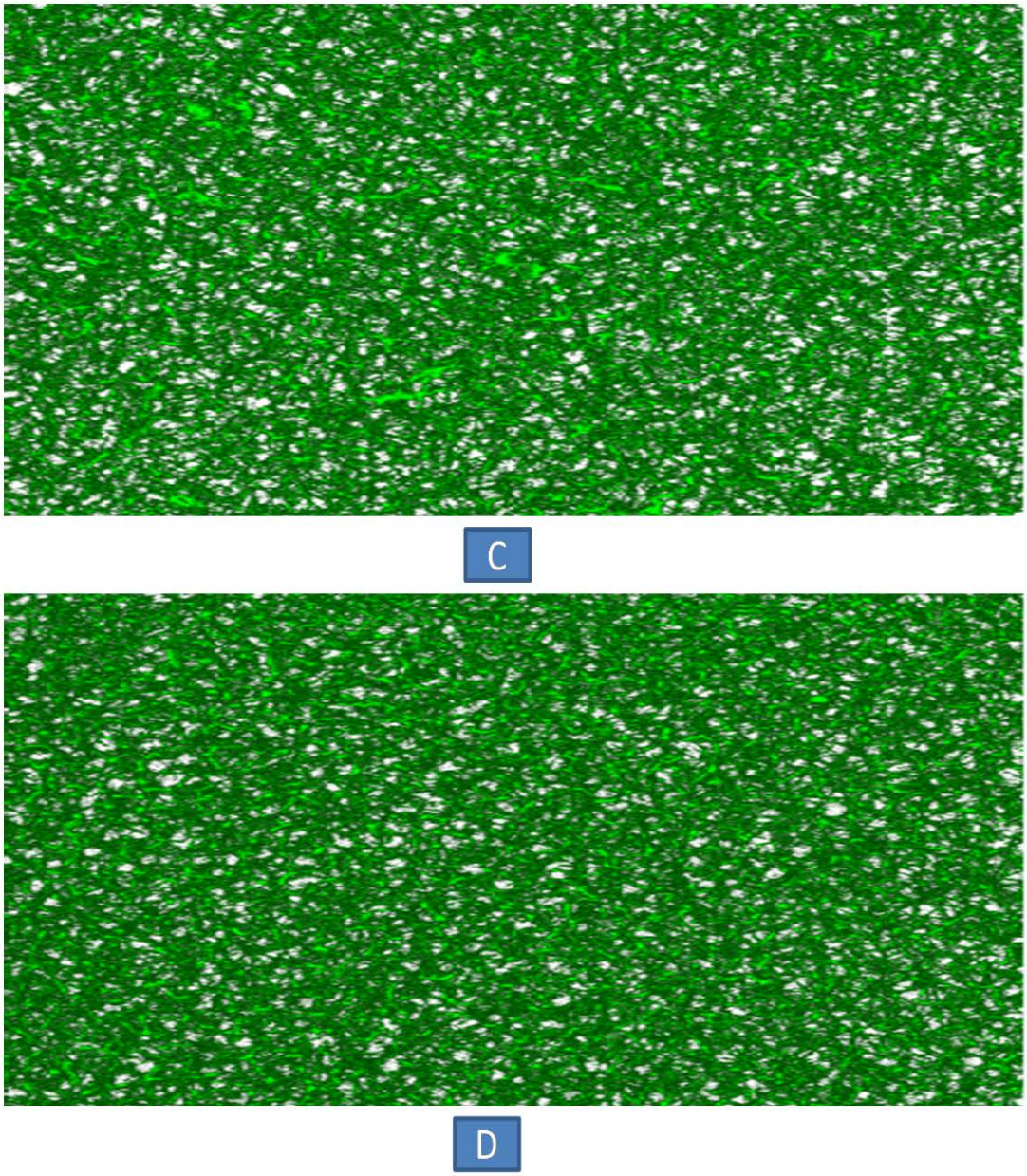


Figure 4.30 Confocal microscopy of 20 µm Z-stack (magnification of 400x) of mature fibrin clot of healthy control. Fibrin clot made from pooled fibrinogenof poor diabetes control (n=12) incubated *ex vivo* with 20 mmol/l glucose (C), glucose+10mg/l aspirin (D).

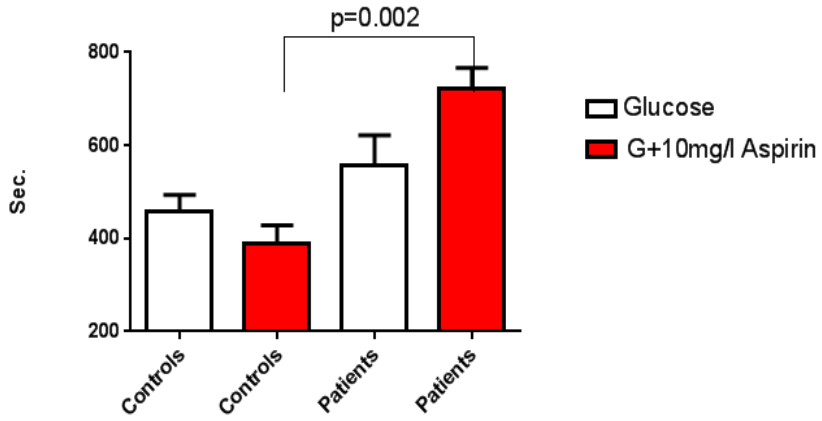


Figure 4.31 Fibrin clot fibrinolysis of mature clots measured using confocal microscopy of (magnification of 400x). The results represent the 3 repeats mean±SEM of 4 independent lysis experiments of clots made from pooled fibrinogen of healthy controls (n=12) or poor diabetes control (n=12) incubated ex vivo with 20 mmol/l glucose, glucose+10mg/l aspirin

4.3.12 Scanning electron microscopy of purified fibrinogen clot after incubation with extra glucose

Scanning electron microscopy showed that in the presence of extra glucose, fibrin fibre thickness of clots made from pooled purified fibrinogen was not affected by aspirin treatment in control or diabetes samples. Also, no difference in fibre thickness was observed comparing healthy control with poor diabetes control samples (Figure 4.32).

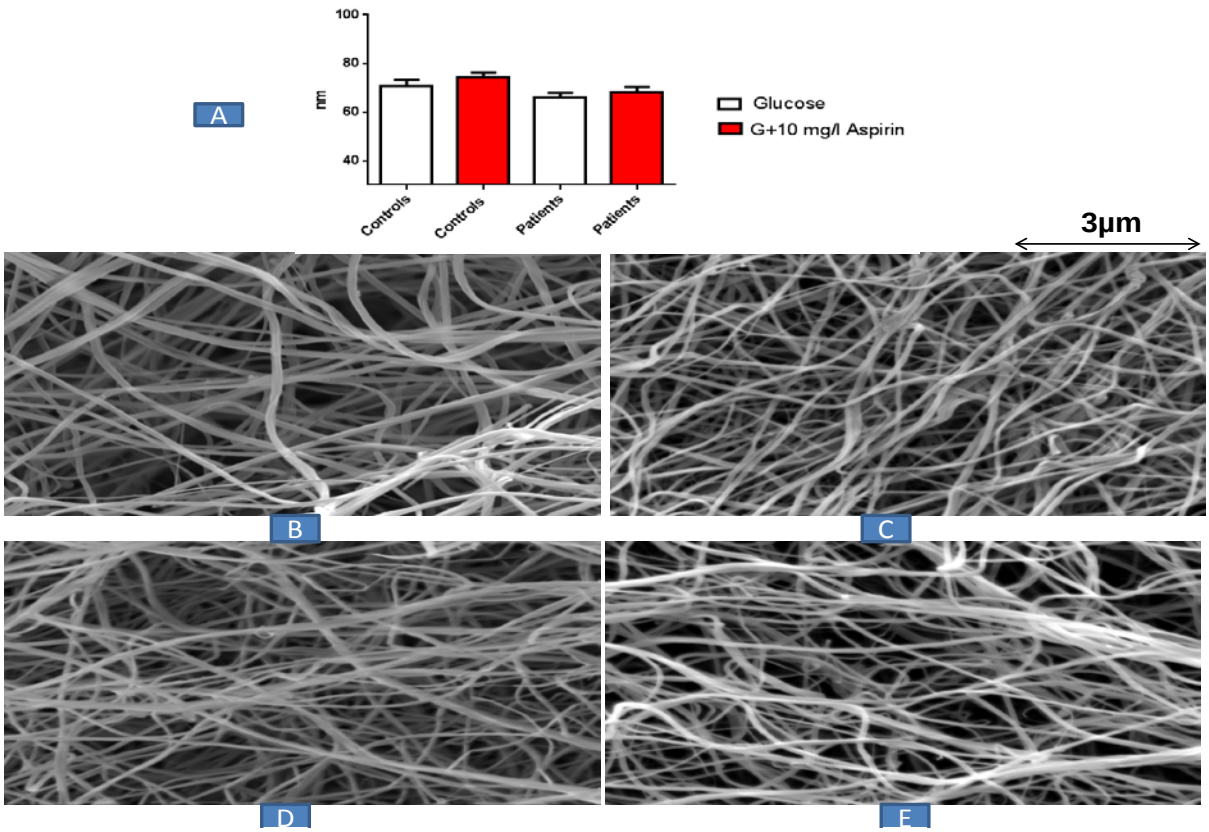


Figure 4.32 Fibrin clot analysis using electron microscopy (magnification of 10000x). Fibrin fibre thickness of clots made of pooled purified fibrinogen of healthy controls or patients with poor diabetes control (n=12 in each pool) after incubation with 20mmol/l glucose and glucose+10mg/l aspirin. Results represent the mean±SEM of 200 readings from 12 images (A). Clot Images of healthy controls after treatment with glucose (B), and after treatment with glucose+10mg/l aspirin (C). D, E, representative images from patients in the glucose and glucose+10 mg/l aspirin.

4.3.13 Fibrinogen purification

Gel electrophoresis of purified fibrinogen, three bands were expected for fibrinogen (corresponding to the α, β and γ; Figure 4.33)

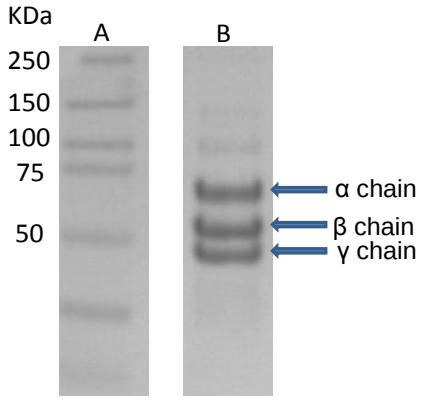


Figure 4.33 SDS-PAGE gel using electrophoresis technique showing purified fibrinogen. Each band of fibrinogen (α, βγ and γ) iswell defined and separated at the expected moleculare weight. There is no smearing or any unexpected bands.

4.3.14 Acetylation of fibrinogen by the ex vivo effect of aspirin

Western blot analysis was conducted to assess protein acetylation using a monoclonal antibody directed against acetylated protein. In the absence of aspirin, a faint band, corresponding to α chain of fibrinogen, was detected in both controls and diabetes samples indicating low level protein acetylation. This band was more obvious in the presence of 1 mg/l aspirin, although it remained faint. This band became intense after treating the samples with 10 mg/l aspirin. Moreover, with high aspirin concentration, a band of ~54 Kda became apparent, corresponding to the β chain of fibrinogen. No acetylation in γ chain was seen. The presence of additional glucose did not appear to affect protein acetylation.

Acetylation of fibrinogen was not different comparing patients with controls (Figure 4.34).

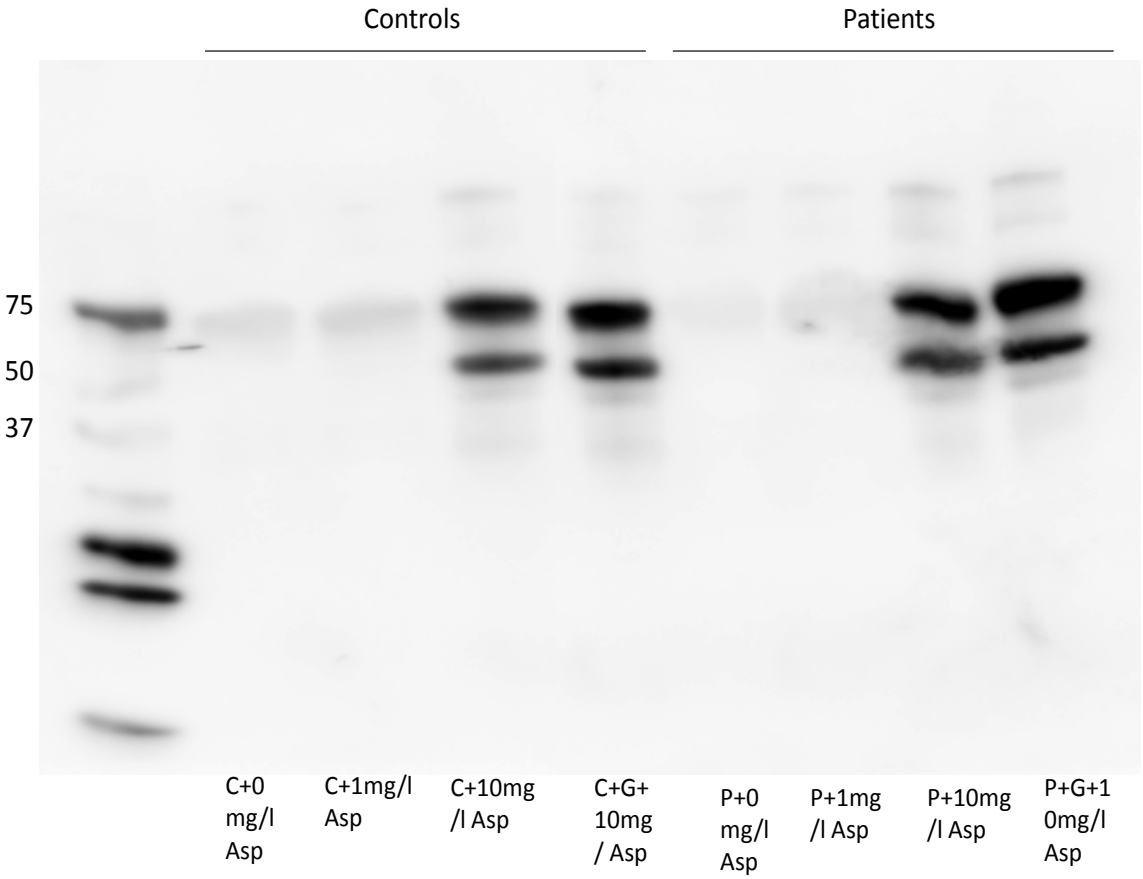


Figure 4.34 Western blotting using antibody against acetylated-lysine residues. Samples were prepared using pooled purified fibrinogen from healthy controls and diabetes patients in the presence and absence of aspirin with and without additional glucose (20 mmol/l). C=controls, P=patients, G=glucose, Asp=aspirin.

4.4 Discussion

In this Chapter I assessed the *ex vivo* effect of low and high aspirin concentration on fibrin clot formation, structure and fibrinolysis using plasma samples or purified fibrinogen. Both healthy controls and patients with diabetes were studied. Moreover, I investigated whether high glucose concentration modulates the effects of aspirin on clot structure and lysis.

There are a number of observations emerging from my studies that can be summarised as follows: i) Diabetes patients exhibit more compact clots with resistance to fibrinolysis compared with healthy controls, ii) Addition of aspirin to whole blood reduced lysis time of plasma clots made from healthy controls but not diabetes patients, iii) The fibrinolytic effects of aspirin were abolished in the presence of high concentrations of glucose, iv) Neither aspirin, nor glucose had an effect on lysis time in clots made from purified fibrinogen, v) background acetylation of fibrinogen was detected in patients and controls, which intensified after exposure to high aspirin concentration but no difference was seen comparing healthy controls with diabetes samples.

Jorneskog 1996 and colleagues were the first to investigate the effect of diabetes on fibrin gel structure. Ten individuals with type 1 diabetes and no cardiovascular history were investigated. None of these patients were on any treatment other than insulin.Compared with 15 healthy control subjects, plasma clots from diabetes patients showed less permeability and more compact structure (Jorneskog et al., 1996). These findings support my data as, I found that clots made from plasma or purified fibrinogen have a more compact fibrin network structure with impaired fibrinolysis compared with controls.

Clot changes in subject with diabetes have been reported in a number of studies (Jorneskog et al., 1996, Dunn et al., 2006, Ajjan and Ariens, 2009, Hess et al., 2012). These changes are related, at least in part, to post-translational modifications in the fibrinogen molecules (Dunn et al., 2006) caused by non enzymatic glycation of the protein(Brownlee et al., 1983). Pieters et al 2008 have shown that improving glycaemic control in patients with type 2 diabetes decreases glycation of fibrinogen and hence improves permeability and lysis

rates in clots made from purified fibrinogen. This is consistent with my findings, which demonstrate that lower HbA1c is associated with less thrombotic clot phenotype(Pieters et al., 2008).

Work from our laboratory has shown that addition of aspirin to culture media of fibrinogen-producing CHO cells, results in fibrinogen acetylation, which in turn affects clot structure and lysis (Ajjan et al., 2009). In particular, aspirin treated fibrinogen resulted in clots that were more porous and easier to lyse. These findings are consistent with my data, which demonstrated that *ex vivo* addition of aspirin to whole blood and facilitated fibrinolysis and affected clot structure, mainly with high aspirin concentrations. After incubation of whole blood withaspirin, fibrinolysis was significantly shorter with high concentration of aspirin. Enhanced lysis by aspirin was also observed using confocal microscopy. However no changes in fibrinolysis were observed, after incubation of purified fibrinogen with aspirin. These findings suggest that incubation of aspirin in whole blood could affect other plasma protein that makes clot fibrinolysis easier while this does not occur with purified fibrinogen. My findings contradict previous reports using recombinant or purified fibrinogen (He et al., 2001). This discrepancy is likely to be related to methodological differences. Aspirin was added to culture media in the publication by Ajjan et al, and therefore exposure of fibrinogen to aspirin was longer. Moreover, it is possible that intracellular acetylation of the protein is important to show an effect. On the other hand, He et al used a purified system and showed an effect for aspirin using a shorter incubation period. The possibility of fibrinogen degradation should be borne in mind given the longer incubation time, which is less likely to occur under tissue culture conditions. Interestingly, a short incubation period in

whole blood was enough to show an effect and in retrospect, I should have had identical incubation periods with aspirin in whole blood and purified fibrinogen. Further work is warranted using purified fibrinogen at different incubation times with aspirin followed by SDS-PAGE analysis to investigate protein integrity and repeat turbidimetric analyses.

I then moved on to study the effects of glucose on response to aspirin treatment. Neir et al 1991 have studied the *ex vivo* effects of glucose on plasma clot characteristics in patients with diabetes. They have shown that fibrin clot characteristics were altered in both healthy controls and patients with diabetes after the addition of high glucose concentrations immediately before the experiments. In healthy control, extra glucose did not affect fibre thickness but permeability and compaction were significantly reduced, indicating the networks were altered because fibrin fibres become more tightly packed. On the other hand, in subjects with uncontrolled diabetes (type of diabetes was unclear), fibre thickness and permeability were reduced but compaction remained relatively unaltered (Nair et al., 1991b). My work failed to show a clear effect of additional glucose on clot structure but I have found that extra glucose can impair the fibrinolytic properties of aspirin, when clots were made from plasma samples, which has not been documented before. However, no such effect was observed when aspirin was incubated with purified fibrinogen, indicating that aspirin affects other components of plasma proteins. However, the failure to detect a difference with purified samples may be due to methodological flaws. In the purified experiments, I used pooled samples and therefore these may not be representative of the diverse population. Also, detection of acetylation of fibrinogen using Western blots and radioactive aspirin techniques strongly

suggests that acetylation of this protein plays a role in the observed effects of aspirin on plasma clot lysis. Future work is required using large number of purified fibrinogen samples in order to establish the exact mechanism for the effect of aspirin on plasma clot lysis.

In conclusion, my data show that the effects of aspirin on plasma clots in diabetes are compromised, which may be one mechanism for the reduced clinical efficacy of this agent in diabetes. The failure to detect an effect for aspirin on clots made from fibrinogen indicates that the observed effects in plasma are related to proteins other than fibrinogen or it may simply be due to poor sensitivity of the experimental conditions given the use of pooled protein. Further work is required to fully understand the mechanisms for the reduced effects of aspirin on plasma clot properties in diabetes.

Chapter 5

Diabetes and *in vivo*

modulation of the fibrin clot by aspirin

5.1 Introduction

As mentioned previously, altered fibrin clot characteristics can predict the future development of cardiovascular disease (Fatah et al., 1996a, Fatah et al., 1992, Collet et al., 2006, Undas et al., 2010a). Diabetes is characterised by more compact fibrin networks and resistance to fibrinolysis, which appear to contribute to increased risk of vascular events in this population (Alzahrani and Ajjan, 2010). Jorneskog and colleagues were the first to report altered clot structure in diabetes. They have demonstrated that *ex vivo* plasma clots from patients with type 1 diabetes are less permeable and have a more compact structure, which is not necessarily related to changes in fibrinogen levels or the presence of vascular complications (Jorneskog et al., 1996). Similarly, clots from subjects with T2DM also display altered characteristics including smaller pore size and increased branch points compared with healthy controls (Dunn et al., 2005). Moreover, there is a relationship between fibrin clot properties in diabetes and the presence of vascular complications (Alzahrani et al., 2012). In those with established vascular disease, diabetes has an additional adverse effect on fibrin network structure, making individuals with diabetes and vascular pathology at a particularly high risk of thrombosis (Neergaard-Petersen et al., 2014a). Therefore, modulation of fibrin clot structure in diabetes may offer a management strategy to reduce cardiovascular risk in this population. Addressing vascular risk factors, including glycaemia, smoking and lipid levels, improves clot structure properties in diabetes (Alzahrani and Ajjan, 2010, Kurdee et al., 2014). However, current therapies do not specifically target the fibrin network directly in order to reduce thrombosis risk and perhaps this may be one strategy that requires further exploration.

Tehrani et al have analysed the effect of low and high dose aspirin on fibrin gel porosity in patients with T1DM. They have shown that only high aspirin was able to modulate fibrin clot permeability. Unexpectedly, only patients with poor glycaemic control showed a significant benefit, whereas in those with good glycaemic control the effect was marginal (Tehrani et al., 2012). However, the study can be criticised for the lack of a control group and the use of a single technique to assess clot structure that is known to have great variability. Therefore, this remains an area in need of further clarification in order to understand the role of aspirin in modulating fibrin-related thrombosis risk in diabetes.

In this chapter, I have assessed the *in vivo*effects of aspirin, using two different doses of 75mg/day and 300 mg/dayon fibrin network characteristics in patients with type 1 diabetes and age/sex matched healthy controls.

5.2 Materials and methods

5.2.1 Patients and controls

The same patients and controls who gave blood for platelet tests (Chapter 2), gave additional blood that was collected in citrated tubes (Sigma), for plasma clot analysis, as well as lithium heparin tubes for fibrinogen purification. Samples from a total of 29 patients and 29 healthy controls were collected.

Sample collection and preparation for clots made from plasma samples

Blood samples were collected from both patients and healthy controls at baseline and after 2 weeks treatment with 75mg/day aspirin. This was followed by a wash out period, after which individuals were treated with 300 mg/day

aspirin for two weeks with samples collected at baseline and end of the two week period. Blood samples were then centrifuged at 3000 rpm for 20 minutes, platelet poor plasma collected, aliquotedand stored at -80°C until analysis (Figure 5.1).

Sample collection and preparation for clots made from purified fibrinogen

Samples were collected (baseline, after 75mg/day and after 300mg/day, as described previously) in lithium heparin and centrifuged for 20 minutes at 3000 rpm. Fibrinogen was subsequently purified from plasma samples as detailed in Chapter 2. Pools of plasma purified fibrinogen were then constructed using 12 samples from patients with diabetes (HbA1c≥8.5%) or 12 samples from healthy controls (Figure 5.1).

5.2.3 Assessment of clot structure

Fibrin clots were made using plasma samples or purified fibrinogen and analysed by turbidimetric assays, laser scanning confocal microscopy and electron scanning microscopy as described in Chapter 2

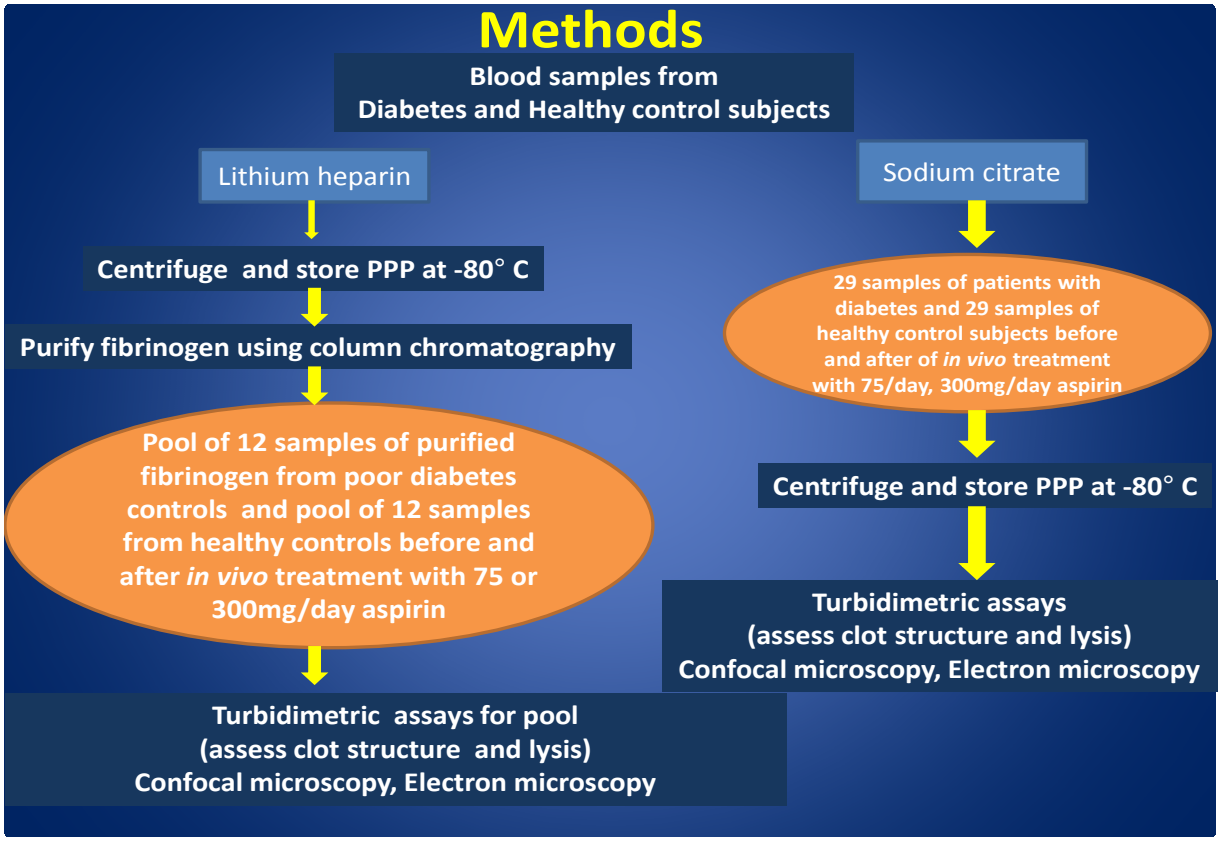


Figure 5.1 Summary of sample selection and preparation. Blood samples from diabetes and controls were collected before and after treatment with aspirin 75mg/day or 300mg/day (two weeks each with two weeks wash out period). For plasma fibrin clot analysis, samples were collected in sodium citrate, whereas lithium heparin tubes were used to collect plasma samples for fibrinogen purification.

5.3 Results

5.3.1 Turbidimetric analysis of plasma fibrin clots

Lag phase

Low and high daily dose of aspirin (75mg/day and 300mg/l respectively) had no effect on lag phase in individuals with diabetes (Figure 5.2A) or in controls (Figure 5.2B). Glycaemic control, assessed as HbA1c, did not influence the effects of aspirin on lag phase (Figure 5.2 C, D respectively).

There was no difference in lag phase between controls and individuals with diabetes at baseline (Figure 5.3).No significant changes have been observed after excluding the two smokers.

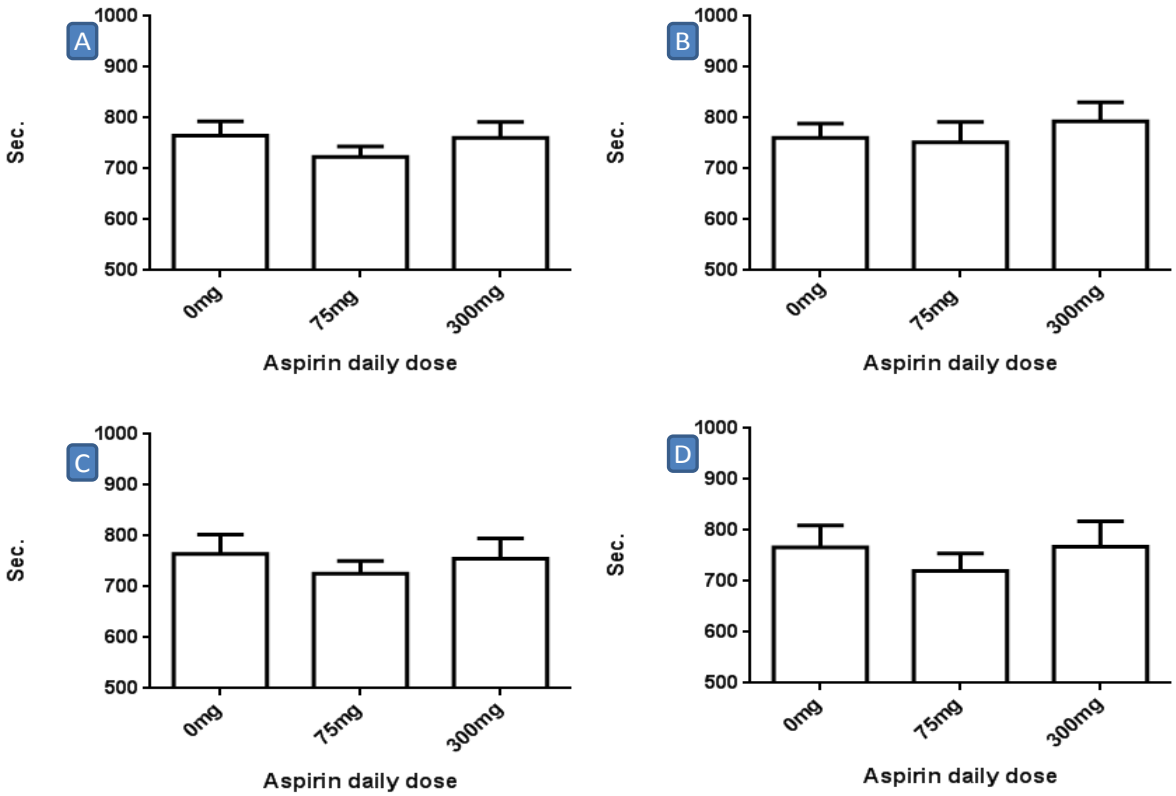


Figure 5.2 Aspirin on plasma clot lag phase using turbidity and lysis technique. Effects of different daily dose of aspirin 0, 75, and 300mg/day on lag phase in subjects with diabetes (A; n=29), healthy controls (B; n=29), subjects with poor diabetes control (C; n=15) and subjects with better diabetes control (D; n=14). Results(measured by seconds) are presented as mean±SEM.

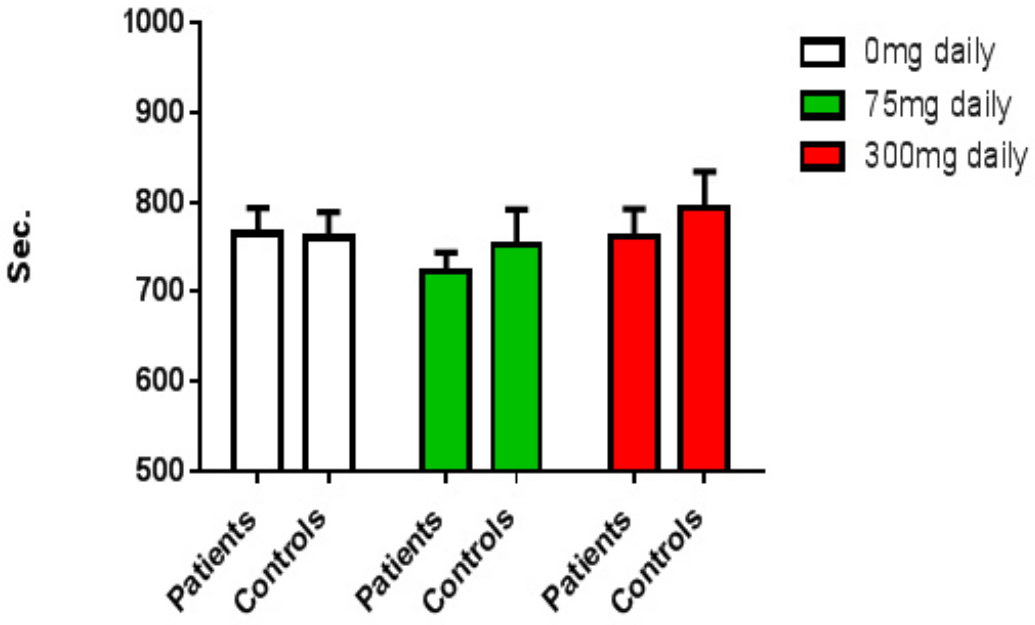


Figure 5.3 Aspirin on plasma clot lag phase using turbidity and lysis technique. Effects of different daily dose of aspirin on lag phase in subjects with diabetes(n=29) and healthy controls (n=29). Results(measured by seconds) are presented as mean±SEM. Sec: seconds.

Maximum turbidity

Low daily dose of aspirin (75 mg/day) and high daily dose (300mg/day) had no effect on fibrin clot maximum absorbance in patients (Figure 5.4A) or in controls (Figure 5.4B). The same observation was found in both individual with poor diabetes control (HbA1c≥8.5%) and individuals with better diabetes control (HbA1c<8.5; Figure 5.4 C, D respectively).

Comparing individuals with diabetes and healthy controls, maximum absorbance was higher in the former group only with 300 mg daily dose of aspirin (0.21±0.01 and 0.16±0.01 respectively p=0.016, Figure 5.5 A). While comparing individuals with poor and better diabetes control, maximum

absorbance was higher in the former group only in the absence of aspirin (0.22±0.15 and 0.18±0.015 respectively p=0.04, Figure 5.5 B). No significant changes have been observed after excluding two smokers.

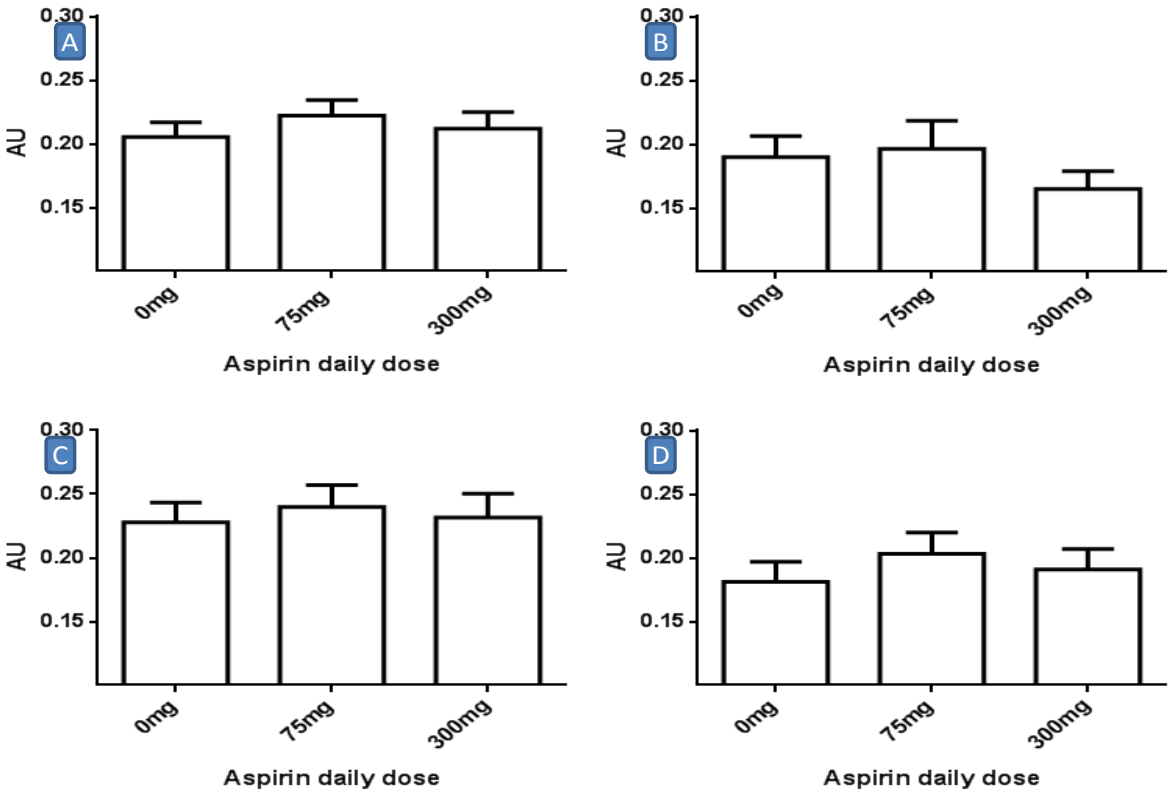


Figure 5.4 Aspirin on plasma clot maximum absorbance using turbidity and lysis technique. Effects of different daily dose of aspirin 0, 75, and 300mg/day on maximum absorbancein subjects with diabetes (A; n=29), healthy controls (B; n=29), subjects with poor diabetes control (C; n=15) and subjects with better diabetes control (D; n=14). Results are presented as mean±SEM. AU: arbitrary units.

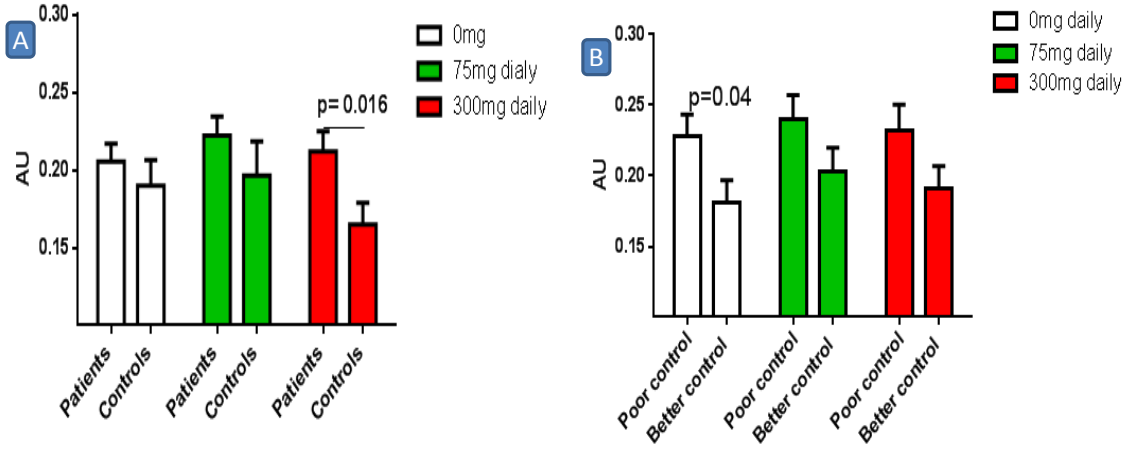


Figure 5.5 Aspirin and plasma clot maximum absorbance using turbidity and lysis technique. Effects of different daily dose of aspirin on maximum absorbance in (A) plasma of subjects with diabetes (n=29) and healthy controls (n=29), (B) plasma of subjects with poor controlled diabetes (n=15) and better controlled diabetes (n=14). Results are presented as mean±SEM.

Lysis time

There was no effect of low or high daily dose of aspirin on clot lysis time in individuals with diabetes (Figure 5.6A). However, in healthy controls, lysis time has significantly reduced from 550±26 before treatment with aspirin to 510±16 after treatment with high dose of aspirin (300mg/day, p=0.02, Figure 5.6B). There was no difference in response in those with poor or better diabetes control (Figure 5.6 C, D). Comparing diabetes subjects with healthy controls, clot lysis time at baseline was longer in the former group compared with the latter but failed to be significant (596±32 and 550±26, respectively), with a

bigger difference detected following highdose aspirin treatment (602±32 and 510±16, respectively; p=0.03,Figure 5.7). No significant changes were observed after excluding the two smokers.

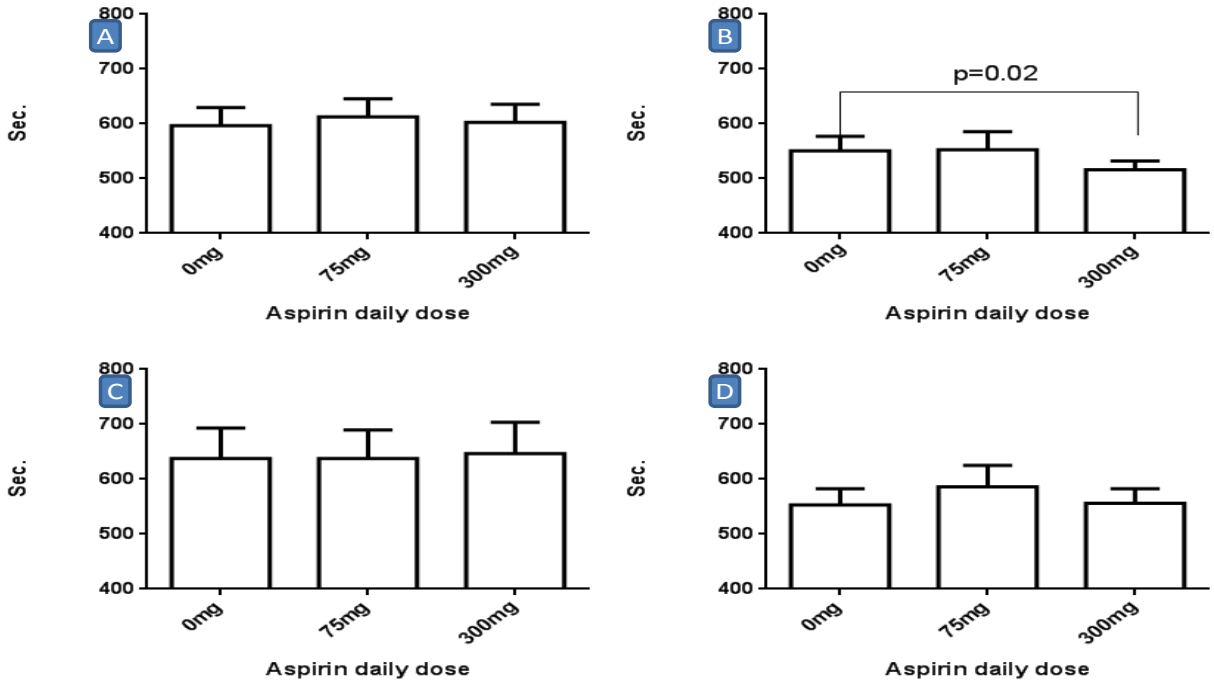


Figure 5.6 Aspirin on plasma clot lysis time using turbidity and lysis technique. Effects of different daily dose of aspirin 0, 75, and 300mg/day on lysis time in subjects with diabetes (A; n=29), healthy controls (B; n=29), subjects with poor diabetes control (C; n=15) and subjects with good diabetes control (D; n=14). Results (measured by seconds) are presented as mean±SEM.

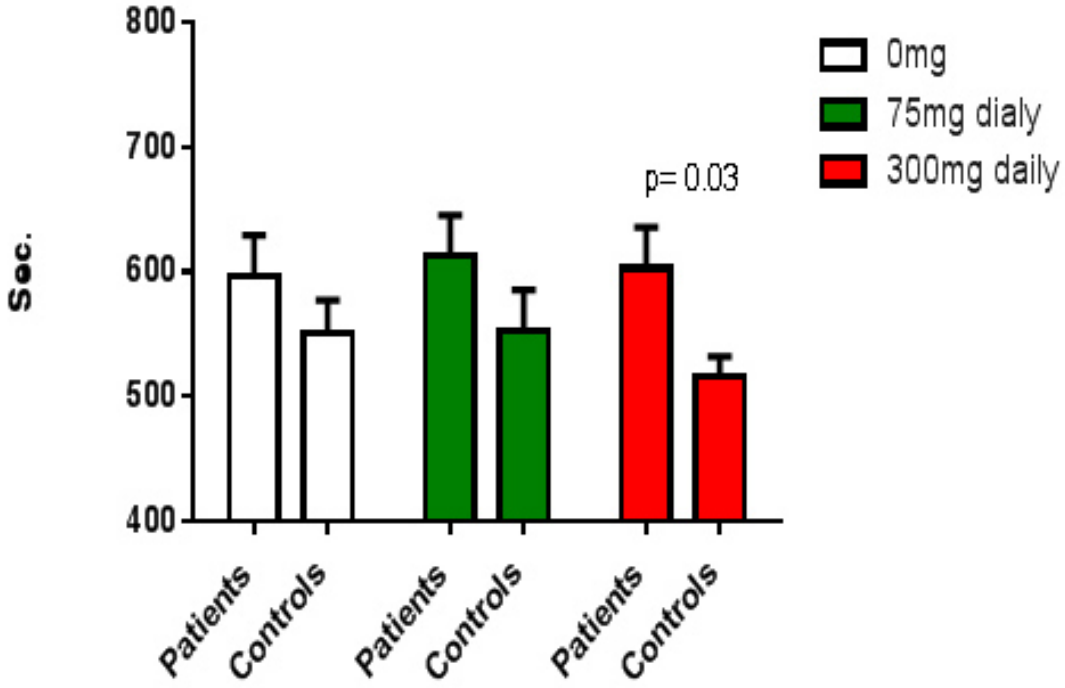


Figure 5.7 Aspirin on plasma clot lysis time using turbidity and lysis technique for patients and controls. Effects of different daily dose of aspirin on lysis in subjects with diabetes (n=29) and healthy control (n=29). Results (measured by seconds) are presentedas mean±SEM.

5.3.2 Scanning confocal microscopy of plasma fibin clots

Fibrin clot structure

Fibrin structure before and after aspirin therapy with 75mg/day and 300mg/day of plasma clots from a pool of healthy controls is shown in Figure 5.8 A-C respectively. Only high dose of aspirin (300mg/day) made the fibrin clots more porous in healthy control (Figure 5.8 C). There was no clear difference in poor control diabetes and healthy controls in relation to plasma clot density (Figure 5.8A, Figure 5.9 D, respectively).

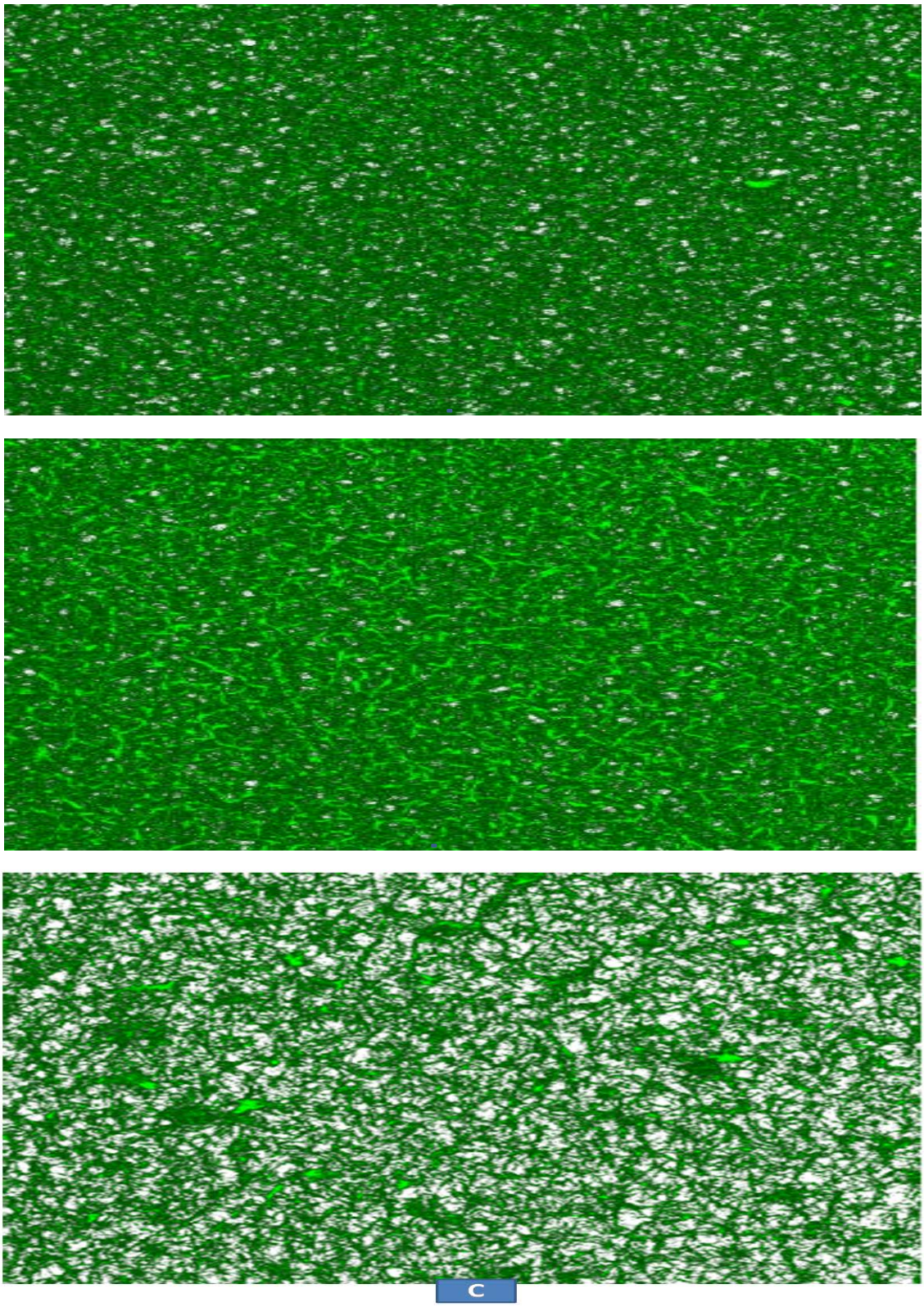


Figure 5.8 Confocal microscopy of 20 µm Z-stack of mature fibrin clot of healthy control (magnification of 400x). Fibrin clots were made from pooled plasma of healthy controls (n=12) before aspirin treatment (A), after 75 mg/day aspirin treatment (B) and after 300 mg/day aspirin treatment (C).

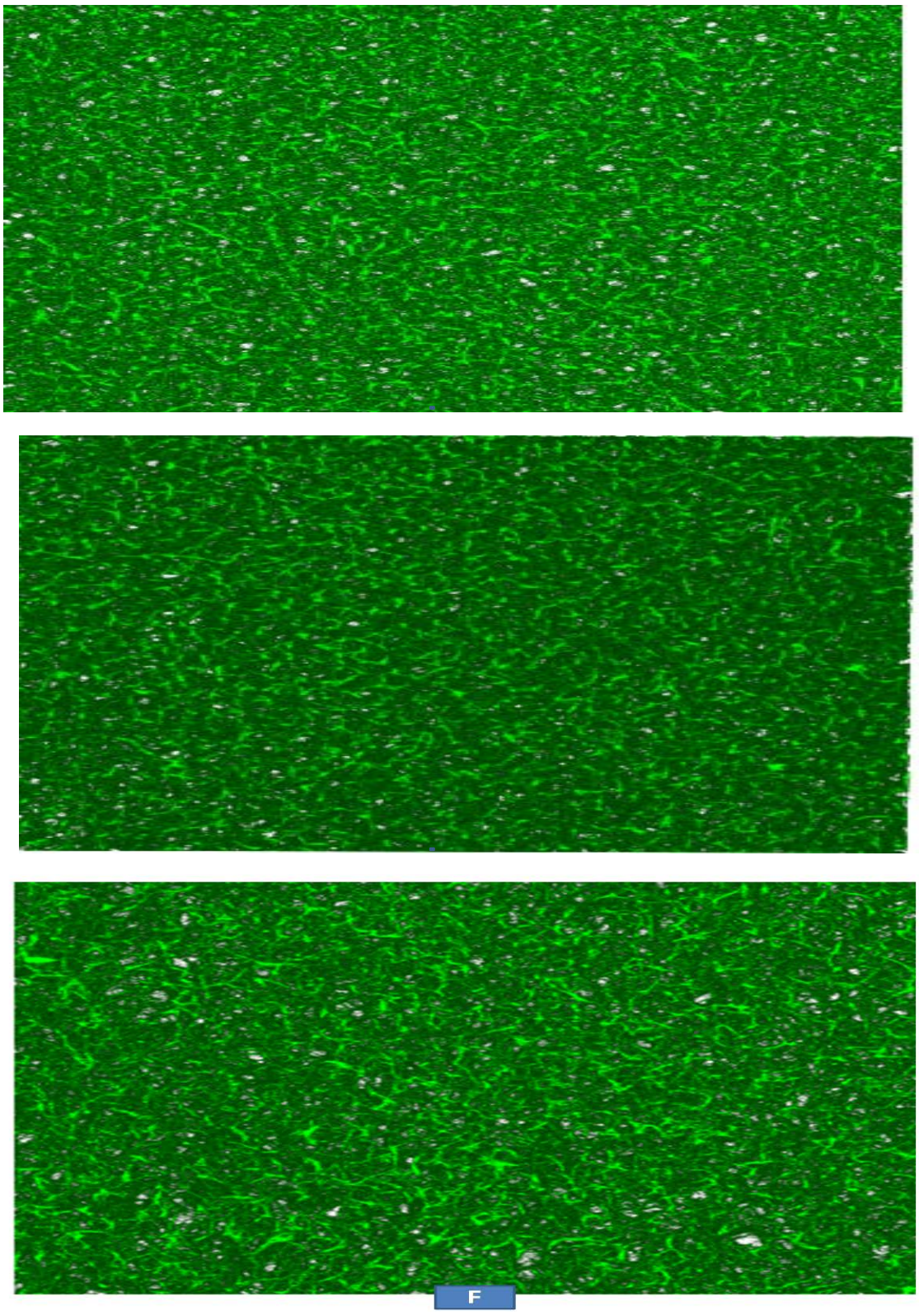


Figure 5.9 Confocal microscopy of 20 µm Z-stack of mature fibrin clot of healthy control (magnification of 400x). Fibrin clots were made from pooled plasma of poor diabetes control (n=12) before aspirin treatment (D), after 75mg/day aspirin treatment (E) and 300mg/day aspirin treatment (F).

Clot lysis analysis

Fibrinolysis of mature clots was observed in real time using laser scanning confocal microscopy. I recorded lysis time in at least four independent experiments for each condition. Low or high dose of aspirin had no effect on clot lysis time of pooled plasma from individuals with poor diabetes control at baseline, following 75 or 300 mg/day aspirin (507±35 sec, 532±49 sec and 507±89, respectively; p>0.1, Figure 5.10A). There was a reduction in lysis time of pooled plasma clots from healthy controls from 556±103 sec before treatment with aspirin to 441±80 sec after treatment with 75mg/day aspirin. (p=0.03, Figure 5.10B). There was also a reduction in lysis time with 300mg/day aspirinbut this failed to reach statistical significance (Figure 5.10B).

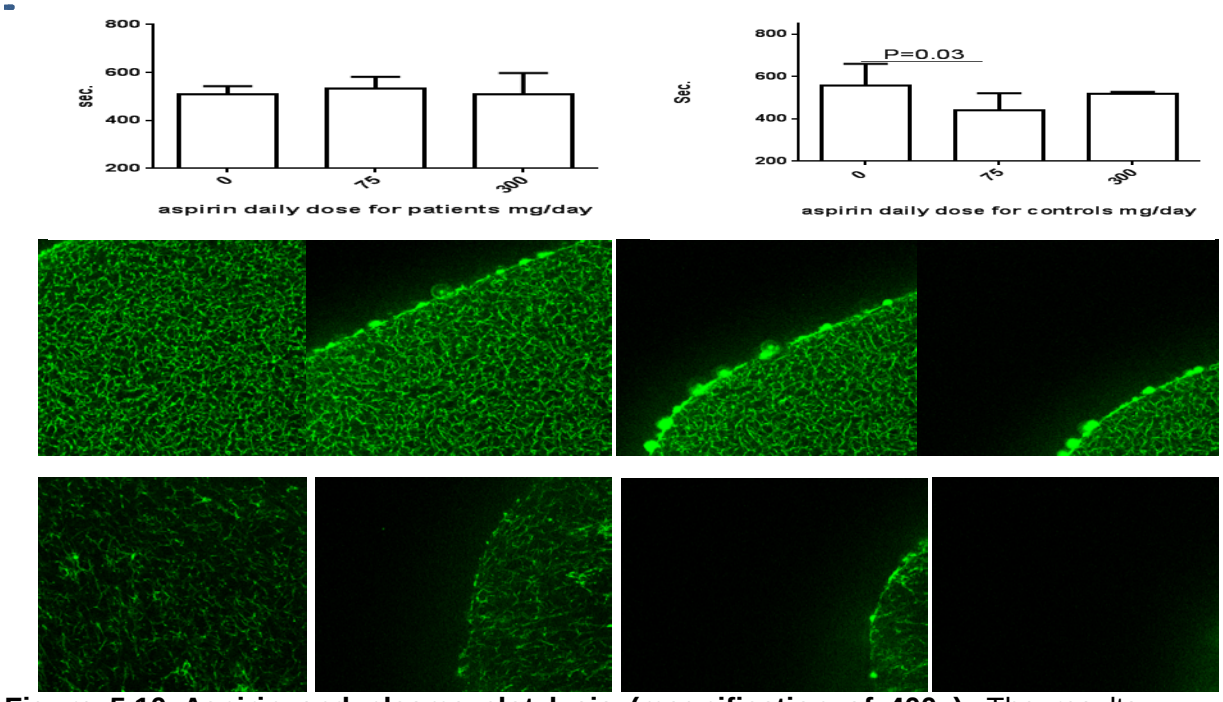


Figure 5.10 Aspirin and plasma clot lysis (magnification of 400x). The results represent the mean±SEM of 4 independent lysis experiments of clots made from pooled plasma samples of subject with poor diabetes control (n=12) (A) or healthy controls (n=12) (B). Representative figures of clot lysis using healthy control plasma at various time points before (C) and after 75 mg/l aspirin treatment (D).

5.3.3 Scanning electron microscopy of plasma fibrin clot

There was no significant effect of aspirin on fibre thickness of clots made from patients with diabetes before, after 75 mg/day with a trend observed with 300 mg/day aspirin (65±1.6 and 68±1.9 and 70±2 nm, respectively; p=0.1 and p=0.09, compared with baseline, Figure 5.11A). There was no significant difference in clots made from pooled plasma of healthy controls before and after aspirin treatment with 75 mg/day (64±1.7nm and 67±3 nm, respectively; p=0.6), whereas fibre thickness increased with 300 mg/day aspirin (73±2 nm, p=0.0006 compared with baseline). Pore size also increased with aspirin treatment in healthy controls but not individuals with diabetes (Figure 5.12).

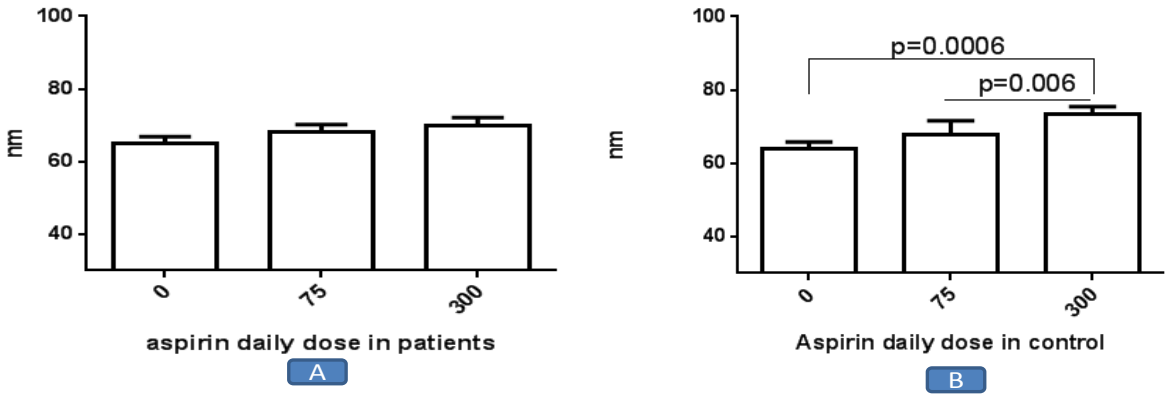


Figure 5.11 The effect of aspirin in fibrin fiber thickness measures (in electron microspore images, magnification of 10000x using ImageJ software). *In vivo* effects of different daily dose of aspirin on fibrin fibre thickness in clots made from pooled plasma of subjects with poor diabetes control (n=12, A) or healthy controls (n=12, B). Results are presentedas mean±SEM.

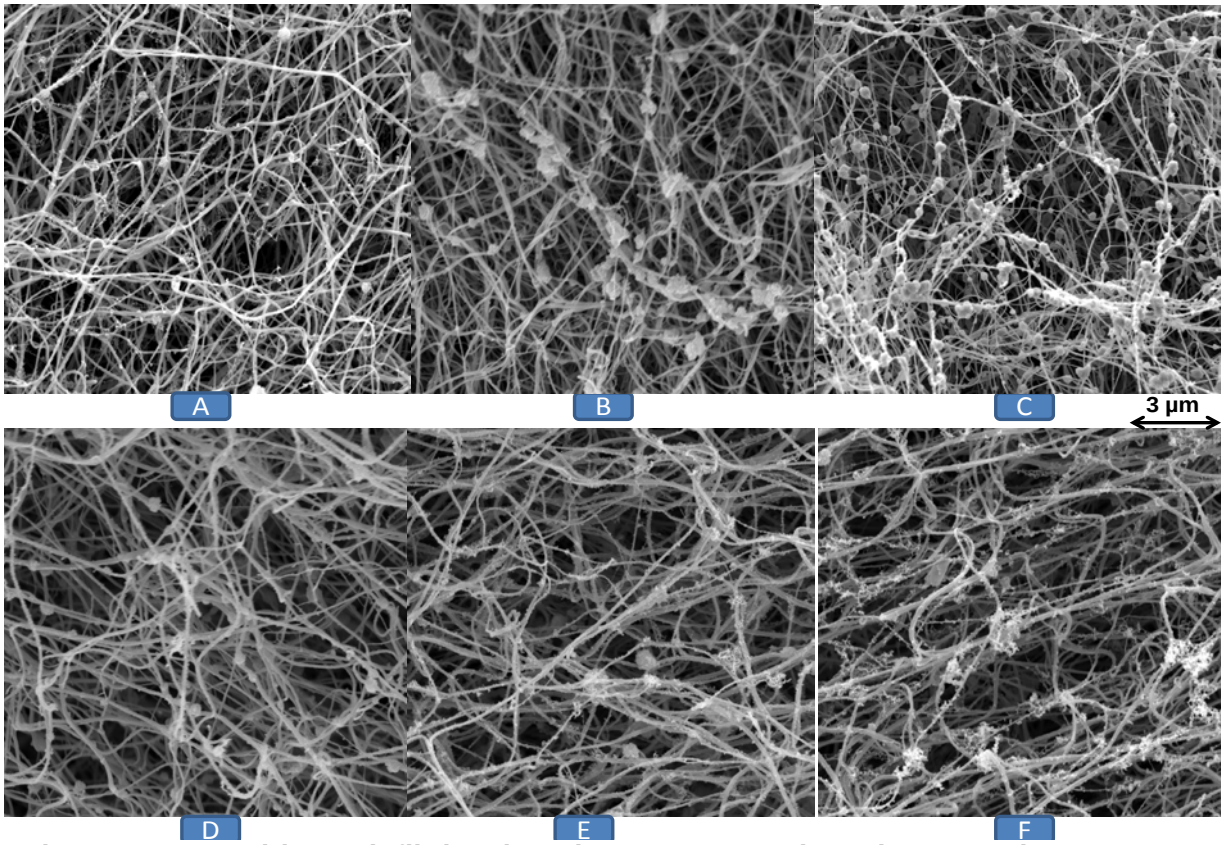


Figure 5.12 Aspirin and fibrin clot ultrastructure using electron microscopy (magnification of 10000x). Clot Images of pooled plasma from healthy controls(n=12) before (A) and after 75 mg/day or 300 mg/day aspirin (B, C). Clot images of pooled plasma from patients with poor diabetes control (n=12) before (D) and after 75 mg/day or 300 mg/day aspirin daily dose (E, F).

5.3.4 Turbidimetric analysis of plasma purified fibrinogen clots

Maximum absorbance

No significant effect of low or high daily dose of aspirin on maximum absorbance in individuals with diabetes was noted (Figure 5.13A). In contrast, aspirin treatment with 75mg/day increased fibrin clot maximum absorbance in controls from 0.04±0.004 to 0.05±0.007 (p=0.02) and to 0.06±0.009 (p=0.02) after treatment with 300mg/day respectively (Figure 5.13B).

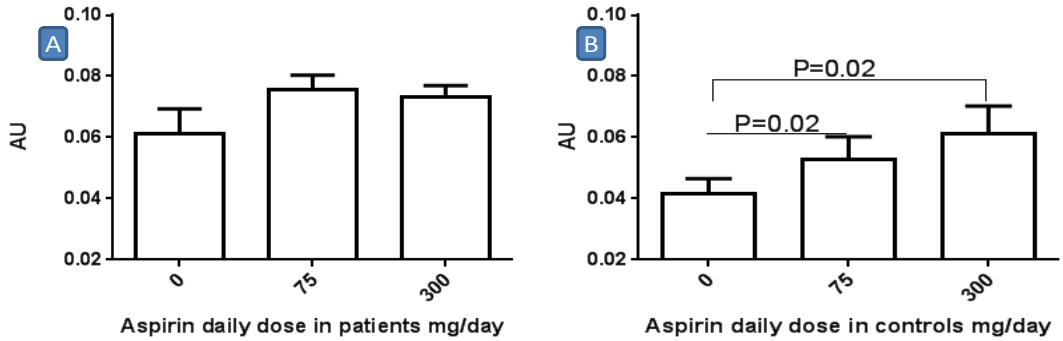


Figure 5.13 Aspirin and clot maximum absorbance using purified fibrinogen (turbidity and lysis technique). Maximum absorbance was measured after daily aspirin administration at 0, 75, and 300mg/day using pooled purified fibrinogen from subjects with diabetes (A; n=12) or healthy controls (B; n=12). Results are presentedas mean±SEM of three independent experiments each conducted in duplicate.

Lysis time

There was no significant difference in lysis time before and after treatment with 75mg/day or 300mg/day in patients with diabetes or in healthy controls (Figure 5.14).

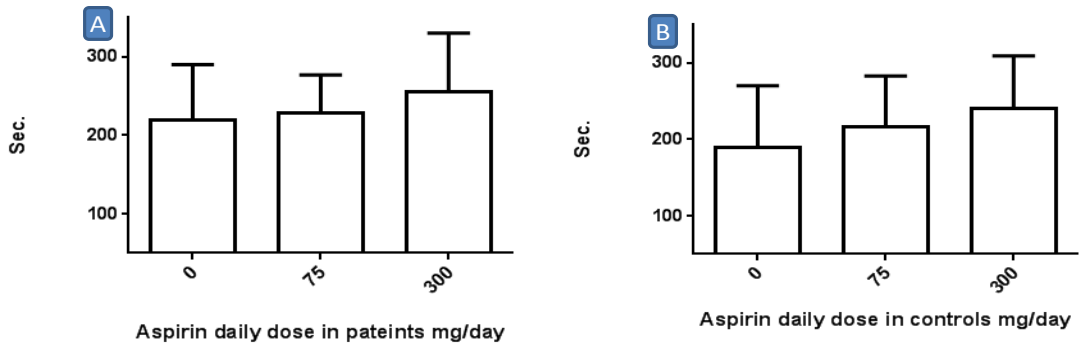


Figure 5.14 Aspirin and clot lysis time of fibrin clot from purified fibrinogen (turbidity and lysis technique). Lysis time was measured after daily aspirin administration at 0, 75, and 300mg/day using pooled purified fibrinogen from subjects with diabetes (A; n=12) or healthy subjects (B; n=12). Results are presented as mean±SEM of three independent experiments each conducted in duplicate.

5.3.5 Scanning confocal analysis of fibre clot from plasma purified fibrinogen clot

Clot image

Fibrin structure before and after aspirin therapy with 75mg/day and 300mg/day of made from healthy control pooled purified fibrinogen is shown in Figure 5.15A-C. Only high dose of aspirin (300mg/day) made the fibrin clots more porous in healthy controls. A similar finding was detectedin patients with poor diabetes control (Figure 5.16 D-F). Without aspirin treatment, there was no clear difference between controls and patients in relation to clot density (Figure 5.15 and 5.16, respectively).

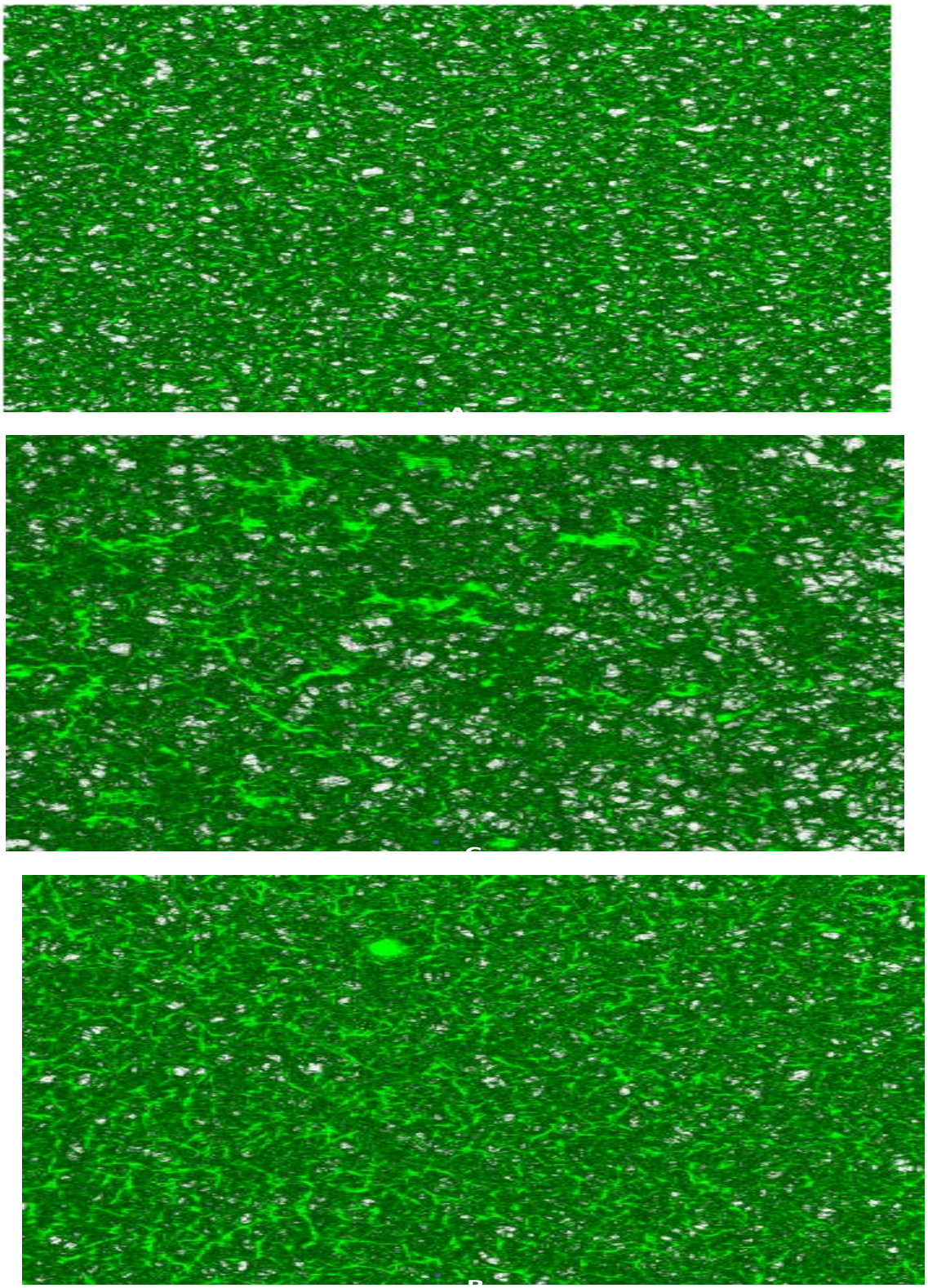


Figure 4.15 Confocal microscopy of 20 µm Z-stack of fibrin clots made from healthy controls purified fibrinogen. Fibrin clots were made from pooled fibrinogen of healthy controls (n=12) before aspirin treatment (A), after 75, 300 mg/day aspirin (B, C; respectively).

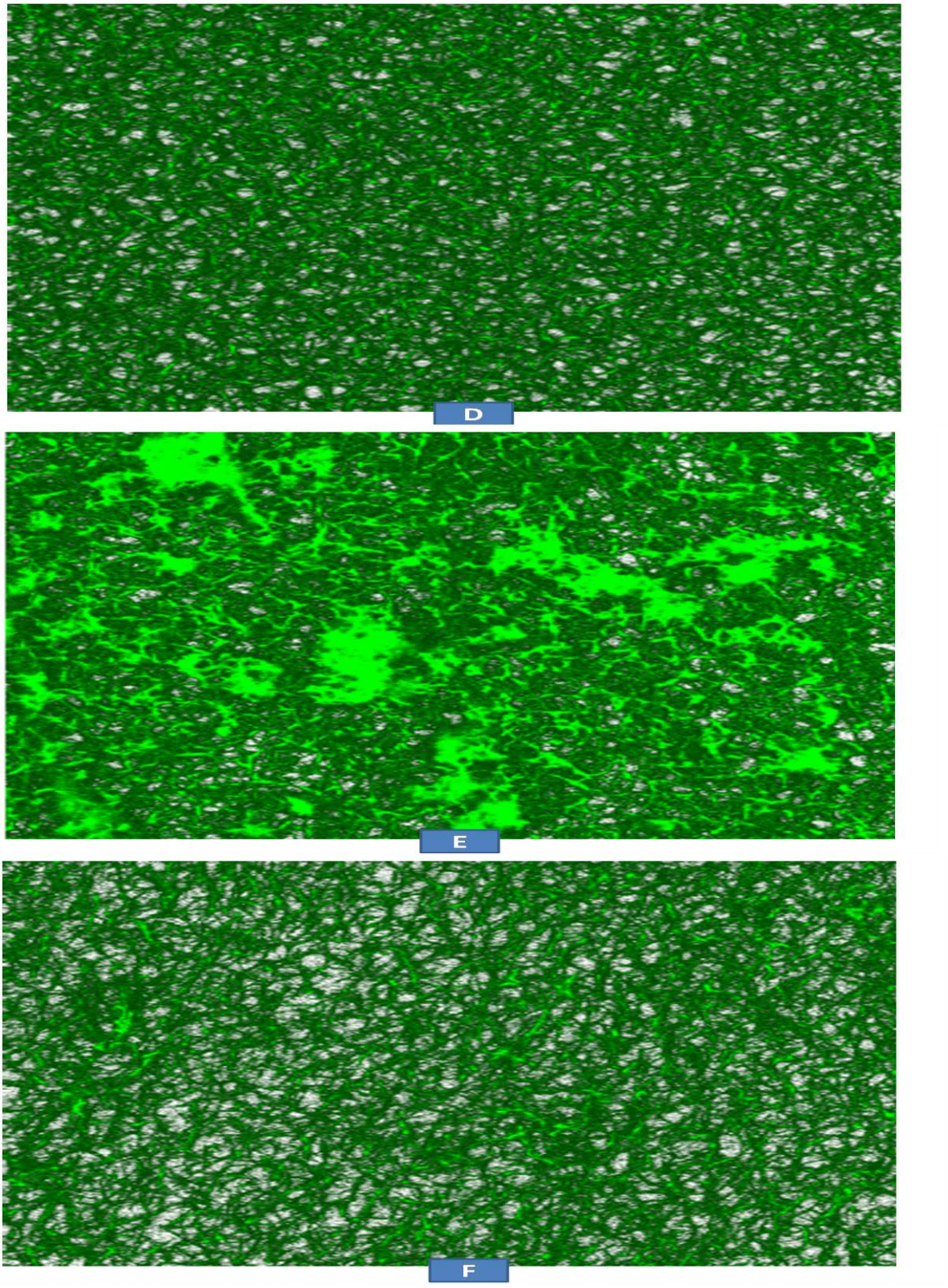


Figure 4.16 Confocal microscopy of 20 µm Z-stack of fibrin clots made from patients with poor diabetes control purified fibrinogen. Fibrin clots were made from pooled fibrinogen of subjects with poor diabetes control (n=12) before aspirin treatment (D) and after 75, 300 mg/day aspirin (E, F; respectively).

Clot lysis analysis

Fibrinolysis observed in real time using laser scanning confocal microscopy. Lysis time readings of different four slides for each pool have been recorded (Figure 5.17 A, B). There was no significant reductionobserved in lysis of clots made from purified fibrinogen of individuals withpooe diabetes control (Figure 5.17A). In healthy controls,lysis time did not show significant changes following aspirin therapy (Figure 5.17B).

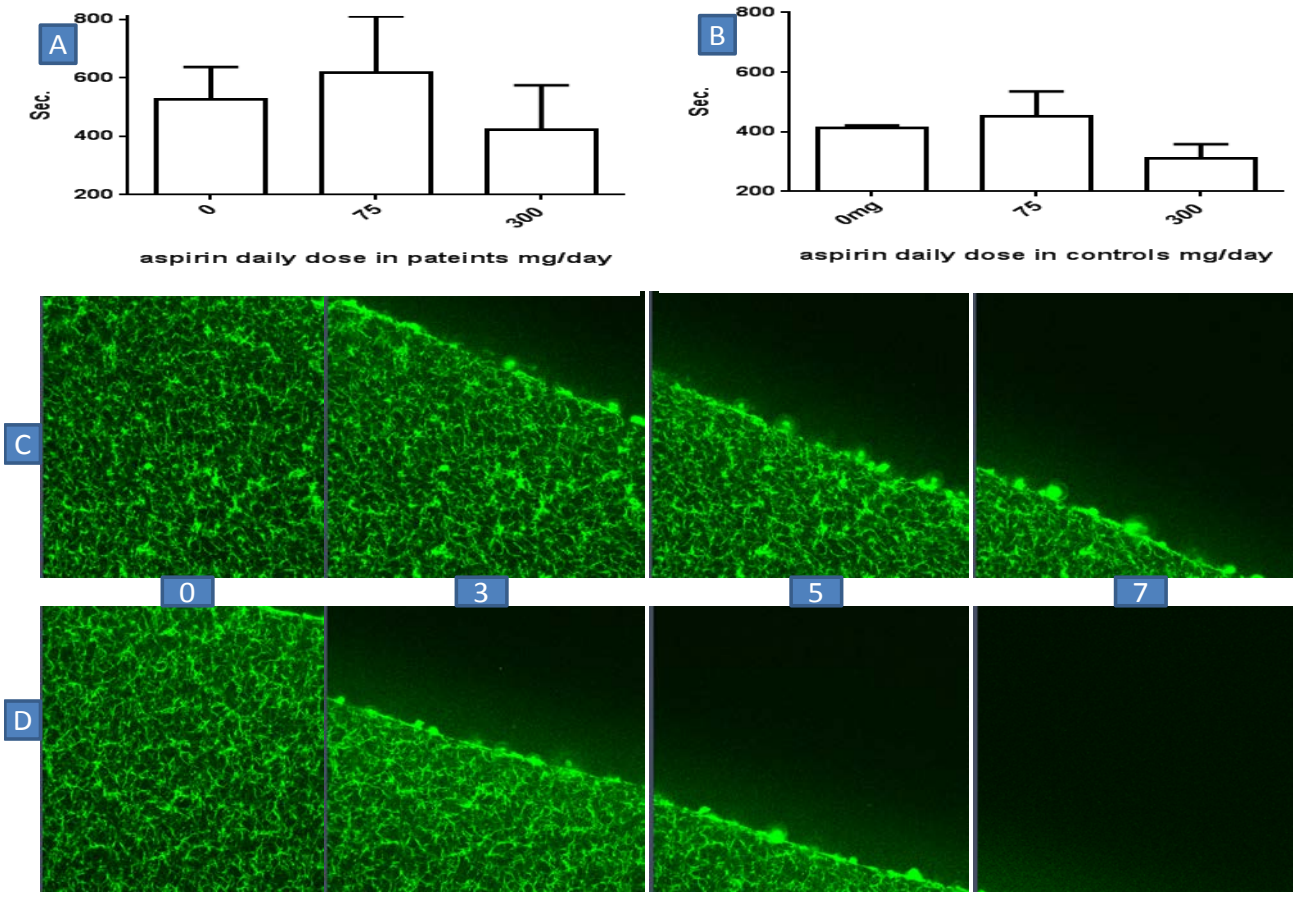


Figure 5.17 Fibrinolysis of mature clots made from purified fibrinogen. Results are presentedas mean±SEM of 4 independent lysis experiments. Clots were made from pooled purified fibrinogen of subjects with poor diabetes control (n=12;A) or pooled protein from healthy controls (n=12; B). Representative figures of clot lysis using control purified fibrinogen at various time points (measured in minutes) before (C) and after 300mg/l aspirin treatment (D).

5.3.6 Scanning electron microscopy of plasma purified fibrinogen clot

Fibrin fibre size analysis

There was no significant effect of aspirin on clots made from patients with poor diabetes control before, after 75 mg/day or 300 mg/day aspirin (66±1.8 and 66±1.9 and 69±1.9 nm, respectively; p=0.6 and p=0.2, Figure 5.18A).

No significant difference in fibrin fibre thickness of clots made from pooled purified fibrinogen from healthy controls before and after aspirin treatment with 75 mg/day (70±1.9 and 73±1.8nm, respectively; p=0.2) while with 300 mg/day aspirin treatment, the fibrin fibre size was significantly increased (77±2.3 nm, p=0.02 compared with baseline). Also, larger pore size was detected in healthy controls following treatment with 300 mg/day aspirin (Figure 5.18).

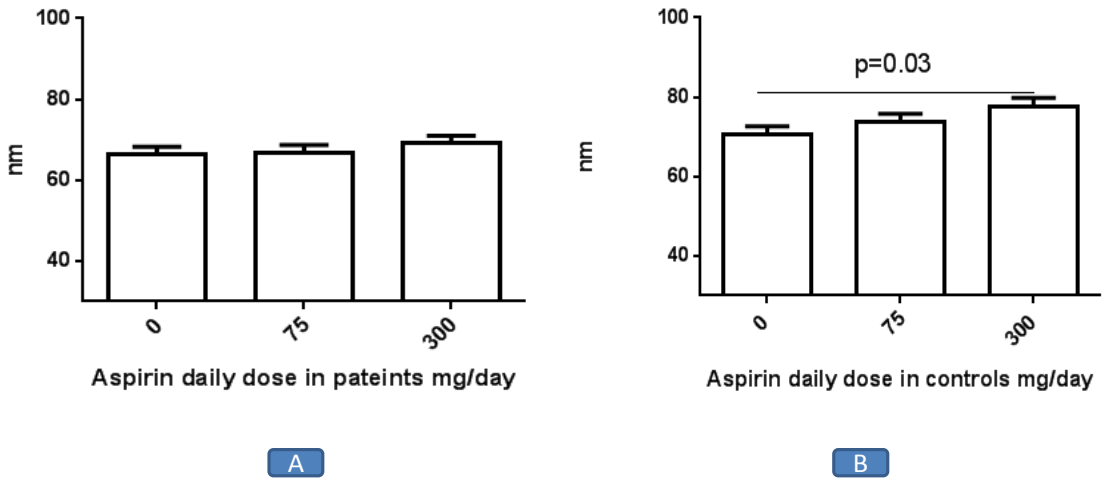


Figure 5.18 Effect of aspirin in purified fibrin fiber thickness measures (in electron microspore images, magnification of 10000x using ImageJ software). Clots were madefrom pooled purified fibrinogen of subjects poor diabetes control (n=12, A) or healthy controls (n=12, B). Results are presentedas mean±SEM of a total of 200 readings.

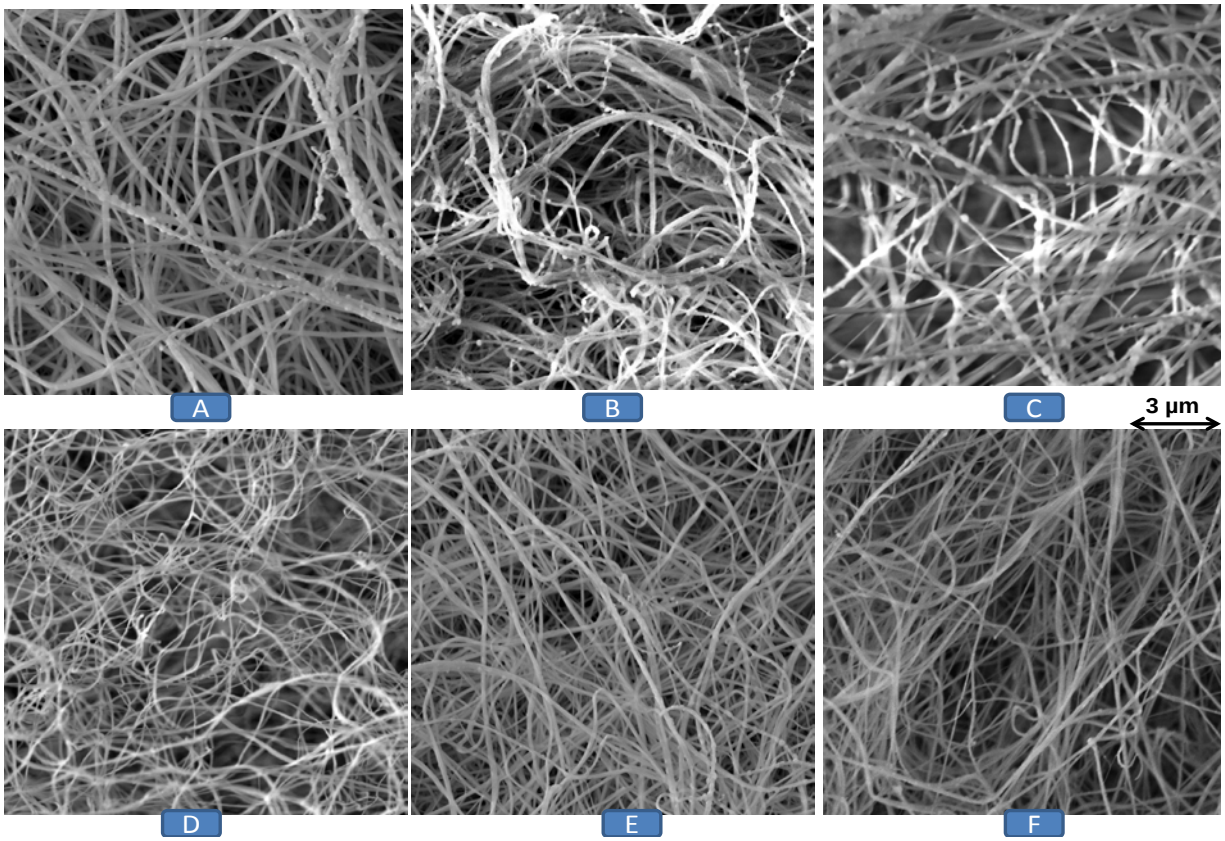


Figure 5.19 Fibrin clot Images using scanning electron microscopy (magnification of 10000x). Clots were made from pooled healthy control fibrinogen (n=12) before (A) and after 75 mg/day or 300 mg/day aspirin therapy (B, C) or poor diabetes control (D, E and F, respectively).

5.3.7 Acetylation of fibrinogen by radioactive aspirin

I further assessed acetylation of pooled fibrinogen from diabetes subjects and controls (n=12 each) using radioactive techniques. Two different formulations of this agent were tested: C14-labelled aspirin at the acetyl group and C14-labelled aspirin at the carboxyl group, which provided back ground radiation and used as a negative control. Experiments were repeated on 3 different occasions and mean difference in radioactive counts were calculated for diabetes and control fibrinogen. I found incorporation of acetyl C14 into all three chains of fibrinogen. There was small decrease of acetyl C14 incorporation into diabetic fibrinogen compared with control protein with reduced α chain acetylation by

18.3%, β chain by 12.7% and γ chain by 20.6%. Data are summarised in Figure 5.20.

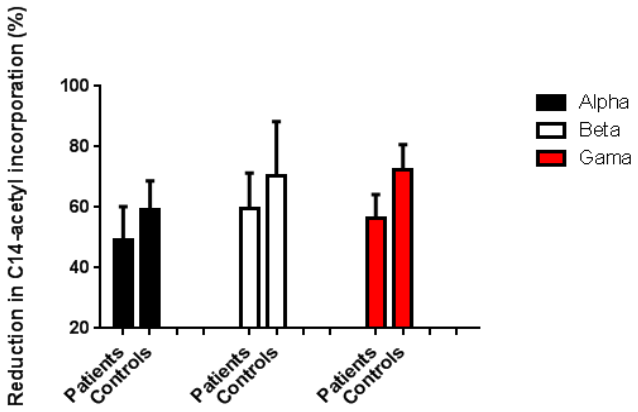


Figure 5.20 Acetylation of purifiedfibrinogen from subjects with poor diabetes control and healthy controls. Mean percentage of acetylation reduction in the different chains of fibrinogen (A) and all chains of fibrinogen (B) from a pool of controls and a pool of patients with diabetes (n=12 for each). Data shown represent the mean±SEN of a 3 independent experiments.

5.4 Discussion

In this Chapter I assessed the *in vivo* effect of low (75mg/day) and high (300mg/day) daily dose of aspirin on fibrin clot formation, structure and fibrinolysis using plasma samples and purified fibrinogen. Both healthy controls and patients with diabetes were studied. There are a number of observations emerging from my studies that can be summarised as follows: i) Diabetes patients exhibit more compact clots compared with healthy controls, ii) In healthy controls, aspirin administration results in altered plasma clot turbidity

and reduced lysis time, an effect that is not found in those with diabetes, iii) Aspirin administration to healthy controls alters the structure of clots made from purified fibrinogen but has no effect in individuals with diabetes.

In order to determine whether aspirin and antioxidant therapy, combined or alone, are more effective in reducing the development of cardiovascular events in patients with diabetes (T1DM or T2DM), a large prospective study found that 100 mg aspirin daily dose in diabetic patients has no effect in primary prevention of cardiovascular events and mortality (Belch et al., 2008). Nearly the same observation was recorded by another large prospective study which demonstrated that low dose of aspirin (81- 100mg/day) as a primary prevention did not reduce the risk of cardiovascular events in patients with T2DM (Ogawa et al., 2008). Aspirin is unique amongst antiplatelet agents by modulating both platelet function and fibrin clot structure/lysis (Ajjan et al., 2009). The mechanisms for clinical aspirin treatment failure in diabetes are not entirely clear and it is possible that an interaction between acetylation and glycation at the level of fibrinogen could modulate the fibrinolytic properties of aspirin, thus contributing to reduced efficacy of this agent in diabetes.

My data show that high dose of aspirin (300mg/day) facilitates fibrinolysis in healthy controlswith no effect observed in patients with diabetes. Interestingly, glycaemic control, measured as HbA1c, had no effect on response to treatment in the diabetes group. It should be noted, however, that the numbers became small once patients were divided into poor and better diabetes control and the study was not powered to investigate at subgroups. Therefore, concrete conclusions regarding interaction between glycaemic control and response to aspirin therapy cannot be made. Interestingly, there was a reduction in

acetylation of diabetic fibrinogen using radioactive techniques, although the magnitude of the effect was small and it does not necessarily explain the difference in plasma clot lysis comparing controls with diabetes patients.

When comparing data generated using clots made from plasma samples with clots made from purified fibrinogen, there were similarities but also notable differences. Aspirin affected structure of clots made from plasma as well as clots made from plasma-purified fibrinogen. However, aspirin only affected plasma clot lysis and had no effect on fibrinolysis in clots made from purified fibrinogen. There is a number of explanations for these results. First, it is possible that aspirin may have affected plasma proteins other than fibrinogen, and therefore it was able to modulate lysis of plasma clots, but not clots made from purified fibrinogen. Second, given that pooled samples were used in the purified experiments, it is possible that some minor differences were missed and ideally the experiments should have been conducted using individual purified fibrinogen samples. However, this was not possible given the limited yield of fibrinogen and the need to perform several repeat experiments to ensure robustness of the data. Third, the failure to detect differences in lysis time using clots made from purified fibrinogen may be related to methodological issues. Although a standard methodology was used, lysis time was relatively short in the purified experiments. In retrospect, I should have experimented with different concentrations of plasminogen and tPA and this is perhaps an area for future research. My work shows that *in vivo* aspirin therapy can modulate structure/and or lysis of fibrin clots made from plasma or purified fibrinogen of healthy controls. In contrast, aspirin treatment has no effect on fibrinolysis in individuals with diabetes regardless of glycaemic control. It was interesting to

note that only high dose aspirin resulted in changes to clot characteristics in healthy controls. Given that aspirin can be used to protect from venous thrombosis (Donohoe et al., 2011), regarded as a fibrin rather than a platelet clot, future research needs to consider whether 300 mg/day aspirin represents a better option in these patients than the standard 75 mg/day dose.

Data generated in this Chapter using *in vivo* studies are largely similar to the pervious Chapter employing *ex vivo* work. Therefore, it can be argued that *ex vivo* studies can replace in vivo work for the assessment of aspirin effects on the fibrin network. It remains unclear, however, whether the effects of aspirin on the fibrin structure are meaningful clinically. There are some cross-sectional studies suggesting that this may indeed be the case but prospective longitudinal studies are requiredin order to draw definitive conclusions.

In summary, the current chapter shows that aspirin affects the clot structure in healthy controls, although high doses of this agent were required, at 300 mg/day, to show an effect. The observed alterations in clot properties by aspirin in healthy control appear to be due to changes in the fibrinogen molecule, although an additional effect on plasma proteins cannot be ruled out. In contrast, aspirin fails to modulate clot structure in individuals with diabetes regardless of the dose of aspirin used or the level of glycaemic control. My work does not rule out, however, that aspirin may be more efficacious in those with near normal blood glucose levels as most of the patients studied had poor glycaemic control.

Chapter 6

Platelet-fibrinogen interaction

6.1 Introduction

Platelet-fibrinogen interaction is essential for the formation of the intravascular thrombus. The interaction of fibrinogen with activated platelets mainly occurs via the integrin receptor complex, glycoprotein (GP) IIbIIIa ($\alpha_{IIb}\beta3$) (Nachman et al., 1984, Phillips et al., 1991). This complex receptor consists of two glycoproteins: IIb (integrin α_{IIb}) and IIIa (integrin β3) (Floyd and Ferro, 2012). In a single platelet, there are about 80,000 (GPIIbIIIa) complex, making it the most predominant receptor on the platelet surface (Wagner et al., 1996). This number could be increased by the internal pool of $\alpha_{IIb}\beta3$ that exist on α-granules inside the platelet, that can move to the surface during platelet activation (Wencel-Drake et al., 1986). In addition to binding to fibrinogen,$\alpha_{IIb}\beta3$ can bind Von Willebrand factor (vWf) which has a significant role in haemostasis, as previously described (section 1.1.2.2).

There are two main sites on fibrinogen that are responsible for binding $\alpha_{IIb}\beta3$ on platelets. It has been shown that the mainsequence mediating fibrinogen binding to platelet was the carboxy-terminal sequence on the gamma chain, $\gamma_{406\text{-}411}$ (Kloczewiak et al., 1984).Arg-Gly-Asp (RGDsequences) also bind platelets and fibrinogen has two of these sequences on each α-chain. The first resides close the N-terminus (AA95-97) with the second towards the C-terminus (AA572-574) (Doolittle et al., 1979, Rixon et al., 1983, Sadler et al., 1985).Plow et al. (1985) have also explored the presence of (RGD) peptide in vWF and investigated its contributions to fibrinogen and vWF binding to platelets. They found that RGD containing peptides contribute toenhancing fibrinogen and vWF binding to platelets (Plow et al., 1985).

As platelets fibrinogen binding is an essential process in the construction of platelet-mediated thrombus, factors that modulate this interaction would have a crucial role in blood thrombus formation. Preliminary work from our laboratory, using recombinant techniques, has shown that exposure of fibrinogen to aspirin alters platelet binding properties of the protein (unpublished data). Given our findings and the general lack of studies adequately addressing platelet-fibrinogen interaction in diabetes, the aims of this Chapter were to:

i) Understand the effects of aspirin on platelet-fibrinogen interaction using fibrinogen purified from healthy controls.

ii) Establish the effects of glucose on this interaction.

iii) Investigate whether post-trasnslational modifications of fibrinogen in diabetes alterinteraction with platelets.

A combination of *in vivo* and *ex vivo* experiments were conducted using fibrinogen purified from healthy controls and patients with diabetes.

6.2 Materials and methods

In order to study platelet-fibrinogen interaction, two techniques were used. We developed a method that relies on labelling fibrinogen and studying its binding to platelets using flow cytometry.Also, static platelet-fibrinogen binding experiment were performed based on the methods for measurement of platelet adhesion in microtiter plates technique (Bellavite, 1993).

6.2.1 Patients and controls

Plasma samples were stored from the same patients and controls who gave blood samples for the platelet test and clot structure (Chapter 2, 3, 4). Platelet

fibrinogen binding studies were conducted in this group using purified fibrinogen.

6.2.2 Purified fibrinogen

Samples collected in lithium heparin for fibrinogen purification as detailed in Chapter 2 (section 2.3). Pools of plasma purified fibrinogen were then constructed using 6 samples from healthy controls and6 samples from type 1 diabetes patients with poor glycaemic control (HbA1c≥8.5%). For *ex vivo* test, different concentrations of aspirin 0 and 10mg in the presence and absence of additional glucose (20 mM) were added to pools of purified fibrinogen. Samples were then incubated for 24 hrs at 4°C.

For *in vivo* test, plasma pools of 6 samples from healthy controls and 6 samples from type 1 diabetes patients with poor glycaemic control (HbA1c≥8.5%) were made before and after treatment of the individuals for 2 weeks with aspirin at 75 mg/day, or 300 mg/day aspirin. Pools of fibrinogen were used for platelet fibrinogen binding using flow cytometry or static platelet experiments.

6.2.3 Labelling of fibrinogen for flow cytometry experiment

In order to measure fibrinogen binding with platelets using flow cytometry technique, pools of fibrinogen were labelled using Alex Flour 647 Monoclonal antibody labelling kit as described in section 2.8.2

6.2.4 Measuring of labelled fibrinogen concentration for flow cytometry experiment

The labelled fibrinogen was measured as described in section 2.8.2

6.2.5 Preparation of platelets and platelets-fibrinogen binding reaction tube

Platelets were collectedform healthy volunteers and platelet-fibrinogen interactions Static experiments were performed in section 2.8.1. Figure 6.1 summarised the methods.

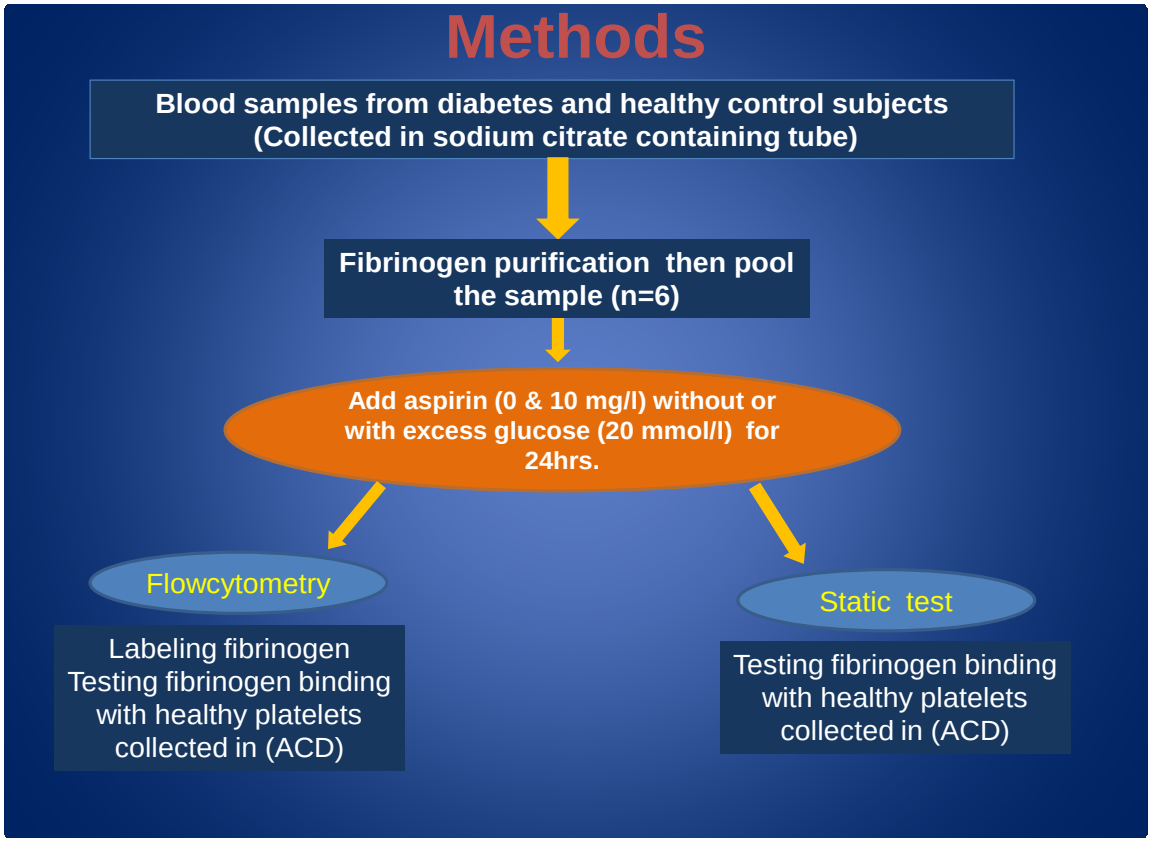


Figure 6.1 Summary of sample selection and preparation. Blood samples from diabetes and controls were collected followed by fibrinogen purification from each sample. Fibrinogen from 6 healthy controls and 6 patients with diabetes were pooled. Pooled fibrinogen samples were then incubated with 0 and 10mg/l of aspirin with and without additional glucose for 24 hrs at 4ºc followed by testing platelet-fibrinogen interactions using healthy control platelets. ACD: Acid citrate dextrose.

6.3 Results

6.3.1 Optimisations of flow cytometry

Conditions for fibrinogen concentration, the effects of ADP, tirofiban, using washed or whole blood platelets were optimised.

Measuring of labelled fibrinogen concentration

There was no significant difference in fibrinogen concentrations between the pool of patients and controls ($p>0.1$; Figure 6.2).

It is worth noting that the final concentration of fibrinogen using protein exposed to aspirin *in vivo* was higher than the concentration of fibrinogen in samples pooled after the *in vitro* addition of aspirin, which is explained by the dilution effect following the addition of aspirin to the samples. Fibrinogen concentrations in the experiments were subsequently kept constant using appropriate dilutions as detailed in Table 2.1

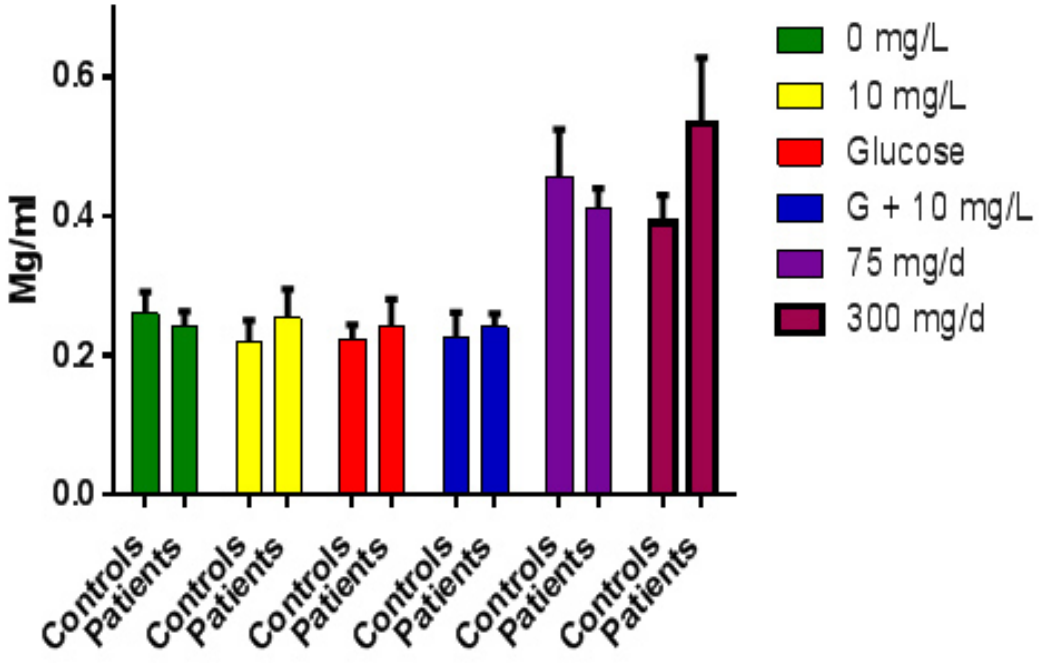


Figure 6.2 Fibrinogen concentration readings using NanoDrop (mg/ml). Readings of fibrinogen concentrations using pools of patients and controls before and after aspirin treatment *ex vivo* or *in vivo*. The effects of additional 20 mmol/l *ex vivo* glucose were also investigated.

Fibrinogen binding with washed or whole blood platelets and the effect of ADP/tirofiban

A number of experiments were conducted to assess platelet interactions with fibrinogen using washed platelets for specific $\alpha_{IIb}\beta 3$-fibrinogen investigations or platelets in their natural environment (whole blood) to demonstrate physiological relevance.

Using whole blood samples, platelets were identified by their characteristic light scattering, and 10,000 events were collected for each sample. In this experiment, 50 µg/ml of labelled fibrinogen was used. Also to test effects of ADP on fibrinogen platelet binding, basal platelet (without added ADP) and activated platelet (after adding 10uM ADP) analyses were conducted in both

whole blood and washed platelets. It appeared that washed platelets did indeed enhance the fluorescence signal, but that was at the expense of an increased background of non-specific binding. The high background was evidenced by a detectable signal that was not decreased by the presence of the $\alpha_{IIb}\beta3$ inhibitor tirofiban. This non-specific binding was present in both methods but was particularly pronounced with washed platelets. ADP treatment resulted in enhanced fluorescence signal using washed platelets or whole blood, emphasising the sensitivity of this technique (Figure 6.3).

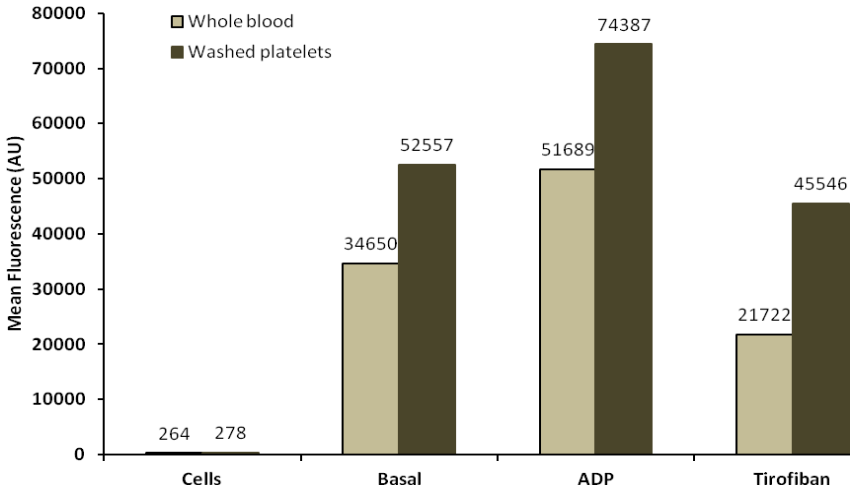


Figure 6.3 Mean fluorescence intensity of platelet fibrinogen binding in flow cytometry using whole blood and washed platelets. Cells represent platelet fluorescence intensity in the absence of fibrinogen. Basal shows fluorescence following incubation of platelets with fibrinogen without any agonist added. Fluorescence increases in the presence of 10 µM ADP, whereas inhibition is evident after incubation with 50 µg/ml Tirofiban, a known $\alpha_{IIb}\beta3$ inhibitor.

6.3.2 Optimisation of static platelet fibrinogen binding

Optimising fibrinogen concentration

Differentconcentrations of fibrinogen were used for coating the wells followed by adding platelets as detailed in section 2.7. Low fibrinogen concentration 0.001 and 0.01mg/ml did not show enough binding with platelets while there was significant binding with 0.1, 0.125, 0.25, 0.5, and 1 mg/ml (Figure 6.4). The readings show the mean±SEM of three independent experiments.

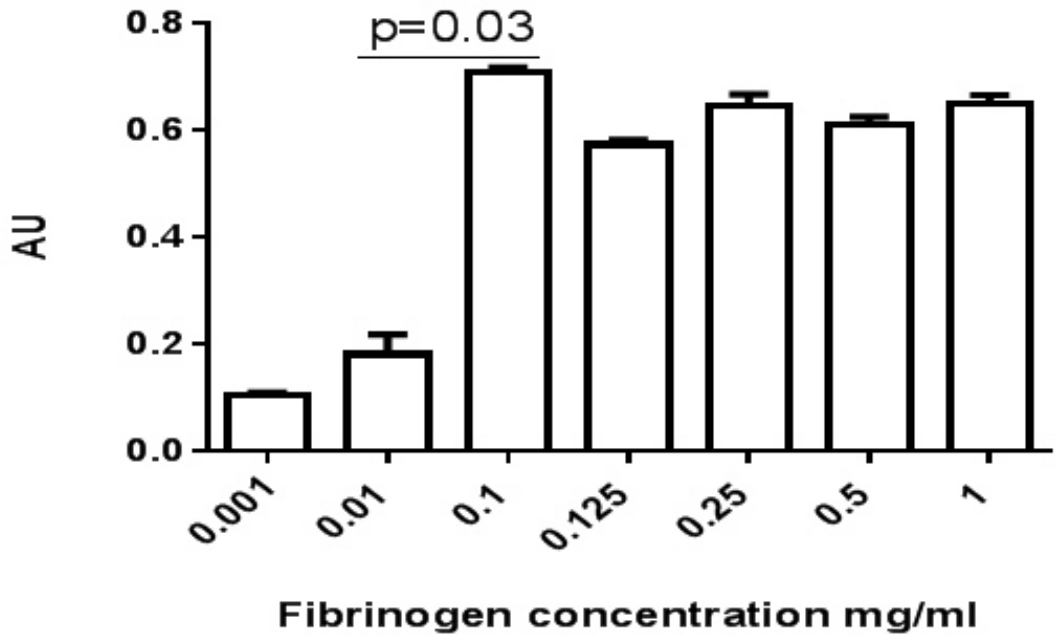


Figure 6.4 Reading of platelet-fibrinogen interaction in static tests. Plates were coated with increasing concentrations of fibrinogen ranging from 0.001 to 1mg/ml. The readings show the mean±SEM of three repeat experiments.

Specificity of platelet binding to fibrinogen

In order to ensure specific platelet binding to fibrinogen and that binding occurs with the integrins $\alpha_{IIb}\beta3$, I used bovine serum albumin (BSA) and C3 as control proteins with and without $\alpha_{IIb}\beta3$ inhibitor tirofiban. The concentration of all these proteins was 1mg/ml. The binding of platelets with BSA and C3 was very low 0.1±0.004 and 0.16±0.04 respectively. Binding of platelets to fibrinogen was 0.65±0.03 and it was inhibited by tirofiban to reach 0.17±0.006 Readings show the mean±SEM of three repeat experiments (Figure 6.5).

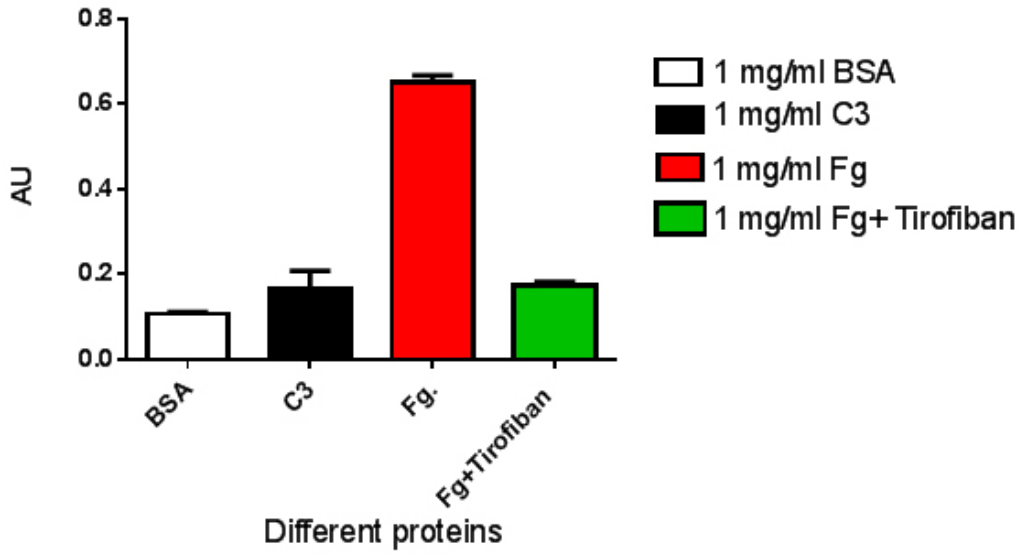


Figure 6.5 Platelet interaction with bovine serum albumin (BSA) complement C3 and fibrinogen. Plates were coated with BSA, C3 or fibrinogen at 1mg/ml. The readings show the mean±SEM of three repeat experiments with tirofiban used to test for $\alpha_{IIb}\beta3$ specificity. AU: arbitrary units

6.3.2 *Ex vivo* effect of aspirin on platelet fibrinogen binding

Fluorescence measurement can be expressed either as the percentage of positive cells above the threshold set with an appropriate negative control or as the mean or median fluorescence intensity (MFI) of a population of cells. The median is the most appropriate measure that shows a non-Gaussian or heterogeneous distribution on the platelet population. Percentage positive data are used primarily to measure an increase in the percentage of platelets that are bound to labelled fibrinogen, whereas MFI is used to quantify the level of expression of fibrinogen that is bound to platelets. Thus, measurement of both was undertaken to analyse the affinity and avidity of platelet fibrinogen binding under these conditions.

Aspirin and glucose reduce binding affinity of healthy control, but not diabetic fibrinogen to platelets in flow cytometry

The concentration of pooled fibrinogen from different conditions was similar ranging between 0.22-0.26 mg/l (Figure 6.6A). Binding of fibrinogen from patients with poor diabetes control to platelets was not affected by the presence of aspirin and/oradditional glucose compared with baseline. Aspirin-treated fibrinogen resulted in a non-significant increase in binding (3.7±4.4% p=0.4) that was not affected by the presence of glucose (3.2±4.5%, p=0.5). Glucose alone had no effect on fibrinogen binding to platelets (-1.6±2.2%, p=0.4, Figure 6.6B). In contrast, aspirin-treated fibrinogen from healthy controls showed decreased platelet binding (-13.3%±2.2 p=0.019) compared with untreated protein, with a further inhibition in the presence of additional glucose (-15.9±2% p=0.0006).

Interestingly, glucose alone also showed an inhibitory effect at10.1±1.5% (p=0.001; Figure 6.6C).

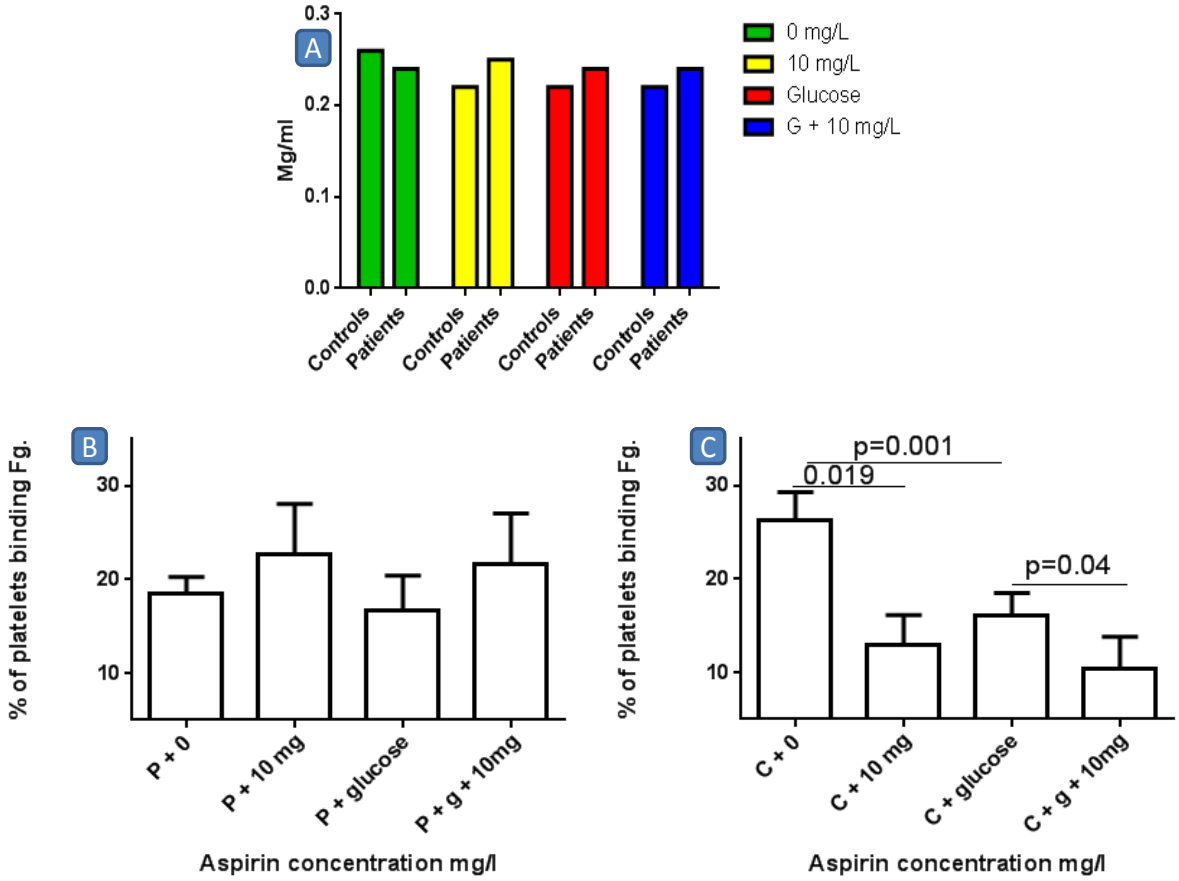


Figure 6.6 The effects of *ex vivo* aspirin treatment on fibrinogen-platelet interactions assessed as percentage of platelet binding. A. Fibrinogen concentration was kept constant in the various experiments. B, C. Percentage of platelets bound topooled purified fibrinogen from patients with poor diabetes control (P, n=6) or healthy controls (Cont., n=6). Purified fibrinogen was incubated without or with 10 mg/l aspirin (asp), 20 mmol/l glucose (glucose) or both (g+asp). The results represent the percentage of cells binding fibrinogen (Fg) corrected for baseline (mean±SEM) of 6 independent experiments.

Aspirin and glucose reduce binding avidity of healthy control, but not diabetic fibrinogen to platelets

Fluorescence intensity of bound fibrinogen was used as an index to estimate the amount of fibrinogen bound per platelet and thereby show binding avidity. Similar to results for the binding affinity, platelet-fibrinogen fluorescence intensity using fibrinogen from diabetes patients was not affected by aspirin and/or glucose therapy compared with baseline. Aspirin-treated fibrinogen from patients with poor diabete controlresulted in a non-significant decrease in fluorescence intensity (-4376±1108, p=0.1) that was not affected by the presence of glucose (2125±697, p=0.09). Glucose alone had no effect on theflorescence intensity of fibrinogen binding to platelets (-702±878% p=0.1, Figure 6.7 A).

In contrast, aspirin-treated fibrinogen from healthy controls showed decreased fluorescence intensity (-4515±1401, p=0.02) compared with untreated protein, with a possible further inhibition in the presence of additional glucose (4941±1375 p=0.016). Glucose alone also showed a significant inhibitory effect (-3625±979 p=0.012) (Figure 6.7 B).

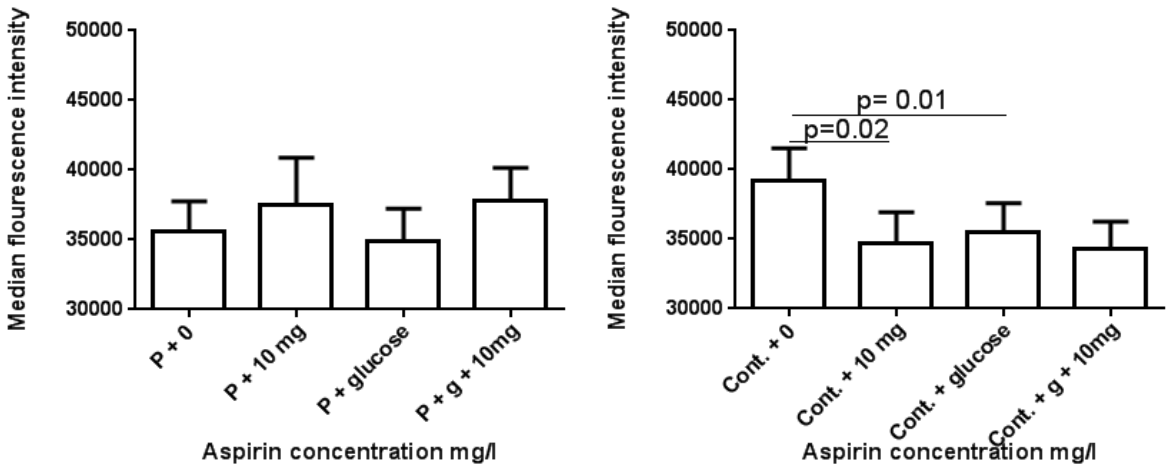


Figure 6.7 Platelet-fibrinogen interaction studies and median fluorescence intensity. Platelet binding of pooled purified fibrinogen was tested in the presence of 10 mg/l aspirin, 20 mmol/l excess glucose or both using protein from patients withpoor diabetes control (A) or healthy controls (B). The results represent the mean±SEM of 6 independent experiments**.**

Ex vivo aspirin and glucose fail to show an effect on platelet binding to fibrinogen in static tests

Binding of fibrinogen from patients with poor diabetes control to platelets was not affected by the presence of aspirin and/oradditional glucose compared with baseline (A). The same observation was shown in controls (Figure 6.8 A, B).

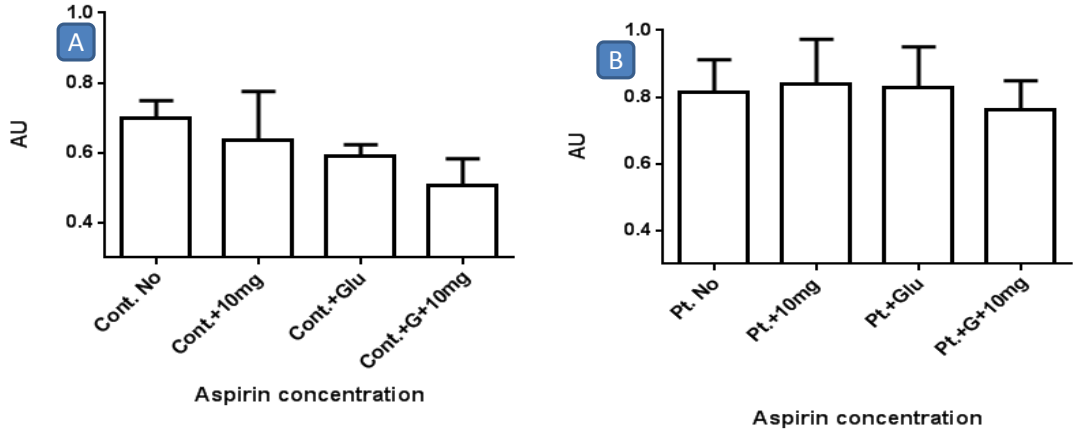


Figure 6.8 Static Platelet-fibrinogen interactions. Platelet binding of pooled purified fibrinogen was tested in the presence of 10 mg/l aspirin (asp), 20 mmol/l excess glucose (Glucose) or both (g+asp) using protein from patients with poor diabetes control (A) or healthy controls (B). The results represent the mean±SEM of 6 independent experiments**.**

6.3.3 The effects of in vivo aspirin administration on platelet fibrinogen binding

In vivo aspirin treatment reduces binding of healthy control, but not diabetic fibrinogen to platelets

The concentration of pooled fibrinogen ranged between 0.4 and 0.5 mg/l and there were no differences between different pools (Figure 6.9 A). Low daily dose of aspirin (75mg/day) did not inhibit the ability of fibrinogen pooled from patients with poor diabetes control to bind platelets. A non-significant increase in fibrinogen binding to platelets was observed after *in vivo* treatment with 75 mg/day of aspirin (3.9±2.9% p=0.2, Figure 6.9 B). However, 300 mg/day of

aspirin showed a significant increase in binding to platelets (10±3.9% p=0.03, Figure 6.11 B).

In contrast, fibrinogen from healthy controls showed a reduction in binding after *in vivo* treatment with 75 mg/day of aspirin (-12.8±4.9% p= 0.048) but not with higher aspirin dose (-2.6±2.9%, Figure 6.8 C).

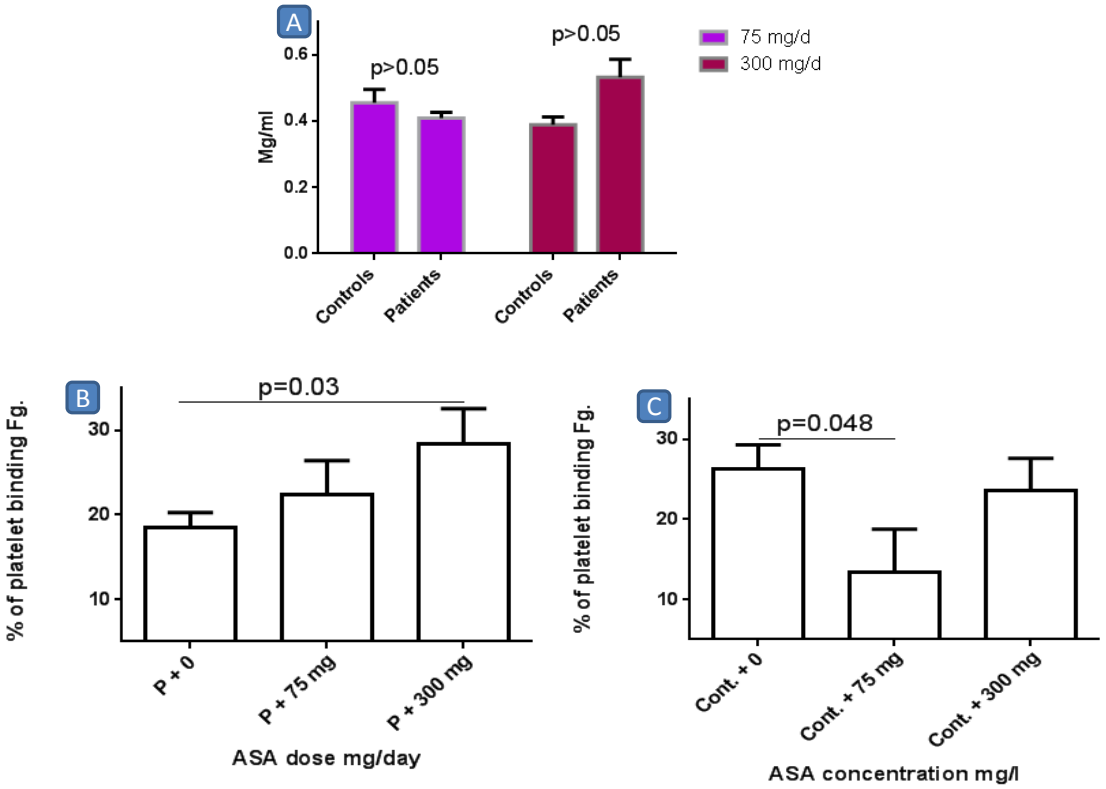


Figure 6.9 The effects of *in vivo* aspirin treatment on fibrinogen-platelet interactions assessed as percentage of platelet binding. A. Comparison of the amount fibrinogen pooled separately from controls and patients with poor diabetes control after treatment with 75 or 300 mg/day with aspirin for two weeks. B. Percentage of platelets bound to purified fibrinogen pooled from individuals with diabetes (n=6) following low and high dose aspirin therapy corrected for baseline. C. Percentage of platelets bound to purified fibrinogen pooled from healthy controls (n=6) following low and high dose aspirin therapy corrected for baseline. The results represent the mean±SEM of 6 independent experiments.

Fluorescence intensity

In patients with poor diabetes control, treated with 75 mg/day, there was an increase in fluorescence intensity, indicating higher binding (2874±917 p=0.026). Treatment with 300mg/day also resulted in a significant increase in fluorescence intensity (4129±766 p=0.003) (Figure 6.10A). In contrast, 75mg/day aspirin treatment in controls showed a non-significant decrease in fluorescence intensity (-5245±3193; p=0.3) with no effect observed following treatment with 300mg/day (-1161±1351; p=0.7; Figure 6.10B).

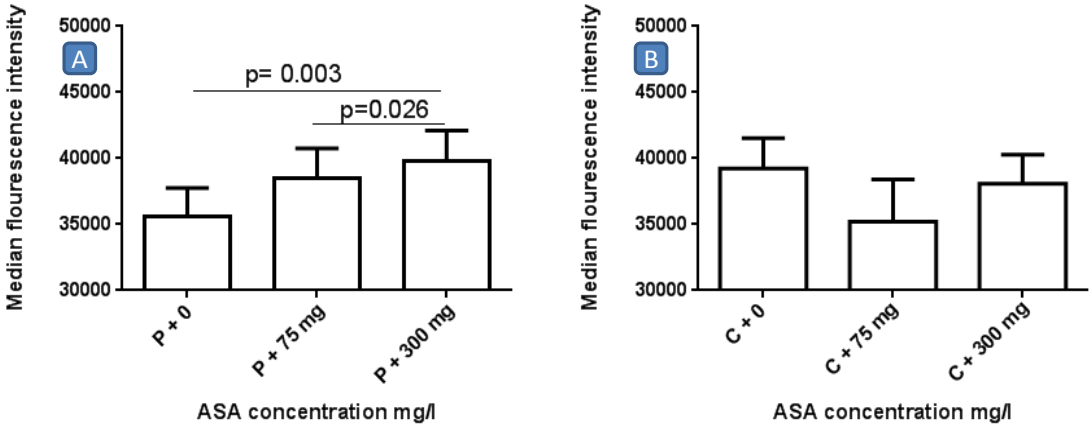


Figure 6.10 The effects of *in vivo* aspirin treatment on fibrinogen-platelet interactions assessed as fluorescent intensity. A. Median fluorescence intensity of platelets bound to purified fibrinogen from pooled patients with poor diabetes control samples (n=6) after aspirin treatment, corrected for baseline. B. Median fluorescence intensity of platelets bound to purified fibrinogen from pooled healthy controls (n=6) after aspirin treatment,corrected for baseline. The results represent the mean±SEM of 6 independent experiments.

In vivo aspirin fails to show an effect on platelet binding to fibrinogen in static tests

Low daily dose of aspirin (75 mg/day) and high daily dose (300 mg/day) did not significantly inhibit the ability of fibrinogen pooled from patients with poor diabetes dibete control to bind platelets (Figure 6.13 A) with similar data produced when healthy control fibrinogen was investigated (Figure 6.13 B).

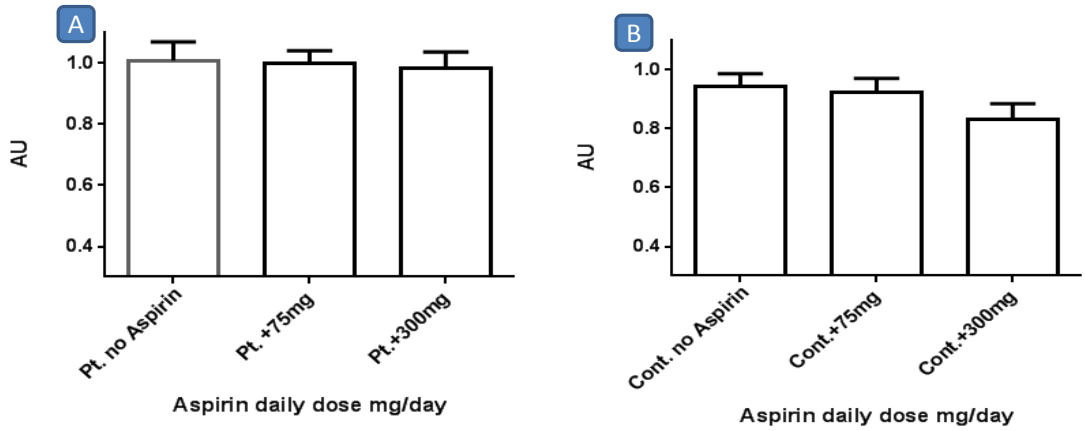


Figure 6.11 The effects of *in vivo* aspirin treatment on static fibrinogen-platelet interactions assessed as aggregation unite (AU). A**.** Platelets bound with purified fibrinogen pooled from individuals with poor diabetes control (n=6) following low and high dose aspirin therapy [P (75) and P (300), respectively], corrected for baseline. B. Platelets bound with purified fibrinogen pooled from healthy controls (n=6) following low and high dose aspirin therapy [Cont (75) and Cont (300), respectively], corrected for baseline. The results represent the mean±SEM of 6 independent experiments.

6.4 Discussion

In this Chapter, I investigated, for the first time, the effect of *ex vivo* aspirin and glucose on platelet fibrinogen interactions using protein purified from patients with diabetes and from healthy controls. Moreover, I investigated whether high glucose concentration modulates the effects of aspirin on platelets fibrinogen binding. This was complemented by *in vivo*studies investigating the effects of low and high daily dose of aspirin (75mg/day and 300mg/day) aspirin on

modulation of fibrinogen-platelet interaction. Platelets used were always from healthy controls and therefore this was kept constant.

This part of the work required significant amount of purified fibrinogen and therefore the number of samples were limited. Given the exploratory nature of these experiments, and lack of previous data, formal power calculation was not possible. Therefore, the results of these experiments must be regarded as preliminary and concrete conclusions cannot be made other than confirming the feasibility of this new methodology. Future work will be needed, using more samples, to confirm the findings and probe into mechanistic explanations for the observed differences.

There are a number of observations emerging from my studies that can be summarised as follows: i) *Ex vivo* aspirin treatment of healthy control fibrinogen reduces platelet binding ii) *Ex vivo* addition of glucose to healthy control fibrinogen reduces platelet binding. iii) Neither aspirin, nor glucose had an effect on binding of fibrinogen from patients with diabetes to platelets iv) *In vivo* treatment with aspirin results in reduced binding of fibrinogen from healthy controls to platelets while this effect of aspirin is abolished in patients with diabetes.v) In contrast to flow cytometry studies, static platelet adhesion tests failed to show an effect for aspirin and/or glucose on fibrinogen binding to platelets.

Free amino groups on lysine in the fibrinogen molecule can participate in various reactions such as crosslinking of fibrin fibres or proteins into the clot by factor XIII (Bjornsson et al., 1989).

Aspirin acetylated fibrinogen forms ε-N-acetyl-lysine in vitro (Bjornsson et al., 1989, Pinckard et al., 1968). Based on SDS-PAGE gels, using [^{14}C], it has been

demonstrated that about 50% of the acetylation occur in the E domain with each of the D domains having about 25%. One third of the total acetyl substitution occur in each of the individual α, β and γ chain (Bjornsson et al., 1989), whereas others have shown this to be slightly different between chains at 41%, 35% and 24% respectively (Svensson et al., 2012).

Lysine residues constitute 7% of the 2964 amino acids of fibrinogen and therefore acetylation of these residues may have an important role in protein function (Bjornsson et al., 1989). Lysine residues involves in acetylation include: αK191, αK208, αK224 aK429, αK457, αK539, αK562, in the α-chain, βK233 in the β-chain and γ K170 and γ K273 in the γ-chain (Svensson et al., 2012). Interestingly, Sevensen and his group reported that glycation occurred at lysine residuse on βK133 and γK75, alternatively γK85, ruling out interaction between acetylation and glycation. However, in vitro glycation is always a challenging technique and the authors should have used samples from diabetes patients, which would have better presented the relevant glycation sites. Indeed others that studied in vivo glycation of fibrinogen documented additional glycation in the three chains of fibrinogen (Dunn et al., 2005).

Using flow cytometry I showed that *ex vivo* treatment of samples as well as in vivo aspirin therapy is associated with reduced platelet binding to fibrinogen using protein from healthy controls. This suggests that fibrinogen acetylation reduces interaction with platelet, highlighting a new mechanism of action for aspirin that has not been described before. However, there are a number of inconsistencies that should be acknowledged. Following high dose in vivo treatment with aspirin, there was no reduction in platelet fibrinogen interaction in control. This was unexpected given the consistent results with low aspirin dose.

Given time restrictions, I was unable to repeat the test using different pool of samples, which would have helped to clarify this point. An alternative explanation is individuals not complying with higher dose of aspirin due to side effects. Another concern was the inability to reproduce the data using static fibrinogen-platelet binding tests. Thisis most likely related to the inferior sensitivity of this method and the inability to detect small differences that are more readily picked up by flow cytometry. In contrast, using fibrinogen from patients with diabetes failed to show an effect of aspirin on platelet binding. If anything there was a small increase compared with baseline by mechanisms that are unclear.This strongly suggests that the effects of aspirin on platelet fibrinogen interaction are blunted or reversed in diabetes possibly due to post-translational modifications of the protein, most probably glycation. It should be noted, however, that these experiments can only be described as preliminary at this stage and more work is needed using larger number of samples from a variety of diabetes patients before concrete conclusions can be made.

Another observation was the effects of glucose on platelet-fibrinogenbinding that was evident in controls but not patients with diabetes. As the glucose incubation time was short, fibrinogen glycation is unlikely to account for these changes. One of thepossible reasons include alteration in fibrin(ogen) conformation resulting from the effect of osmotic changes making the protein less amenable to interact with platelets (Rand, 2004). This was not seen in those with diabetes who already had elevated levels of glucose in their blood. One caveat of the study is the use of platelets from healthy controls. This was necessary to keep variables to a minimum but in real life healthy control platelets will not encounter diabetic fibrinogen and, therefore, the system used

is artificial. There are precedents to this, however, as complement C3 from healthy volunteers was used to assess the effect of this molecule on fibrin clot lysis using fibrinogen from patients with diabetes and controls (Hess, Diabetologia 2012). Further experiments will be required comparing fibrinogen-platelet interactions in diabetes and healthy controls using cells and proteins from the same individuals.

In conclusion, these preliminary results suggest that *ex vivo* aspirin treatment of fibrinogen reduces platelet-fibrinogen interaction in healthy controls but not in patients with diabetes. It shows that the *in vivo* data largely mirror my *ex vivo* findings except for the high dose aspirin. Reason for the latter are not entirely clear (discussed above) and will require further investigation.

The effects of glucose on platelet fibrinogen interaction in controls but not in patients with diabetes indicates that instantaneous high glucose is not necessarily prothrombotic and it is long-term hyperglycaemia, leading to post-translational protein modifications, which is the main culprit for increased thrombosis risk in diabetes.

Finally, it remains unclear whether the observed differences in platelet fibrinogen interactions comparing healthy controls with diabetes patients are clinically significant and further studies are needed to address this point.

Conclusions and future work

Cardiovascular disease (CVD) remains a major cause of mortality and morbidity worldwide, a trend that is likely to continue in the near future (Franco et al., 2011), despite efforts to control various risk factors (Padmanabhan et al., 2010). One of the most important risk factors of CVD is diabetes mellitus which is becoming the epidemic of the 21st with an increasing number of individuals diagnosed with this condition.

Patients with diabetes have 2-4 times higher cardiovascular mortality compared with the general population, which is due to a number of factors including more extensive vascular disease and increased thrombosis potential. The latter occurs secondary to changes in clotting factor plasma level/activity, impaired fibrinolysis and increased platelet reactivity (Alzahrani and Ajjan, 2010, Angiolillo et al., 2005).

Anti-platelet therapy remains a cornerstone in the management of individuals with established CVD. Aspirin was the first anti-platelet agent used for the management of atherothrombotic events. This agent does not only affect platelet function, by reducing thromboxane A2 synthesis, but can also acetylate fibrinogen resulting in the formation of a more porous fibrin network that is easier to lyse.

The reduced clinical and/or clinical efficacy of aspirin is more commonly documented in patients with diabetes (Ferreiro et al., 2010). The exact mechanisms for this phenomenon are unknown but there are a number of hypotheses that were put forward, related to a compromise in the effects of this agent on both the cellular and fluid phase of coagulation, secondary to long-term hyperglycaemia.

The main aim of my work was to investigate the molecular basis for aspirin treatment failure in diabetes and the main findings are discussed below.

1. Effects of glycation and blood glucose levels on platelet response to aspirin therapy

The reasons for aspirin resistance can perhaps be broadly categorised into pharmacokinetic (insufficient blood concentration of aspirin) andpharmacodynamic factors (incomplete suppression of platelet activity despite sufficient plasma drug levels (Benedek et al., 1995, Cerletti et al., 2003), (Weber et al., 2002). Inter-individual variability, secondary to co-morbidities, various treatment and other unmodifiable factors, is likely to play a role in the heterogeneous response to aspirin treatment. In order to address pharmacokinetic change and limit inter-individual variability, I have developed an *ex vivo* system to dissect out the role of medium term and instantaneous hyperglycaemia on response to aspirin. This is why I concentrated on type 1 diabetes patients, not on any treatment other than replacement doses of insulin and conducted a series of *ex vivo* and in vivo studies.

My data show, for the first time, that both glycation and high glucose levels interfere with the action of aspirin. Although low aspirin concentration inhibited platelet response to AA stimulation, this effect was compromised in patients with poor glycaemia control. In contrast, higher aspirin concentrations inhibited AA-induced stimulation of platelet aggregation in both diabetes patients and controls and were not affected by glycaemic control. This fits nicely with the recent hypothesis that the effects of aspirin do not last 24 hours in diabetes High aspirin concentration, achieved early following aspirin administration,

overcomes the effects of protein glycation and results in good suppression of platelet reactivity. However, as the day progresses and given the short half-life of aspirin, newly synthesised platelets escape the inhibitory response of low aspirin concentration, creating a thrombotic environment.

Moreover, modulation of response to aspirin by high glucose levels indicates that early control of diabetes following an ischaemic event is important to optimise response to aspirin therapy.

Interestingly, minor elevation in HbA1c was not associated with decreased response to aspirin. This suggests that only individuals with very poor diabetes control (HbA1c≥8.5%) have reduced response to aspirin thus further explaining some of the contradictory data on aspirin resistance.

Future work is required to establish the optimum glycaemic control following acute vascular ischaemia. To complicate matters, hypoglycaemia (resulting from over-treatment of hyperglycaemia) is in itself prothrombotic and therefore developing appropriate glucose monitoring strategies is paramount in order to fully understand the role of glycaemia in response to aspirin therapy. This is made possible with recently approved new glucose monitoring devices, such as Libre (Abbott), and studies in this area are awaited with interest. Also, studies assessing biochemical effects of aspirin should not only concentrate on obtaining samples at peak aspirin concentration but also at trough drug levels, which would give a better indication of platelet suppression over 24 hours.

2. Diabetes, blood glucose and modulation of the fibrin clot by aspirin

Clot changes in subject with diabetes have been reported in a number of studies (Jorneskog et al., 1996, Dunn et al., 2006, Ajjan and Ariens, 2009, Hess

et al., 2012). These changes are related, at least in part, to post-translational modifications in the fibrinogen molecules (Dunn et al., 2006) caused by non-enzymatic glycation of the protein(Brownlee et al., 1983). Work from our laboratory has shown that theaddition of aspirin to culture media of fibrinogen-producing CHO cells, results in fibrinogen acetylation, which in turn affects clot structure and lysis (Ajjan et al., 2009). These findings are consistent with my data, which demonstrated that *ex vivo* addition of aspirin to whole blood facilitated fibrinolysis in plasma system. However, aspirin had little effect when purified fibrinogen was used. These findings suggest that incubation of aspirin in whole blood could affect other plasma protein that alter fibrinolysis while this does not occur with purified fibrinogen. Interestingly, a short incubation period in whole blood was enough to show an effect and in retrospect, I should have had identical incubation periods with aspirin in whole blood and purified fibrinogen. In other words, the longer incubation period with fibrinogen may have induced some protein degradation and therefore failing to show an effect for aspirin on clot lysis. Alternatively, it is possible that the conditions used for the turbidimetric experiments were not sensitive enough to detect changes in fibrinolysis by aspirin and perhaps a series of experiments are needed using decreasing concentrations of tPA and/or plasminogen in order to detect a difference.

My work failed to show a clear effect of additional glucose on clot structure but I have found that extra glucose can impair the fibrinolytic properties of aspirin, when clots were made from plasma samples, an observation that has not been documented before. However, no such effect was observed when aspirin was incubated with purified fibrinogen, indicating, yet again, that aspirin affects other

components of plasma proteins. However, the failure to detect a difference with purified samples may be due to methodological flaws mentioned above.

Having an *in vitro* system mimicking the *in vivo* situation would help to further study interactions between glycation and acetylation in relation to the effects on fibrinogen. I considered growing CHO cells with different glucose concentrations in the presence and absence of aspirin to further investigate interactions between fibrinogen glycation/acetylation. However, preliminary experiments indicated that CHO cell growth is compromised in the presence of low glucose concentration (these cells are usually grown in the presence of >16 mmol/l glucose) and therefore I did not pursue this avenue.

Further work is required incubating aspirin with whole blood and purified fibrinogen for identical periods of time before concluding that in such a system the fibrinolytic effects of aspirin are related to an effect on plasma proteins other than fibrinogen. Also, further work is warranted using purified fibrinogen at different incubation times with aspirin followed by SDS-PAGE analysis to investigate changes in protein integrity.

3. Diabetes, blood glycation and *in vivo* modulation of platelet function and fibrin clot by aspirin

Given that samples were taken within two hours of aspirin administration, there was no difference in platelet inhibition comparing control and diabetes group. This is consistent with my ex vivo data showing that high aspirin concentration causes similar inhibition of platelet function in control and diabetes subjects. In retrospect, I should have arranged for an extra sample to be taken before

aspirin dose to investigate platelet inhibition with low aspirin concentration and this remains an area for future research as detailed above.

My data also show that high dose of aspirin (300 mg/day) facilitates fibrinolysis in healthy controls with no effect observed in patients with diabetes. Interestingly, glycaemic control had no effect on response to treatment in the diabetes group. It should be noted, however, that the numbers became small once patients were divided into poor and better diabetes control and the study was not powered for subgroup analysis. Therefore, concrete conclusions regarding interaction between glycaemic control and response to aspirin therapy cannot be made. Most importantly, however, data from *in vivo* work largely mirror my *ex vivo* findings opening the door for further *ex vivo* experiments aiming to identify those with reduced response to aspirin therapy.

When comparing data generated using clots made from plasma samples with clots made from purified fibrinogen, there were similarities but also notable differences. It is possible that aspirin may have affected plasma proteins other than fibrinogen, consistent with the explanations given for the ex vivo studies above. Alternatively, given that pooled samples were used in the purified experiments, it is possible that some minor differences were missed. Although a standard methodology was used, lysis time was relatively short in the purified experiments.In retrospect, I should have experimented with different concentrations of plasminogen and tPA, and this is perhaps an area for future research.

4. Platelet-fibrinogen interaction

As platelet fibrinogen binding is an essential process in the construction of platelet-mediated thrombus, factors that modulate this interaction would have a crucial role in blood thrombus formation. Preliminary work from our laboratory, using recombinant techniques, has shown that exposure of fibrinogen to aspirin alters platelet binding properties of the protein (unpublished data). Given our findings and the general lack of studies adequately addressing platelet-fibrinogen interaction in diabetes, I investigated, for the first time, the effect of *ex vivo* aspirin and glucose on platelet fibrinogen interactions using protein purified from patients with diabetes and from healthy controls. Moreover, I investigated whether high glucose concentration modulates the effects of aspirin on platelets fibrinogen binding. This was complemented by *in vivo* studies investigating the effects of low and high daily dose of aspirin (75 mg/day and 300 mg/day) aspirin on modulation of fibrinogen-platelet interaction. Platelets used were always from healthy controls and therefore this was kept constant.

The preliminary findings using flow cytometry, showed that *ex vivo* treatment of samples as well as *in vivo* aspirin therapy is associated with reduced platelet binding to fibrinogen using protein from healthy controls. This suggests that fibrinogen acetylation reduces interaction with platelet, highlighting a new mechanism of action for aspirin that has neverbeen described before. In contrast, aspirin failed to reduce binding of diabetic fibrinogen to healthy platelets. This strongly suggests that the effects of aspirin on platelet fibrinogen interaction are blunted or even reversed in diabetes due to post-translational modifications of the protein, most probably glycation. Naturally, further experiments are needed using different pools and also individual fibrinogen

samples from a variety of diabetes patients and different controls before concrete conclusions can be made. Nevertheless, these findings provide a potential third mode of action for aspirin, which is compromised in diabetes, and indicates that studying biochemical efficacy of this agent by monitoring platelet function (usually at a single time point) is both inadequate and misleading.

Another observation was the effects of glucose on platelet-fibrinogen binding that was evident in controls but not patients with diabetes. As the glucose incubation time was short, fibrinogen glycation is unlikely to account for these changes. One possible reasonincludes alteration in fibrin(ogen) conformation resulting from the effect of osmotic changes making the protein less amenable to interact with platelets (Rand, 2004). The counter-argument, however, is that this was not seenin diabetes samples whichhave already been exposed to elevated levels of glucose. It should be noted that these are just a preliminary findings and future work warranted using higher number of samples to.

To minimise confounding factors, I always used platelets from healthy donors in my experiments. This can be criticised as “diabetic” fibrinogen will not encounter “healthy” platelets *in vivo*. Therefore, further experiments will be required comparing fibrinogen-platelet interactions in diabetes and healthy controls using cells and proteins from the same individuals.

In summary, my studies answer questions but equally generate a number of interesting questions. In particular, further studies are needed to study the cellular and protein arm of coagulation at trough levels of aspirin. Also, more work is needed to investigate a potential third mode of action of aspirin, affecting platelet-fibrinogen interactions, and further work is required to understand whether this pathway is affected in diabetes. Finally, appropriately

designed glycaemic studies are required to understand glucose levels at which aspirin can have optimal effects in individuals with diabetes.

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