The effect of blood glucose control on fibrin network characteristics of African subjects with uncontrolled type 2 diabetes

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Namukolo M Covic

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I dedicate this PhD thesis to all women who decide to pursue further studies after raising their children. The courage to do it in spite of the challenges will forever be a source of inspiration for me.

Abstract

Type 2 diabetes is a growing health problem worldwide. People affected face increased cardiovascular (CVD) disease risk. Cardiovascular disease is a recognised leading cause of mortality among people with type 2 diabetes. It is suspected that alterations in fibrin network structure may, in part, contribute to the increased CVD risk. A possible mechanism contributing to the altered fibrin network structure is the non-enzymatic glycation of fibrinogen due to continuous exposure to high glucose levels in the diabetic condition.

Twenty Black South Africans with uncontrolled type 2 diabetes were recruited for the study and 20 age and BMI matched non-diabetic volunteers were included as a reference group. The diabetic volunteers were treated with insulin under out-patient conditions to control both fasting and post-prandial glucose in order to determine if glycaemic control would reduce fibrinogen glycation and improve fibrin network structure. Blood samples of the diabetic volunteers were drawn at the beginning and the end of the study once glycaemic control was achieved and maintained for a further 8-day period. Blood samples were collected from the non-diabetic volunteers who underwent no intervention at times comparable to those of the matched diabetic volunteers. Fibrin network structure variables were measured both in plasma and in fibrinogen purified from the volunteers' plasma. The purified fibrinogen results would indicate the individual effects of fibrinogen glycation, while the plasma results would indicate the contribution of the effect of fibrinogen glycation on fibrin network structure in the presence of other plasma constituents.

There was no difference in fibrinogen concentration between the two groups (4.25 vs 4.02 g/l, respectively) and the fibrinogen concentrations were higher than expected for the population group. The uncontrolled diabetic volunteers at baseline had higher fibrinogen glycation than the non-diabetic volunteers (7.84 vs 3.89 mol glucose/mol fibrinogen, respectively; p=0.0002). Fibrinogen glycation in

the diabetic volunteers was significantly reduced with achievement of glycaemic control (7.84 to 5.24 mol glucose/mol fibrinogen; p=0.0007).

In the purified fibrinogen model, permeability improved in the diabetic group after achievement of glycaemic control (p=0.02). The rate of lateral aggregation (slope) for the diabetic volunteers was higher than for non-diabetic volunteers at baseline. The slope correlated positively with fibrinogen glycation (r=0.47; p=0.01) and glycaemic control measured by HbA1c (r=0.59; p=0.001) and venous glucose (r=0.51; p=0.005).

In the plasma model, clot rigidity (p=0.013) and time taken for the proto-fibrils to reach a sufficient length for lateral aggregation to take place (lag-time) (p=0.03) increased, in the diabetic group, with glycaemic control. None of the fibrin network structure variables correlated with glycaemic control or fibrinogen glycation. Permeability, slope and fibre size did however, correlate with fibrinogen concentration.

Fibrinogen glycation was reduced by glycaemic control resulting in alterations to fibrin network structure. From the purified fibrinogen model, reduction in fibrinogen glycation resulted in an improvement in clot permeability, but when other plasma constituents were introduced, in the plasma model, these effects were obscured. The high fibrinogen concentrations that prevailed in the study population may have masked the effect of fibrinogen glycation in the plasma model. Having done this intervention under out-patient conditions makes these results applicable to the general diabetic population.

Opsomming

Tipe2-diabetes is wêreldwyd 'n groeiende gesondheidsprobleem. Diabete het verhoogde kardiovaskulêre siekterisiko (KVS). Kardiovaskulêre siektes is van die grootste oorsake van mortaliteit in tipe 2 diabetes. Dit blyk dat veranderinge in fibriennetwerk-struktuur bydra tot hierdie verhoogde KVS-risiko. 'n Moontlike meganisme wat tot die verandering in fibriennetwerk-struktuur in diabete bydra, is die nie-ensiematiese glikosilering van fibrinogeen as gevolg van blootstelling aan volgehoue hoë bloedglukose.

Twintig swart Suid-Afrikaanse ongekontroleerde tipe 2-diabete is in die studie ingesluit. Twintig nie-diabete wat volgens ouderdom en liggaams-massa-indeks met die diabete afgepaar is, is as verwysingsgroep ingesluit. Die diabete is as buite-pasiënte met insulien behandel om sodoende beide vastende en post-prandiale glukosevlakke te kontroleer. So kon bepaal word of glukemiese beheer, fibrinogeen-glikosilering sal verlaag en gevolglik die fibriennetwerk-strukture soul verbeter. Bloedmonsters is by die diabete gekry, beide aan die begin en aan die einde van die studie nadat glukosebeheer verkry en vir 'n periode van 8 dae volgehou is. Bloedmonsters is van die nie-diabete, wat geen intervensie ondergaan het nie, gekry op tye wat ooreenstem met dié van die diabete. Fibriennetwerk-struktuur-veranderlikes is in beide plasma en fibrinogeen wat uit deelnemerplasma geïsoleer is, gemeet. Resultate van die geïsoleerde fibrinogeenmodel illustreer die individuele effek van fibrinogeen-glikosilering op fibriennetwerk-strukture. Resultate van die plasmamodel illustreer die bydra van die effek van fibrinogeen-glikosilering op fibriennetwerk-strukture in die teenwoordigheid van ander plasmakomponente.

Daar was geen verskil in die fibrinogeenkonsentrasie tussen die twee groepe nie (4.25 teenoor 4.02g/l onderskeidelik) hoewel dit hoër as verwag was, vir hierdie populasie. Die ongekontroleerde diabete het hoër vlakke van fibrinogeen-glikosilering as die nie-diabete getoon (7.84 teenoor 3.89 mol glukose / mol

fibrinogeen; p=0.0002). Fibrinogeen-glikosilering het egter betekenisvol gedaal in die diabete met die bereiking van glukosebeheer (7.84 na 5.24 mol glukose / mol fibrinogeen; p=0.0007).

In die geïsoleerde fibrinogeenmodel het die permeabiliteit van die fibriennetwerke, in die diabete, verbeter met bereiking van glukosebeheer (p=0.02). Die tempo van laterale aggregering (helling) was hoër in die diabete in vergelyking met die nie-diabete tydens die aanvang van die studie. Die tempo van laterale aggregering het positief gekorreleer met fibrinogeen-glikosilering (r=0.47; p=0.01) en glukosebeheer soos gereflekteer deur HbA1c (r=0.59; p=0.001) en veneuse glukose (r=0.51; p=0.005) waardes.

In die plasmamodel, het rigiditeit van die klont (p=0.013) en die tyd nodig vir die protofibrille om voldoende lengte te bereik om lateraal te aggregeer (p=0.03), beide verhoog in die diabete met die bereiking van glukosebeheer. Nie een van die fibriennetwerk-struktuur-veranderlikes het met glukosebeheer of fibrinogeen-glikosilering gekorreleer nie. Die tempo van laterale aggregering en veselgrootte het wel met fibrinogeenkonsentrasie gekorreleer.

Glukose beheer het 'n verlaging in fibrinogeen-glikosilering tot gevolg gehad, met 'n daaropvolgende verandering in fibriennetwerk-struktuur. In die geïsoleerde fibrinogeenmodel, het 'n verlaging in fibrinogeen-glikosilering, verhoogde permeabiliteit van die fibriennetwerk tot gevolg gehad. In die plasmamodel waar ander plasmakomponente egter teenwoordig was, soos byvoorbeeld die hoë fibrinogeenkonsentrasie van die populasie, is die effek van fibrinogeen-glikosilering op fibriennetwerke egter daardeur verskans. Die feit dat hierdie studie onder buite-pasiënt-omstandighede uitgevoer is, maak die resultate dus van toepassing op die tipe 2-diabetiese gemeenskap in die breë.

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List of abbreviations

ACE	Angiotensin converting enzyme inhibitors
AGEs	Advanced glycation end products
ANOVA	Analysis of variance
Apo B	Apo-protein B
APS	Ammonium persulfate
ARIC	Atherosclerosis Risk in Communities
BMI	Basal metabolic rate
$CaCl_2$	Calcium chloride
CHD	Coronary heart disease
CNBr	Cyanogen bromide
CVD	Cardiovascular disease
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Glu	Glutamine
HbA1c	Glycated haemoglobin
HCl	Hydrochloric acid
HDL-C	High density lipoprotein-cholesterol
HOMA	Homeostasis model assessment
Ks	Permeation coefficient
LDL-C	Low density lipoprotein-cholesterol
Lys	Lysine
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium hydrogen carbonate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
PAI-1	Plasminogen activator inhibitor-1
$PAI\text{-}1_{act}$	Plasminogen activator inhibitor-1 activity
PI	Plasmin inhibitor

List of abbreviations continued

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAFIa	Thrombin activatable fibrinolytic inhibitor
TEMED	Tetramethylethylene di-amine
THUSA	Transition and health during urbanisation of South Africans
t-PA	Tissue plasminogen activator
Tris	2-Amino-2-(hydroxymethyl)-1,3- propanediol
VLDL-C	Very low density lipoprotein-cholesterol
WHO	World Health Organisation

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Chapter 1: Introduction

1.1 Background

Diabetes is a growing world health concern. It has been estimated that the worldwide total of people with diabetes may more than double to 366 million by 2030 from 171 million in 2000. In Sub-Saharan Africa, a similar trend is also expected (Wild, Roglic, Green, Sicree, & King, 2004).

Cardiovascular disease (CVD) has been shown to have up to a fourfold prevalence among people with diabetes (Stamler, Vaccaro, Neaton, & Wentworth, 1993; Wei, Gaskill, Haffner, & Stern, 1998) and CVD has been recognised for some time now as a leading cause of mortality among type 2 diabetes patients (Bathesda, 2005; Kannel, D'Agostino, Wilson, Belanger, & Gagnon, 1990; Kannel & McGee, 1979). South African statistics are already showing that diabetes deserves serious attention, as it has been ranked among the top eight leading causes of death among adults from 1997 to 2001 (Statistics South Africa, 2002). Among South African women, diabetes ranked even higher as the fifth leading cause of death in women above fifty years old (Statistics South Africa, 2002).

A number of reasons have been put forward for the observed high CVD risk and mortality affecting people with diabetes. These include the hyperglycaemia itself (Middelbeek & Horton, 2007), possible elevation of plasma fibrinogen (Saito, Folsom, Brancati, Duncan, Chambless, & and McGovern, 2000), hyperlipedaemia (Asia-Pacific Cohort Studies Collaboration, 2007), hypercoagulability (Barazzoni, Zanetti, Davanzo, Kiwanuka, Carraro, Tiengo, & Tessari, 2000; Ceriello, Giacomello, Stel, Motz, Taboga, Tonutti, Pirisi, Falleti, & Bartoli, 1995), hypofibrinolytic activity (Dunn *et al.*, 2006; Geiger & Binder, 1991), enhanced platelet activity (Vinik, Erbas, Park, Nolan, & and Pittenger, 2001), the presence of oxidative stress (Yamagishi, Fujimori, Yonekura, Yamamoto, & Yamamoto, 1998), the presence of insulin resistance (Takanashi & Inukai, 2000) and glycation of

proteins that are involved in coagulation, including fibrinogen (Brownlee, Vlassara, & Cerami, 1983; Dunn, Ariens, & Grant, 2005; Dunn *et al.*, 2006). Each of these factors may contribute both individually and in combination with other factors to the observed increase in CVD risk in diabetic patients. Black South Africans face an additional factor, the prevalence of high fibrinogen levels in the general population (Pieters & Vorster, 2008), and fibrinogen itself has been recognised as an independent CVD risk marker (Dunn & Grant, 2005).

This thesis focuses particularly on the possible role of fibrinogen glycation on fibrin network structure. It is suspected that the increased CVD risk in those with diabetes may, in part, be due to alterations in fibrin network structure (Jorneskog, Egberg, Fagrell, Fatah, Hessel, Johnsson, Brismar, & Blomback, 1996; Nair, Azhar, Wilson, & Dhall, 1991). Fibrinogen as the substrate for fibrin formation, plays an important role in clot formation. The removal of fibrinopeptides A and B by the enzyme thrombin from the fibrinogen molecule leads to formation of fibrin monomers which then polymerise in a middle to end staggered fashion, forming double stranded proto-fibrils (Mosesson, 1998). These proto-fibrils then aggregate laterally to form fibres of varying thicknesses and branching densities depending on the polymerisation conditions that prevail, finally producing a fibrin network that forms the scaffold that supports blood clots (Weisel, Veklich, & Gorkun, 1993). While fibrinogen as a glycoprotein, consists of four clusters of carbohydrate (Weisel, 2005), continued exposure to hyperglycaemia, in diabetes, has been reported to result in non-enzymatic addition of further glucose units to fibrinogen. The fibrinogen glycation likely takes place at lysine residues on the proto-fibrils (Brownlee *et al.*, 1983; Lütjens, te Velde, vd Veen, & vd, 1985). The non-enzymatic fibrinogen glycation is suspected to be one of the mechanisms contributing to alterations in fibrin network structure in diabetes (Jorneskog *et al.*, 1996; Nair *et al.*, 1991).

Several fibrin network structure variables have been investigated in this study in association with the possible effects of non-enzymatic fibrinogen glycation on the functional structure of fibrin in diabetes. The variables that were investigated in this study include **permeability** of the fibrin networks which gives an indication of the porosity of a network; **compaction** which measures the volume of fluid released

when a fibrin network collapses under a specific centrifugal force and is an indication of clot rigidity; and **terbidimetric analysis** including lag-time, slope and maximum absorbance which are an indication of the kinetics of clot formation under given conditions. **Lag-time** gives the time taken for fibrin proto-fibrils to reach a sufficient length for lateral aggregation to take place and may also give an indication of the rate of proto-fibril formation; **slope** gives the rate of lateral aggregation during the clotting process and **maximum absorbance** gives an indication of the average fibre size of fibrin fibres.

Nair *et al.* (1991) reported reduced permeability and compaction of fibrin fibres prepared from diabetic plasma indicating reduced porosity and increased clot rigidity, respectively, in diabetic patients compared to non-diabetic control subjects. They also reported fibre thickness in uncontrolled diabetic patients to be reduced. Jorneskog *et al.* (1996) working with type 1 diabetes also reported reduced permeability in the diabetic patients in comparison to non-diabetic controls and in an intervention study (Jorneskog, Hansson, Wallen, Yngen, & Blomback, 2003) involving continuous subcutaneous infusion with insulin, they reported that the permeability improved as a result of the intervention. Because both Nair *et al.* (1991) and Jorneskog *et al.* (2003) worked with fibrin networks developed in plasma, the influence of other plasma constituents on their results cannot be ruled out. In addition, neither group measured the levels of fibrinogen glycation involved in their studies. More recently, Dunn *et al.* (2005) carried out investigations using fibrinogen isolated from patients with type 2 diabetes. They too, reported reduced permeability of the fibrin networks compared to fibrin from non-diabetic subjects. In addition they reported an increased rate of proto-fibril formation, a higher maximum absorbance (an indication of fibre size), and higher fibre density and number of branch points in the patients with type 2 diabetes compared to control subjects. While by using purified fibrinogen, Dunn *et al.* (2005) excluded the influence of other plasma constituents, they too, did not measure the level of fibrinogen glycation in their study.

While some work has been done on fibrin network structure and diabetes, no intervention study has been done with type 2 diabetes that has investigated fibrinogen glycation and possible alterations in fibrin network structure

characteristics in association with glycaemic control. Thus the following have never been investigated:

a. The relationship between fibrinogen glycation and fibrin network structure.

b. Whether Intervention to bring about glycaemic control would significantly reduce fibrinogen glycation and whether this in turn would result in significant alterations of the fibrin network structures formed.

Of further significance is that this study was done with type 2 diabetic patients on an out-patient basis allowing for the results of the study to have relevance to the general public.

1.2 Research questions and objectives

The objectives of this study were to:

- determine whether there exists a difference in the fasting plasma fibrinogen levels between black South African uncontrolled type 2 diabetic and non-diabetic volunteers

- determine whether there exists a difference in the glycation of fibrinogen between black South African uncontrolled type 2 diabetic and non-diabetic volunteers

- determine whether there exists a difference in the selected fibrin network structure variables between black South African uncontrolled type 2 diabetic and non-diabetic volunteers

- determine whether blood glucose (glycaemic) control intervention, by means of insulin treatment on an out-patient basis, will result in any changes to

levels of glycation of fibrinogen in Black South Africans with uncontrolled type 2 diabetes

- determine whether glycaemic control intervention, by means of insulin treatment, will result in any changes in fibrin network structure characteristics in Black South Africans with uncontrolled type 2 diabetes

The selected fibrin network structure variables to be investigated are permeability, compaction, lag-time, slope and maximum absorbance.

By measuring these variables using a plasma model as well as a purified fibrinogen model, the individual effect of different levels of fibrinogen glycation on the variables could be determined. While the plasma model would present the effect of fibrinogen glycation in the presence of other plasma constituents, the purified fibrinogen model would present the influence of fibrinogen glycation in the absence of the plasma constituents. Figure 1.1 is a schematic diagram representing the two models used.

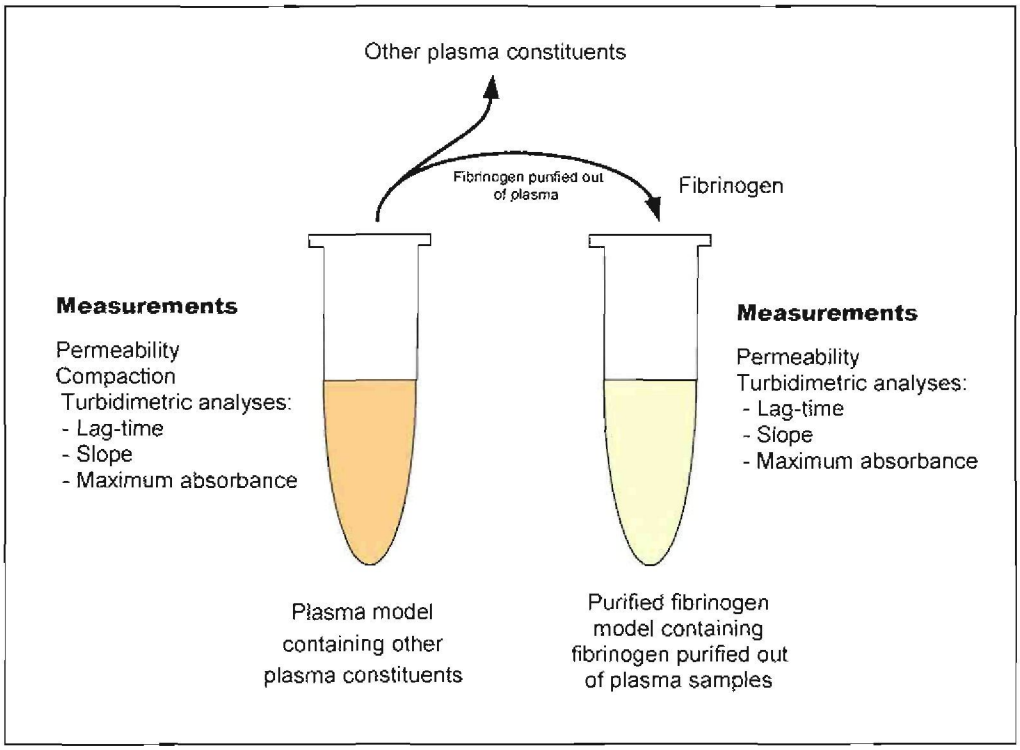

Figure 1.1. Schematic diagram of the model systems used in this study

Table 1.1 shows the level of involvement of the student in this project.

Table 1.1. Level of involvement of the student in the project

Name	**Role**	**Involvement**
Namukolo Covic	PhD student	– Protocol writing – Recruitment of volunteers – Preparation of blood samples for analysis and storage – Plasma fibrinogen analysis – Permeability analysis – Compaction analysis – Turbidimetric analysis – Statistical analysis
Dr. Marlien Pieters	Promoter	– Providing guidance to the student at all stages of the project (protocol writing, planning, and execution of the intervention, sample analysis, data analysis, statistics and report writing) – Fibrinogen purification – Fibrinogen glycation analysis
Prof. Johann Jerling	Co-promoter	– Providing guidance to the student at all stages of the project (protocol writing, planning, execution of the intervention and report writing)
Dr. Dannie van Zyl	Specialist physician	– Recruitment of volunteers – Administration of intervention – Preparation of blood samples for analysis and storage
Prof. Paul Rheader	Specialist physician	– Recruitment of volunteers – Administration of intervention – Preparation of blood samples for analysis and storage

1.3 Structure of the Thesis

Chapter 1 gives background information for the thesis to help put the research topic into perspective. It also includes the objectives of the study and the structure of the thesis.

Chapter 2 is the literature review section of this thesis where a review of the literature is given, in order to provide background information on fibrin network structure and diabetes. In addition to presenting diabetes as a public health concern this chapter gives information on how diabetes is thought to increase CVD risk for those who suffer from it. Background information on fibrinogen and fibrin network structure is also given as well as ways in which diabetes may influence both fibrinogen and the fibrin network structure prepared from it.

Chapter 3 describes how the research data was collected, including details on the selection and exclusion criteria for the volunteers who were recruited for the study. The study design and the process followed for the collection of blood samples are described and the analytical methods used for purifying the fibrinogen and investigating the fibrin network structure variables, as well as, the statistical analyses done on the data are also given.

Chapter 4 presents the baseline characteristics of the study population and the results that were generated from the study. These are presented in tables and figures either as means or medians (25^{th}, 75^{th} percentile) of the data. The changes that may have taken place from baseline to end as a result of the intervention are also presented. The tables and figures in the results section are presented at the end of the section (Section 4.13) and not in the order in which they are cited in the text. This format has been followed in order to present the tables and figures in a way that allows for easier reading due to the nature of the data presented.

Chapter 5 discusses the results generated from this study and compares them with reported results from the literature where this was available. Possible interpretations and explanations for the results are also given.

Chapter 6 gives conclusions of what the effects of glycaemic control on the fibrin network structure variables studied were and gives suggestions for possible further research to help explain further what these effects are.

The **bibliography** has been included ahead of the **annexure** in order to facilitate more logical page numbering because the annexure includes copies of two papers that have been published based on the work done on this study.

Chapter 2: Literature review

2.1 Introduction

Diabetes is a major public health concern. It has been projected that the total number of people with diabetes worldwide would increase from 171 million in 2000 to 366 million by 2030 and for Sub-Saharan Africa, it was estimated that the number of people with diabetes would increase by one hundred and sixty one percent by 2030 (Wild *et al.*, 2004). For South Africa, the increase was projected to go from 814 thousand to 1.3 million by 2030 (WHO, 2007). The Statistics South Africa report on causes of death from 1997 to 2001 lists diabetes among the eight leading causes of death among adults and the fifth for women over fifty years of age (Statistics South Africa, 2002).

Diabetic patients have been shown to face a two to fourfold increase in risk of developing cardiovascular disease (CVD) (Stamler *et al.*, 1993; Wei *et al.*, 1998). The increase in risk of developing CVD in diabetes has been shown to be multifaceted. Some of the reasons put forward include, an increase in plasma levels of fibrinogen and other pro-coagulant factors (Saito *et al.*, 2000), inadequate fibrinolytic activity (Aso, Matsumoto, Fujiwara, Tayama, Inukai, & Takemura, 2002; Barazzoni *et al.*, 2000; Geiger & Binder, 1991), insulin resistance (Ceriello & Motz, 2004; Takanashi & Inukai, 2000), and increased plasma levels of advanced glycation end products due to oxidative stress (Ceriello *et al.*, 1995). Hyperlipidemia has also been considered to be a key factor in the development of diabetic complications (Gugliucci, 2000).

Apart from these, the fibrin network structures formed in diabetes have been shown to be altered (Dunn *et al.*, 2005; Jorneskog *et al.*, 1996). Some of these alterations have been considered as possible mechanisms involved in the increased CVD risk faced by diabetic subjects, in that the clots formed may be more resistant to fibrinolysis (Dunn *et al.*, 2005; Jorneskog *et al.*, 1996).

This chapter reviews the literature indicating a connection between diabetes and aspects of CVD risk. In particular it looks at some haemostatic factors, particularly fibrinogen, as well as the changes that seem to take place in fibrin network structure in diabetes as a result of hyperglycaemia.

2.2 Diabetes and CVD risk

2.2.1. Influence of diabetes on morbidity and mortality

The main cause of mortality and morbidity among type 2 diabetic patients is CVD (Kannel & McGee, 1979). Myocardial infarction is an attribute of the increased mortality rate in diabetic subjects and it is estimated that the risk of death due to myocardial infarction in diabetic patients is one and half to double that of its non-diabetic counterparts (Aronson, Raffield, & Chessebro, 1997). The American National Institute of Health Report on national estimates on diabetes (Bathesda, 2005), indicated that heart disease and stroke accounted for sixty five percent of the deaths in people with diabetes and that adults with diabetes had a two to four times higher rate of death from heart disease than those without diabetes. Similar statistics on the African continent are not readily available. As part of the Framingham study, Kannel *et al.* (1990) reported that type 2 diabetes predisposed subjects to all 408 major cardiovascular disease outcomes considered in the study. In trying to establish reasons why diabetes confers such a high risk of CVD to those who suffer from it, researchers have sought to find answers from different directions including, hyperglycaemia (Dunn *et al.*, 2005; Jorneskog *et al.*, 1996), hyperlipidaemia (Ceriello, 2003; Middelbeek & Horton, 2007), hypercoagulability (Ceriello, Giugliano, Quatraro, Dello, Marchi, & Torella, 1989) (Takanashi & Inukai, 2000), hypofibrinolytic activity (Geiger & Binder, 1991), platelet activity (Vinik *et al.*, 2001), oxidative stress (Yamagishi *et al.*, 1998), insulin resistance (Takanashi & Inukai, 2000) and glycation of proteins (Brownlee *et al.*, 1983; Dunn *et al.*, 2005).

Many studies have shown that diabetes is a hypercoagulable state (Carr, 2001; Gugliucci & Ghitescu, 2002). This would be in line with why individuals with diabetes would have such high CVD mortality and morbidity rates. When young men were infused with glucose to maintain glucose levels at 11.1 mmol/l, the induced

hyperglycaemia was reported to increase activation of the tissue factor pathway of blood coagulation based on increases in plasma levels of Factor VIIa, factor VIIc and tissue factor pathway inhibitor (Rao, Chouhan, Chen, Sun, & and Boden, 1999). Markers of a hypercoagulable state have been shown to be elevated in diabetes, including elevated plasma levels of fibrinogen (Barazzoni *et al.*, 2000; Ganda & Arkin, 1992; Kannel *et al.*, 1990) and thrombin (Ceriello *et al.*, 1995). In addition, elevated plasminogen activator inhibitor-1 (PAI-1) (Collier, Rumley, Paterson, Leach, Lowe, & and Small, 1992a; Folsom, Wu, Conlan, Finch, Davis, Marcucci, Sorlie, & Szklo, 1992), results in hypofibrinolysis which further contributes to the hypercoagulable state. The hypercoagulable state in type 2 diabetes will be discussed in greater detail in section 2.3.

Both clinical and epidemiological studies have shown that hyperglycaemia, itself is an independent CVD risk factor (Middelbeek & Horton, 2007). Meigs *et al.* (2002) was able to show that 2 hour post-challenge hyperglycaemia was an independent risk factor for CVD in the Framingham study. Gresele *et al.* (2003), observed an increased platelet activation, *in vivo* and *in vitro*, as a result of acute short-term hyperglycaemia in type 2 diabetic patients. They suggested that acute short-term hyperglycaemia, by facilitating platelet activation, may play a role in bringing about vascular occlusions. Even among non-diabetic people, hyperglycaemia has been shown to be an independent CVD risk. A ten-year follow-up study involving non-diabetic individuals, reported baseline impaired glucose tolerance to be an independent CVD risk factor in people who did not progress to diabetes during the follow-up period (Qiao, Jousilahti, & Tuomilehto, 2003). A meta-analysis of non-diabetic reference groups from 38 prospective studies, in which CVD incidence or mortality were used as endpoints, reported that people with the highest post-challenge blood glucose levels (8.3 to 10.8 mmol/l) showed a 27 percent higher risk of CVD than people who had the lowest glucose levels (3.3-5.9 mmol/l) (Levitan, Song, Ford, & Liu, 2004). More recently, Barr *et al.* (2007) from the Australian Diabetes Obesity and Lifestyle Study, involving 10 428 participants, over a five year follow-up period, did not find impaired glucose tolerance to be an independent CVD risk factor but reported both *Diabetes mellitus* and impaired fasting glucose to be independent predictors of death from CVD, after adjusting for age, sex and other traditional CVD risk factors. A study aimed at optimising the identification of future

diabetic patients (Rijkelijkhuizen, Nijpels, Heine, Bouter, Stehouwer, & Dekker, 2007), investigated the effect of lowering the American Diabetes Association cut-off point for impaired fasting glucose from 6.1 mmol/l to 5.6 mmol/l. Over a follow-up period from 1996 to 2005, they reported that subjects with impaired fasting glucose of 6.1 mmol/l had a higher risk of CVD than those with normal fasting glucose (<5.6 mmol/l), while those with impaired fasting glucose of 5.6 mmol/l did not differ in CVD risk from those with normal fasting glucose.

The hyperglycaemia in the diabetic condition contributes changes to circulating plasma proteins that can alter the manner in which these proteins function. The effects of hyperglycaemia are important factors of discussion in this section because many proteins with a haemostatic function are affected. Austin *et al.* (1987), reported that glycation of plasma proteins was much greater in diabetic plasma than non-diabetic plasma. Since the structure of a protein is associated with its function one would, therefore, expect altered functionality for glycated proteins. Geiger and Binder (1986) reported that control plasminogen incorporated 14C-glucose into its structure in a dose dependent manner. They also reported that the *in vitro* glycation of control plasminogen resulted in functional abnormalities of the plasminogen similar to those reported for plasminogen obtained from diabetic patients. Plasminogen is the precursor for plasmin which brings about fibrin degradation. An alteration in the structure of this protein leading to functional changes may, therefore, have haemostatic implications in the diabetic subjects.

Several studies have shown changes in functional outcomes of glycated proteins such as fibrinogen (Bobbink, Tekelenburg, Sixma, de Boer, Banga, & de Groot, 1997; Brownlee, Vlassara, & Cerami, 1984; Brownlee *et al.*, 1983; Geiger & Binder, 1986). Hatton (1993) in a study involving rabbit fibrinogen, reported that glycated fibrinogen was preferentially distributed in the extra cellular compartment while unglycated fibrinogen was found preferentially in the intracellular compartment. They concluded that the increased uptake of glycated fibrinogen into vessel walls might contribute towards the greater risk of atherosclerotic disease progression that has been associated with poor glycaemic control.

2.2.2 Influence of hyperglycaemia on plasma lipids

Abnormalities of the lipid profile of type 2 diabetic patients is an important factor that contributes to increased CVD risk (Battisti, Palmisano, & Keane, 2003). Bruckert *et al.* (2007), based on data from the Pan-European Survey, reported low high density lipoprotein-cholesterol (HDL-C) to be common among European, type 2 diabetes patients and the Thailand Diabetes Registry Project reported dyslipidaemia in eighty percent of the patients in a cross sectional study involving 9 419 diabetic patients (Pratipanawatr, Chetthakul, Bunnang, Ngarmukos, Benjasuratwong, Leelawatana, Kosachunhaun, Plengvidhya, Deerochanawong, Suwanwalaikorn, Krittiyawong, Mongkolsomlit, & Komoltri, 2006). The lipid profile of individuals with type 1 diabetes and type 2 diabetes is not the same. Type 1 diabetic patients usually have normal HDL-C and low density lipoprotein-cholesterol (LDL-C) accompanied by high triglycerides levels (O'Brien, Nguyen, & Zimmerrman, 1998). Those with type 2 diabetes, on the other hand, tend to have reduced HDL-C, high triglycerides and a tendency for normal LDL-C with a shift in particle size of the LDL-C fraction toward more small, dense LDL-C particles (Farmer, 2007; Pratipanawatr *et al.*, 2006). Studies have shown that the decreased HDL-C, high LDL-C and triglycerides increase the risk of CVD in type 2 diabetic patients. The Asia-Pacific Cohort Studies Collaboration (Asia-Pacific Cohort Studies Collaboration, 2007) analysed data from thirty studies in the Asia-Pacific region and reported total cholesterol to be positively correlated with coronary heart disease and ischemic stroke for both those with and without diabetes. A large study of available data sets from, the Framingham Cohort Study, the Framingham Offspring Study, The Lipid Research Clinics Prevalence Follow-up Study and the Multiple Risk Factors Intervention Trials Usual Care Group, was used to assess the role of non-HDL-C and LDL-C, together, in predicting coronary heart disease (CVD) death (Reaven, 2002), among people with diabetes (Liu, Sempos, Donahue, Dorn, Trevisan, & Grundy, 2005). Non HDL-C is calculated by subtracting HDL-C from total cholesterol (Havel & Frost, 2001). The study by Liu *et al.* (2005) reported non-HDL-C to be stronger at predicting death from CHD among diabetic patients than LDL-C. Havel and Frost (2001) recommended the use of non-HDL-C in hypertriglyceridemic patients as a CVD risk marker and for evaluating cholesterol-lowering treatment effectiveness. The major apo-protein in chylomicrons, very low density lipoproteins-cholesterol (VLDL-C), intermediate

density lipoprotein-C (IDL-C) and LDL-C is apo-protein B (apo B) and there is one apo B molecule per LDL-C and VLDL-C particle making apo B useful for estimating particle numbers of these lipid fractions (Rader, Hoeg, & Brewer, 1994). Since both LDL-C and VLDL-C are artherogenic, an estimation of apo B gives a combined effect of these two fractions and provides a useful way of estimating artherogenic cholesterol risk (Rader *et al.*, 1994). Although LDL-C has been used as the main target in the treatment of dyslipidaemia, the Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Expert Panel on Detection, 2001) reported non HDL-C and apo B to have been shown to predict CVD events better (Bittner, Hardison, Kelsey, Weiner, Jacobs, & Sopko, 2002; Cui, Blumenthlaal, Flaws, Whiteman, Langenberg, & Bachorik, 2001; Expert Panel on Detection, 2001). Non HDL-C and apo B were reported to be the same, as risk makers of CVD, in hypertriglyceridemic type 2 diabetic patients but apo B was able to identify additional high risk candidates among type 2 diabetic patients with normal triglyceride levels (Wagner, Pérez, Zapico, & Ordónez-Llanos, 2003).

2.2.3 Advanced glycation end products and insulin resistance

Hyperglycaemia has also been associated with increased levels of advanced glycation end products (AGEs) in diabetes due to oxidative stress in the condition (Brownlee, 2005). Yamagishi (1998) suggested that AGEs may have the ability to cause platelet aggregation and fibrin stabilization and Enomoto *et al.* (2006) were able to demonstrate a positive association between fibrinogen and PAI-1 with serum AGEs Levels pointing to a possibility of AGEs being in some way associated with thrombogenesis in humans.

Ceriello and Motz (2004) extensively reviewed research work that indicated that oxidative stress was the common, persistent pathogenic factor mediating the appearance of insulin resistance as well as the passage from insulin resistance to overt diabetes, via impaired glucose tolerance. High plasma insulin may contribute to some of the prothrombotic problems experienced in diabetes. Around eighty to ninety percent of subjects with type 2 diabetes are reported to have insulin resistance (Dunn & Grant, 2005). Insulin resistance is characterized by elevated levels of insulin in the blood. Both insulin resistance and type 2 diabetes have been

associated with the development of endothelial dysfunction (Brownlee, 2005) and enhanced platelet aggregation and activation (Vinik *et al.*, 2001; Yamagishi *et al.*, 1998). The endothelium plays a role in maintaining haemostatic balance because it produces inhibitors of blood coagulation and platelet aggregation (Mosesson, 2005). It is also involved in modulating vessel tone and plays an important role in preventing contact between haemostatic blood components and reactive sub-endothelial structures that promote coagulation when there is vessel damage (Colman, Clows, George, Hirsh, & Marder, 2001). A connection has now been established between insulin resistance and increased free fatty acids in-flow into cells, leading to oxidative stress, through overproduction of reactive oxygen species (Brownlee, 2005). In addition, the following prothrombotic markers have also been shown to increase in association with insulin resistance, plasma fibrinogen, Von Willebrand factor and the anti fibrinolytic factor PAI-1 (Brownlee, 2005). As part of the Insulin Resistance Atherosclerosis Study, Haffner (1999) came to the conclusion that insulin-resistant type 2 diabetic subjects had a more atherogenic cardiovascular risk factor profile than insulin-sensitive type 2 diabetic subjects and that this was only partially related to increased obesity and an adverse body fat distribution.

The connection between diabetes and CVD risk is further confirmed by the fact that intensive diabetes therapy has been shown to have long-term beneficial effects by reducing the risk of developing CVD. As part of the Diabetes Control and Complications Trial (Nathan, Cleary, Backlund, Genuth, Lachin, Orchard, Raskin, & Zinman, 2005), during the mean 17 years of follow-up, a forty two percent decrease in any CVD event and a fifty percent decrease in risk of non-fatal myocardial infarction, stroke, or death from CVD was reported in type 1 diabetes. In this study, Intensive diabetic therapy was also reported to reduce the risk of developing retinopathy by seventy six percent.

The proteins involved in coagulation and fibrinolytic processes do not act independently of each other and work in concert to bring about haemostatic balance in the body. When the level of any of these factors changes, it invariably can affect different stages in these processes, bringing about an imbalance that becomes manifested in some anomaly or other. Some of these haemostatic factors and how they seem to contribute to CVD risk are discussed in the next section.

2.3 Haemostasis and its role in CVD risk

There are many haemostatic variables that play a role in the diabetic condition, some of which have been alluded to above. These factors include fibrinogen, thrombin, plasminogen, PAI-1, tissue plasmin activator (t-PA) and a variety of other molecules that are involved in, or in one way or another, influence the coagulation or fibrinolytic processes (Carr, 2001). The following haemostatic CVD risk markers have been shown by some researchers to be associated with diabetes: increased fibrinogen levels (Barazzoni *et al.*, 2000; Ceriello, Taboga, Falleti, De Stasio, Motz, Lizzio, Gonano, & and Bartoli, 1994; Festa, D'Agostino, Jr., Mykkanen, Tracy, Zaccaro, Hales, & Haffner, 1999; Schalkwijk, Poland, van Dijk, & et.al, 1999), increased PAI-1 (Collier *et al.*, 1992a), increased thrombin generation (Ford, Singh, Kitchen, Makris, Ward, & Preston, 1991) and reduced plasmin generation (Dunn *et al.*, 2006). In addition, the fibrin network structure may be altered in such a way that the rate of fibrinolysis may be reduced (Dunn *et al.*, 2005; Jorneskog *et al.*, 2003). Fibrin network structure and diabetes will be discussed in more detail in section 2.5.

Thrombin plays a critical role in haemostasis. It removes fibrinopeptides A and B from the fibrinogen molecule to form fibrin monomers that subsequently aggregate, forming the fibrin network structure (Weisel *et al.*, 1993). Plasma levels of fibrinopeptide A can, therefore, be used as an indicator of thrombin activity. Ceriello *et al.* (1995) showed that hyperglycaemia may induce thrombin formation. Ceriello *et al.* (1989) were also able to demonstrate a direct role for hyperglycaemia as a stimulus for thrombin activation. They observed an increase in fibrinopeptide A concentration parallel to sustained, induced hyperglycaemia, in healthy individuals. The authors also reported that when blood glucose levels returned to normal fibrinopeptide A values reacted in kind. The study also reported that even mild hyperglycaemia, induced by glucagon infusion, resulted in significant increases in fibrinopeptide A levels. This would indicate an enhancement of coagulation activity, *in vivo,* by glycaemia, by possibly accelerating the rate at which fibrinogen would be converted to fibrin monomers in the coagulation process. Ford *et al.* (1991) used fibrinopeptide A as an indicator of thrombin formation. They reported fibrinopeptide

A to be higher in both type 1 and type 2 diabetic subjects, with or without complications, when compared to controls. This would indicate that even those without complications would already have been in a form of hypercoagulable state, which could put them at increased CVD risk. Wolberg *et al.* (2003) showed that elevated prothrombin levels, the precursor of thrombin, lead to the formation of clots with reduced mass-to-length ratios compared to normal clots. These types of clots may be more resistant to fibrinolysis.

Fibrinogen is a substrate for thrombin in the clotting cascade. It is widely accepted that fibrinogen is strongly, consistently and independently related to CVD (Koenig, 2003) and has been described as a powerful independent risk marker for CVD in the general population (Dunn & Grant, 2005). A large meta-analysis study by Danesh *et al.* (2005) reported a moderately strong association between apparently healthy plasma fibrinogen levels and coronary heart disease, stroke and other vascular mortality in a wide range of circumstances in healthy middle aged adults. Data that describe the possible impact of diabetes on plasma fibrinogen levels are inconsistent. Increased plasma fibrinogen levels have been observed by researchers in type 1 diabetes (Ceriello *et al.*, 1994; Schalkwijk *et al.*, 1999) and type 2 diabetes (Barazzoni *et al.*, 2000; Festa *et al.*, 1999). Some have reported similar levels of fibrinogen between the controls in both type 1 (Jorneskog *et al.*, 1996; Majkowska, Mamos, Fuchs, Pynka, Jastrzebska, Krzyzanowska, & Czekalski, 1994) and type 2 diabetic patients (Missov, Stolk, van der Bom, Hofman, Bots, Pols, & Grobbee, 1996). However, the available evidence for an association between type 2 diabetes and elevated fibrinogen levels is quite strong. Barazzoni *et al.* (2000), reported increased fibrinogen production, *in vivo*, in type 2 diabetic patients with normoalbuminuria and without complications. Donders *et al.* (1993) also reported elevated levels of fibrinogen as well as fibrin monomers, thrombin-antithrombin III complex and factor VIIIc in diabetic patients, indicating an activated coagulation system in the group. Asakawa *et al.* (2000) reported elevated levels of fibrinogen in patients with type 2 diabetic subjects, with the levels being significantly higher in those with complications than those without. The duration of diabetes was much longer in patients with than without complications. Ford *et al.* (1991) reported elevated levels of fibrinogen in both type 1 and type 2 diabetic patients but with no significant differences between those with and without complications. Ganda and

Arkin (1992) also reported elevated fibrinogen levels in both type 1 and type 2 diabetic subjects but that of the type 2 diabetic subjects were disproportionately elevated. As part of the Framingham Study (Kannel *et al.*, 1990), which looked at the influence of fibrinogen on the risk of CVD, over a 16 year follow-up, a rise in fibrinogen levels throughout the range of blood sugar levels was observed.

The elevation of fibrinogen in diabetes is probably affected by many variables such as genetic predisposition and many metabolic factors such as those associated with insulin resistance and oxidative stress. Since elevated fibrinogen levels in the general population have been associated with increased CVD risk (Fatah, Silveira, Tornvall, Karpe, Blomback, & Hamsten, 1996; Koenig, 2003) diabetic subjects who exhibit elevated fibrinogen levels are also at increased risk of CVD. Increased production of fibrinogen in type 2 diabetes may, therefore, be a contributing factor to increased CVD risk in patients who exhibit elevated fibrinogen levels. The observed enhancement of coagulation activity in type 2 diabetes already mentioned above may also lead to increased fibrinogen production and removal. It should also be kept in mind that fibrinogen is an acute phase protein (Colley, Fleck, Goode, Muller, & Myers, 1983; Festa, D'Agastino, Tracy, & Haffner, 2002), therefore, type 2 diabetic patients with micro or macro-vascular complications may exhibit increased levels of fibrinogen due, in part, to inflammatory responses.

In addition to a hypercoagulable state diabetes also presents a hypofibrinolytic state. A balance between fibrin formation and fibrinolysis maintains the blood in a fluid state, so that clots are formed when required to prevent blood loss but once the healing process is on its way, the dissolution of the clot would take place (Weisel, 2005). Plasmin breaks down fibrin. Plasminogen, the zymogen from which plasmin is formed, is bound to fibrin during its formation along with t-PA via their respective fibrin binding sites (Carr, 2001). This allows for the conversion of plasminogen to plasmin to occur on the fibrin surface. Plasmin then breaks down fibrin, releasing fibrin degradation products. Plasmin generation through this process has been shown to be decreased in diabetic subjects (Dunn *et al.*, 2006). Plasminogen activator inhibitor-1 (PAI-1) which inhibits t-PA has been shown to increase in type 2 diabetic subjects (Collier *et al.*, 1992a), pointing toward a possible decrease in fibrinolytic activity in this group. Reduced fibrinolysis may lead to persistence of

clots that are formed in the cardiovascular system and this may in turn lead to an increased risk of thrombotic events in diabetic patients.

Some researchers have suggested that both fibrinolytic and coagulation activities are enhanced in type 2 diabetes (Aso *et al.*, 2002; Conti, Marongui, Mameli, Mamusa, Cambuli, Cossu, Sorano, Biondi, Cirillo, & Balestrieri, 1989), but that the increase in fibrinolytic activity does not seem to compensate adequately for the increased coagulation. Aso *et al.* (2002) reported that the increase in fibrinolytic activity was less in obese diabetic patients than in lean patients. Although Conti *et al.* (1989) reported an inadequate compensation of the fibrinolytic system for the increase in coagulation, they did not find any correlations between glycaemic control and fibrinopeptide A and B release, or HbA1c, as would be expected. Avellone *et al.* (1994), based on differing plasma levels of a number of clotting/fibrinolytic factors, including, fibrinogen, plasminogen, pre and post venous occlusion PAI-1 and pre and post occlusion t-PA levels, demonstrated an impairment of haemostatic and fibrinolytic mechanisms which they felt may play a key role in the pathogenesis of atherosclerotic vascular complications in obesity and type 2 diabetes. Majkowska *et al.* (1994), on the other hand, did not find that glycaemic control influenced fibrinogen or antithrombin-III levels. In the same study they, however, reported that PAI-1 activity was diminished in relation to hyperglycaemia. After reviewing work done on PAI-1 in association with type 2 diabetes and CVD risk, Sobel (2002) hypothesized that PAI-1 can create conditions favourable for the formation of unstable, lipid-laden atherosclerotic plaques, making people with diabetes highly susceptible to rupture of vulnerable plaques and acute coronary syndromes. The studies listed here point toward a reduced level of fibrinolysis being associated with diabetes. This may lead to the persistence of blood clots in the cardiovascular system once formed.

The diabetic condition can, therefore, be affected by many other factors present in plasma besides the hyperglycaemia itself. Many studies have shown changes in levels of both coagulation factors and fibrinolytic factors in plasma of diabetic subjects and there have also been significant associations between some of these factors and conditions like insulin resistance and dyslipidemia (Ceriello & Motz, 2004), all conditions that are associated with diabetes and which are also highly

associated with CVD risk. Plasma can, therefore, have varying levels of the different haemostatic components that can lead to different outcomes for the diabetic subject, depending also on the patho-physiological condition and genetic predisposition the person happens to be in. Patho-physiological conditions such as insulin resistance, oxidative stress and metabolic syndrome may enhance the expression of certain outcomes because of their close association with diabetes and CVD risk. These outcomes vary from changes in levels of the haemostatic factors in plasma to a modification of the molecular structure by glycation or other related processes.

The molecule fibrinogen is central to the fibrin network structure, which forms the scaffold of blood clots. A closer look at this molecule and the changes in functionality that may arise from the diabetic condition is, therefore, useful to be able to understand how diabetes may affect the functionality of the fibrin networks that are formed.

2.4 Fibrinogen and fibrin network structure

2.4.1 Molecular structure of fibrinogen

Fibrinogen, as the glycoprotein molecule from which fibrin monomers are formed, is a critical molecule for fibrin network formation. Fibrinogen is an elongated molecule about 45nm long and has three nodular regions which are globular in nature, one in the middle and one at each end (Weisel, 2005). Figure 2.1 is a diagram of a fibrinogen molecule (Pathology Online, 2007). The middle region is called the E region and is joined to the two distal D regions by α helical coiled-coil structures of the constituent polypeptide chains (Weisel, 2005). The molecule is made up of a total of 6 polypeptide chains which form two identical subsets of three chains each (Blomback, Hessel, & Hogg, 1976; Henschen, Lottspeich, & Kehl, 1983; Hoeprich & Doolittle, 1983). The three chains in each subunit are named Aα, Bβ and γ chains and are joined in the central E region by disulfide bridges (Hoeprich & Doolittle, 1983), one between the two Aα chains and two between the two γ chains (Weisel, 2005). The E-region contains the N-termini of all the 6 polypeptide chains and each

of the three chains in each subunit intertwine to form the α-helical coiled-coils joining the E and D regions (Weisel, 2005).

The shorter Bβ and γ chains terminate in the D regions but the longer Aα chains extend from the D regions back towards the E-region where they interact with one another (Veklich, Gorkun, Medved, Nieuwenhuizen, & Weisel, 1993). The Aα chains terminate in the αC domain. The primary structure of fibrinogen polypeptide chains is composed of 610, 461 and 411 amino acid residues for the Aα, Bβ and γ chains, respectively (Henschen *et al.*, 1983), resulting in a molecule that contains 2 964 amino acids and a relative molecular mass of 329 818 (Standeven, Ariens, & Grant, 2005). There are four clusters of carbohydrate, one on each of the Bβ and γ chains as a result of which the total relative molecular mass of the fibrinogen molecule is 340 000 (Weisel, 2005). The clusters of carbohydrate make fibrinogen a glycoprotein.

This general structure of the fibrinogen molecule is related to its function in that the molecule, as described above, is soluble making it easily transported in plasma. The molecule also has several binding sites for molecules involved in coagulation and fibrinolysis thereby facilitating both processes (Mosesson, 2005). It also has complementary binding sites in the E and D domains that are used in the polymerisation process (Mosesson, 2005). When a short sequence of amino acids is removed from the N-terminal of the Aα and Bβ chains, the specific polymerisation binding sites on the fibrinogen molecule are exposed, leading to the polymerisation of the resulting fibrin monomers (Standeven *et al.*, 2005). The short sequences of amino acids removed are called fibrinopeptides A and B respectively, and are shown in Fig. 2.1.

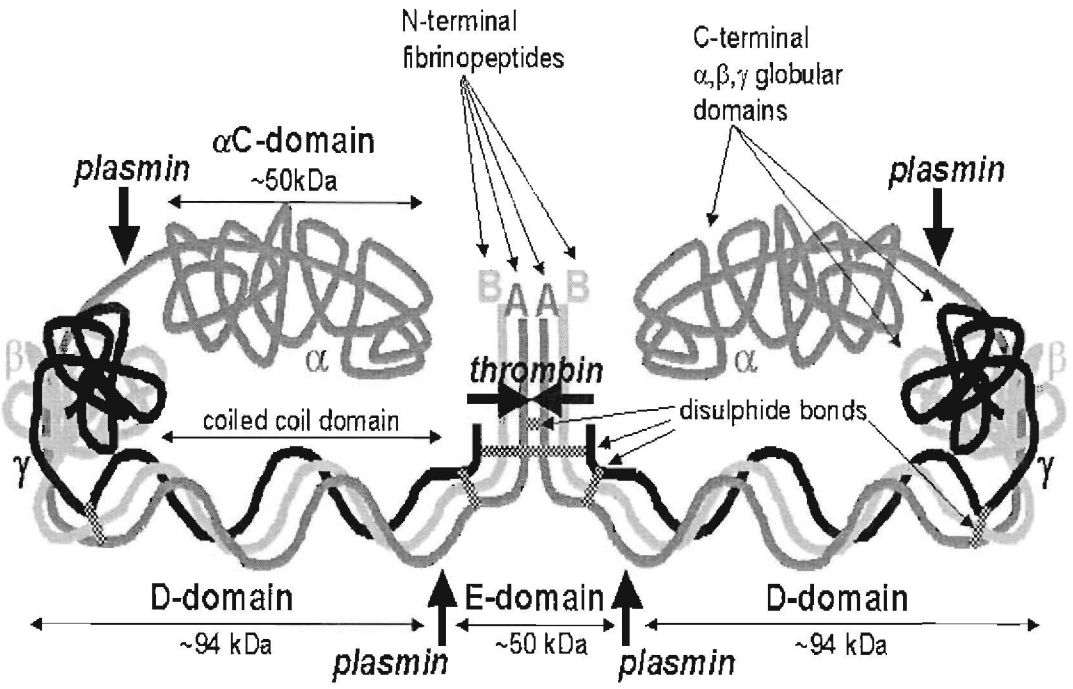

Figure 2.1. A diagram of the fibrinogen molecule showing the elongated nature of the molecule and the Aα, Bβ and γ polypeptide chains that the molecule is made up of (Pathology Outlines, 2007).

The removal of fibrinopeptide A from the N-terminal of the Aα chains in the E region exposes binding sites where adjacent fibrin monomers can bind to complementary binding sites in the D regions of the adjacent fibrin monomers (Mosesson, 1998). This results in a double stranded proto-fibril in which there is a middle-to-end staggered arrangement (Fig. 2.2). Fibrinopeptide B is cleaved more slowly and happens after polymerisation has began (Weisel, 2005). The removal of fibrinopeptide B from the Bβ chain also in the E region exposes further binding sites, which also have complementary binding sites in the D region. The binding that takes place after removal of fibrinopeptide B, though not absolutely required for lateral fibril and fibre aggregation, facilitates this process (Weisel *et al.*, 1993), perhaps

through cooperative interactions resulting from alignment of the D regions of adjacent fibrils in the fibrin polymer (Shainoff & Dardik, 1983).

2.4.2 General overview of fibrin formation and lysis

In general overview, the roles that the haemostatic variables mentioned earlier play in the formation and lysis of the fibrin network structure are as follows. Fibrinogen is converted to fibrin mononers when fibrinopeptides A and B are removed from fibrinogen by thrombin, as discussed above (Mosesson, 1998). The fibrin monomers then polymerise to form the fibrin network structure that forms the scaffold of a blood clot (Mosesson, 1998; Weisel *et al.*, 1993).

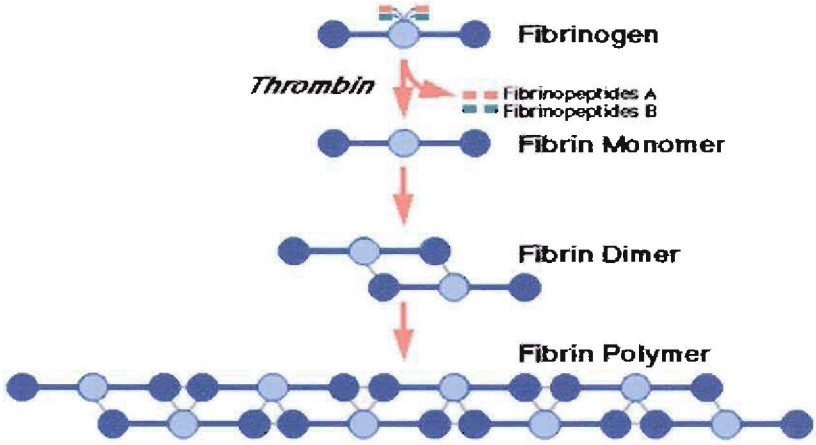

Figure 2.2. An illustration of the staggered nature of the fibrin double stranded proto-fibrils formed during fibrin polymerisation after fibrinopeptides A and B removal (Tollefsen Lab, 2001)

Thrombin is formed from prothrombin through activation of the prothrombinase complex that is generated from the activation of both the intrinsic and the extrinsic pathways of the blood coagulation system (Colman *et al.*, 2001). In addition to its involvement in the formation of fibrin monomers, thrombin also removes activation peptides from factor XIII in the presence of calcium, resulting in the formation of activated factor XIIIa (Greenberg, Miraglia, Ricles, & Shuman, 1985). The fibrin network structure is stabilised by cross-linking of adjacent proto-fibrils by factor XIIIa (Siebelist, Meh, & Mosesson, 1996). To ensure that clot lysis will take place

efficiently when it should, fibrinolytic factors are also incorporated into the fibrin network structure at formation (Weisel, 2005). The fibrinolytic protease plasmin is generated from plasminogen by tissue plasminogen activator (t-PA), a reaction that is facilitated by the binding of both plasminogen and t-PA to specific binding sites on fibrin (Weisel, 2005). Antifibrinolytic factors like PAI-1, thrombin activated fibrinolytic inhibitor (Sakharov, Plow, & Rijken, 1997) and α2-macroglobulin can block the generation of plasmin (Weisel, 2005). The activity of t-PA is inhibited by PAI-1 (Stringer & Pannekoek, 1995). Plasmin inhibitor (PI) inhibits the binding of plasmin to fibrin and also directly inhibits the activity of plasmin itself (Dunn *et al.*, 2006). The plasma protein α2-macroglobulin also inhibits plasmin and activated thrombin activated fibrinolytic inhibitor (TAFIa) reduces the rate at which fibrin enhances the activation of plasminogen by t-PA, through the elimination of binding sites for plasminogen from partially degraded fibrin (Sakharov *et al.*, 1997). Fig. 2.2 from Dunn *et al.* (2006) is a schematic representation of the process described above.

2.4.3 Cross-linking of fibrin by factor XIII

The fibrin network structure that is formed is stabilised by the cross-linking of the α and γ chains by Factor XIIIa (Lorand, 2001) shortly after proto-fibril formation (Standeven *et al.*, 2005). The precursor of factor XIIIa, factor XIII is a transglutaminase that is activated by thrombin to factor XIIIa and the activation is enhanced by fibrin formation (Standeven *et al.*, 2005). The existence of γ-γ dimers (Chen & Doolittle, 1971; Ryan, Mockros, Weisel, & Lorand, 1999), α-α oligomers and polymers (Ryan *et al.*, 1999; Sobel & Gawinowicz, 1996) and even α-γ linked heterodimers (Ryan *et al.*, 1999; Siebenlist & Mosesson, 1996) as a result of factor XIII cross-linking, have all been observed. γ- Chain cross-links are covalent bonds between γ-lys406 of one γ-chain and Glu398 of another (Chen & Doolittle, 1971), though there is a difference of opinion on whether the cross-linking takes place laterally, (Weisel, 2004) or transversely (Mosesson, 2004). In the case of α-chain cross-linking, multiple cross-linking sites are possible between the α-chains (Lorand, 2001; Sobel & Gawinowicz, 1996). The α-γ- cross-links involve both γGlu398 and 399 being linked with αLys413 and 418, with αLys413 being a possible additional

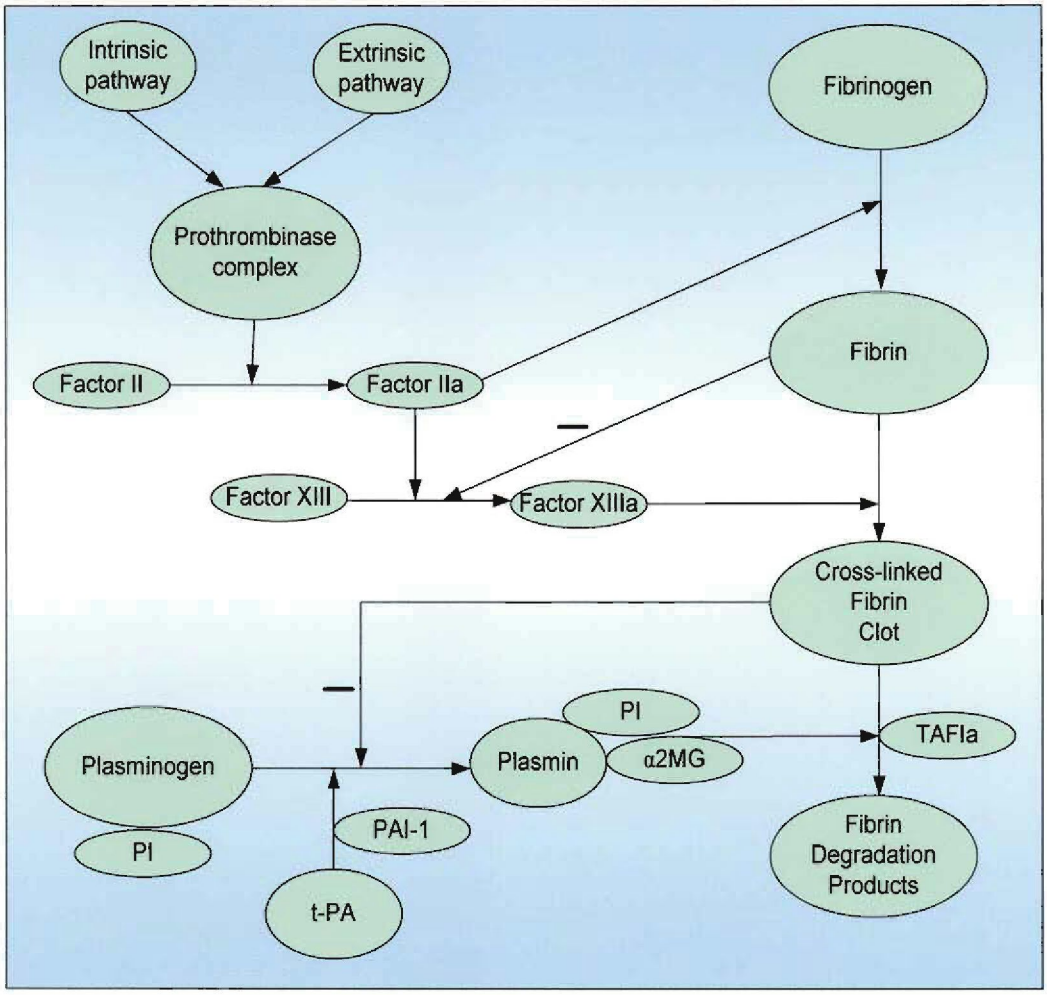

Figure 2.3 Simple schematic diagram of the process of formation of the fibrin network structure showing the main coagulation and anticoagulation factors (Dunn *et al.*, 2006)

linking site (Lorand, 2001). The morphological changes to the fibrin network structure brought about by factor XIIIa cross-linking was reported to be small compared to changes brought about by changes in thrombin, fibrinogen and calcium chloride concentration (Ryan *et al.*, 1999). Ryan *et al.* (1999) also reported that γ-chain cross-linking had a greater effect than α-chain cross-linking on the fibrin network structure. They did, however, attribute the slight reduction in fibre diameter

that they observed to factor XIIIa cross-linking and that this could have been due to a tightening of the lateral bonds between monomers as a result of the cross-linking. The visco-elastic properties of fibrin fibres have been attributed, in part, to the factor XIII cross-linkage (Mosesson, 2005; Weisel, 2005). Factor XIIIa cross-linking was reported to increase flexural fibre stiffness by stabilizing inter-fibril interactions (Ryan *et al.*, 1999) and the ability of a fibrin network to withstand the shear forces of blood flow, *in vivo*, have been attributed to the rigidity of the network which arises, in part, from the cross-linking (Nair & Shats, 1997).

2.4.4 Lateral aggregation and branching of fibrin fibres

The proto-fibrils that are formed in the polymerisation process aggregate laterally to form fibres (Mosesson, 2005; Weisel, 2005). The thickness of these fibres is limited by the fact that as the fibres become thicker, the transverse path of the twisted fibres increases and reaches a point where the energy required to stretch an additional proto-fibril becomes more than the bond energy (Weisel, 2005). The conditions of polymerisation can vary the thickness of the fibres formed. If the conditions of polymerisation prevailing favour lateral aggregation, then thicker fibres composed of fewer branch points form, where as, if the prevailing conditions favour branching, then thinner fibres with a larger number of branch points would form (Ryan *et al.*, 1999). Ryan *et al.* (1999) reported that wider fibres were formed only when branching was reduced and longer fibres were associated with wider fibre diameters. It has been suggested that the carbohydrate structures that form part of the fibrinogen molecule help to modulate the extent of lateral aggregation and that they may also significantly increase fibrinogen's solubility (Weisel, 2005).

The fibrils that form in the polymerisation process described branch to form the three dimensional structure of the fibrin network (Standeven *et al.*, 2005; Weisel, 2005). There are two main types of branching that have been identified. One type is where two double-stranded proto-fibrils converge to form a tetra-molecular branch-point (Mosesson, 1998) or bilateral branch junction (Mosesson, 2005). The other is when a double stranded fibril branches, forming an equilateral branch junction (Mosesson, 2005). The equilateral branch point is probably initiated when a fibrin monomer in a double strand diverges from the double strand to interact with a

second monomer resulting in the formation of another double stranded proto-fibril as a result of which a tri-molecular branch point forms (Weisel, 2005). Ryan *et al.* (1999) reported the equilateral type of branching to have been more frequent than the bilateral branching. Fig 2.3 shows a simple diagram of the different types of branching as illustrated by Mosesson (2005).

Branching and fibre diameter also play an important role in determining final rigidity (Ryan *et al.*, 1999). Ryan *et al.* (1999) reported that a greater amount of branching was associated with greater stiffness of networks, while the reduction in fibre thickness that was associated with increased branch density resulted in an increase in softness of the networks. This study reported that networks which had the highest amounts of stiffness were those that showed intermediate branching densities and fibre diameters.

The diabetic condition and the accompanying differences in many of the haemostatic factors that are involved in coagulation and fibrinolysis, provide a physiological environment that has been shown to be associated with a significant increase in CVD risk. This increase in CVD risk has been attributed, in part, to changes in the fibrin network structures formed by diabetic subjects and section 2.5 reviews some of the effects of diabetes on fibrin network structure.

2.5 Fibrinogen and fibrin network structure in diabetes

The fibrin network structure may be modified in diabetes. Some researchers have reported that clots formed from fibrinogen obtained from diabetic subjects show changes that may influence either thrombus formation or the susceptibility of the thrombi to lytic processes (Dunn & Ariens, 2004). The fibrin network structure variables that have been investigated include, permeability (Dunn *et al.*, 2005; Jorneskog *et al.*, 1996; Jorneskog *et al.*, 2003; Nair *et al.*, 1991); fibre diameter and mass-length ratio (Dunn *et al.*, 2005; Jorneskog *et al.*, 2003; Nair *et al.*, 1991); fibre density and branching (Dunn *et al.*, 2005); factor XIII cross-linking of the networks (Dunn *et al.*, 2006; Lütjens, Jonkhoff-Slok, Sandkuijl, vd Veen, & vd, 1988); and

susceptibility of networks to fibrinolysis (Aso *et al.*, 2002; Dunn *et al.*, 2006; Geiger & Binder, 1986; van Wersch, Westerhuis, & Venekamp, 1990).

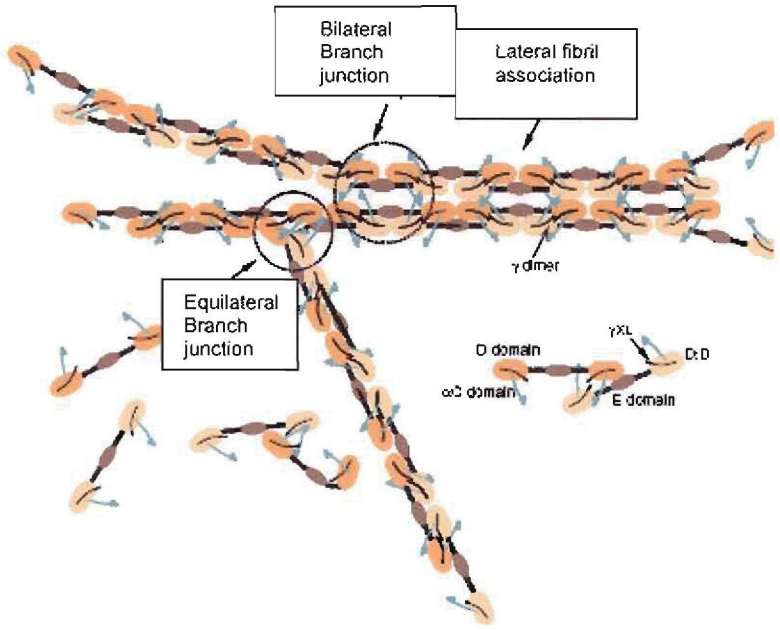

Figure 2.4. A simple diagram illustrating fibrin polymers and possible fibre branch types. The distal D domains are orange and yellow and the central E domains are brown (Mosesson, 2005).

2.5.1 Kinetics of fibrin formation

Elevation of fibrinogen levels in association with diabetes was discussed in section 2.3. The kinetics of clot formation may be altered in situations of high fibrinogen levels. Working with patients who had suffered myocardial infarction, Fatah *et al.* (1992) reported that patients with elevated fibrinogen levels, had lower gel porosity compared with patients with normal fibrinogen levels and controls. For diabetic subjects with elevated fibrinogen levels, the increased fibrinogen may thus contribute to changes in the fibrin network structure as this could lead to a faster

clotting rate (provided the thrombin concentration is not limiting) and formation of less porous networks (Fatah, Hamsten, Blomback, & Blomback, 1992), with thinner fibres (Ryan *et al.*, 1999). In addition to faster clotting rates, elevated fibrinogen levels may also lead to a possible reduction in lysis rates as a result of formation of denser networks (Collet, Park, Lesty, Soria, Soria, Montalescot, & Weisel, 2000).

Information regarding the effect of diabetes on the rate of polymerisation has been obtained from turbidimetric measurements during the polymerisation process. The lag-time in the turbidity development represents the time required for fibrin proto-fibrils to grow to a sufficient length for lateral aggregation to take place (Dunn *et al.*, 2005). Robinson *et al.* (1983) showed that the clotting activity of fibrinogen purified from subjects with diabetes was significantly higher than that of fibrinogen purified from non-diabetic subjects. Dunn *et al.* (2005), in work with type 2 diabetes, reported that the lag-time was reduced indicating that diabetes, in this case, was associated with faster clotting time. Lütjens *et al.* (1988) on the other hand, reported no difference in lag-time for type 1 diabetes despite a significant increase in glycation of the fibrinogen in their study.

2.5.2 Fibre diameter

Fibre diameter is another factor of fibrin network structure that seems to be influenced by diabetes even though there is much less agreement in the literature over what this effect may be. Nair *et al.* (1991) reported the thickness of fibres prepared from plasma of uncontrolled diabetic subjects to be significantly reduced. Jorneskog *et al.* (1996), on the other hand, working with purified fibrinogen from type 1 diabetic subjects, did not find a difference in fibre thickness or mass-length ratio, while Dunn *et al.* (2005), working with fibrinogen purified from type 2 diabetic plasma, reported a non significant increase in fibre diameter and a significantly denser network. In general, thinner fibres are lysed faster than thicker fibres (Weisel, 2005). However, fibre density of networks rather than fibre diameter on its own, seems to influence the rate of clot lysis (Collet *et al.*, 2000) and as such, the diabetic subjects forming more compact fibrin network structures would be expected to experience lower clot lysis rates and the associated increase in CVD risk. Collet *et al.* (2000) reported that fibrin networks made up of thicker fibres were lysed faster

than networks made from thinner fibres, influenced not so much by the thickness of the fibres but rather by the density or compaction of the networks formed.

2.5.3 Factor XIII cross-linking

Factor XII cross-linking stabilizes the fibrin networks formed from fibrinogen. It is not clear what the effect of diabetes on factor XIII cross-linking is. Dunn *et al.* (2005) reported that the rate of α-chain cross-linkage was significantly higher in diabetic subjects while that of γ-chain cross-linkage was unchanged. Lütjens (1988), on the other hand, reported a decrease in α-chain cross-linking. In *in vitro* studies no differences were reported between α-chain and γ-chain cross-linking (Krantz, Lober, Thiele, & Teuscher, 1987; Ney, Pasqua, Colley, Guthrow, & Pizzo, 1985). More research is required to determine the precise effect that fibrinogen glycation may have on factor-XIII cross-linking.

2.5.4 Permeability of fibrin networks

The permeability of fibrin networks is measured to indicate the size of pores of the networks, and a decrease in pore size is associated with lower permeability. Jorneskog *et al.* (1996) reported lower fibrin gel porosity in type 1 diabetes patients in spite of the fact that they had normal plasma fibrinogen levels and irrespective of the presence or absence of microangiopathy. They attributed the abnormal gel structure to possible glycation of fibrin and fibrinogen as a result of long-term hyperglycaemia. Dunn *et al.* (2005), working with type 2 diabetes reported a decrease in permeability of fibrin networks prepared from fibrinogen purified from the diabetic subjects. Nair *et al.* (1991) reported reduced permeability when glucose was added to fibrin networks *in vitro*. Both Dunn *et al.* (2005) and Nair *et al.* (1991) reported the fibrin network structures of the diabetic subjects to be more dense and less porous. These results seem to indicate an association between the diabetic condition and a decrease in permeability resulting from the smaller pores that are associated with more dense networks. The cause of this change in fibrin network structure in diabetes is not clear but it has been suggested that it may be influenced by glycation of fibrinogen and fibrin as a result of hyperglycaemia (Jorneskog *et al.*, 1996). Both Dunn *et al.* (2005) and Jorneskog *et al.* (1996) observed that permeability was negatively correlated to glycaemic control as indicated by HbA1c.

2.5.5 The balance between fibrin formation and fibrinolysis

As earlier indicated there should be a dynamic balance between coagulation/fibrin network structure formation and clot lysis/fibrinolysis in order to maintain blood in a fluid state and avoid either thrombolic or hemorrhagic problems. Fibrinolytic molecules attach to fibrin during its formation (Gabriel, Muga, & Boothroyd, 1992; Standeven *et al.*, 2005) to ensure this kind of balance. Changes in how this happens as a result of hyperglycaemia may influence the lysis rate of fibrin networks formed. Dunn *et al.* (2006) reported that lysis of clots formed from fibrinogen purified from diabetic blood was significantly slower than that of controls. Further, their results pointed towards some possible mechanism by which the reduced fibrinolytic activity may arise. In the diabetic subjects, they reported that plasmin generation at the fibrin surface was significantly reduced and the equilibrium binding affinity of both t-PA and Glu- plasminogen to fibrin was reduced. In addition, cross-linkage of plasmin inhibitor by factor XIII to fibrin was enhanced in the diabetic subjects compared to controls. Each one of these differences can lead to a reduced rate of fibrinolysis of the fibrin network structures formed from the diabetic fibrinogen.

2.5.6 Glycation as a possible mechanism affecting fibrin network structure in diabetes

Glycation of proteins involved in haemostasis has been investigated at as a possible mechanism by which diabetes may affect fibrin network structure. As discussed in section 2.4.1, the molecular structure of fibrinogen includes four clusters of carbohydrates, one on each of the Bβ and γ-chains making it a glycoprotein (Weisel, 2005). The non-enzymatic addition of further glucose units to fibrinogen (non-enzymatic glycation) as a result of continual exposure to hyperglycaemia, over time, takes place *in vivo* (Hammer, John, Flynn, Bellingham, & Leslie, 1989; Lütjens *et al.*, 1985). This non-enzymatic glycation of fibrinogen may contribute to observed alterations of fibrin network structures in diabetes. Based on work involving acetylation and carbamylation (Brownlee *et al.*, 1983) and estimation of the amount of glucose bound to fibrinogen (Lütjens *et al.*, 1985), it has been suggested that glycation of fibrinogen takes place at lysine residues and that this glycation may be

responsible for the observed changes in functional properties of fibrinogen. The glycation of not only fibrinogen but also the various other proteins involved in coagulation and fibrinolysis may change the tertiary and/or quaternary structures of proteins, to the extent that functional changes of the affected proteins take place. *In vitro* studies (Nair *et al.*, 1991) have demonstrated that the non-enzymatic glycation of fibrinogen is dose dependent and *in vivo,* it has been shown that fibrinogen isolated from diabetic plasma is glycated to a greater extent than fibrinogen isolated from non-diabetic plasma (Ardawi, Nasrat, Mira, & Fatani, 1990; Hammer *et al.*, 1989).

The possible effect of fibrinogen glycation on fibrin network structure has been implied from studies that have shown that the permeability of fibrin networks of people with diabetes is reduced compared to those of people without diabetes (Dunn *et al.*, 2005; Jorneskog *et al.*, 1996; Nair *et al.*, 1991). Nair *et al.* (1991) and Jorneskog *et al.* (1996) analysed permeability using fibrin networks prepared in plasma while Dunn *et al.* (2005) used purified fibrinogen, and in both situations, the permeability of diabetic fibrin networks was reduced. Since the study done by Dunn *et al.* (2005) used purified fibrinogen and, therefore excluded, the possible influence of other plasma constituents, it is possible that fibrinogen glycation affected the fibrin network structure resulting in the reduced permeability observed. Jorneskog *et al.* (2003) carried out an intervention study of type 1 diabetes patients in which the patients were treated with continuous sub-cutaneous insulin infusion. The results of this study showed a significant improvement of permeability as a result of the intervention. However, Jorneskog *et al.* (2003) did not measure the level of glycation in the study and the possible effect of other plasma factors on the permeability cannot be ruled out, because the permeability measurement was done in plasma. There has been no intervention study that has measured the levels of fibrinogen glycation before and after intervention in either type 1 or type two diabetes that has investigated the possible effect of fibrinogen glycation, specifically on fibrin network structure variables.

Other fibrin network structure variables that have been studied with respect to the possible effect of non-enzymatic glycation of fibrinogen in diabetes are compaction (Nair *et al.*, 1991), lag-time, fibre size, fibre density per unit volume and number of

branch points per unit volume (Dunn *et al.*, 2005). Each one of these variables was reported to be affected by diabetes, but the levels of glycation of fibrinogen were not measured and it has never been established whether a reduction in fibrinogen glycation through intervention would also result in changes in the fibrin network variables that have been shown to be affected by diabetes.

Additional functional properties of fibrin have been shown to be affected by non-enzymatic glycation. In *in-vitro* experiments, Brownlee *et al.* (1983) demonstrated that non-enzymatic glycation reduces the susceptibility of fibrin to plasmin breakdown and that conditions that increase the rate of protein glycation resulted in correspondingly greater degrees of resistance of the fibrin to breakdown by plasmin. They, therefore, hypothesized that reduced fibrin degradation, *in vivo,* could contribute to observed accumulation of fibrin and proteins in tissues susceptible to diabetic complications.

Because non enzymatic glycation also affects other proteins involved in coagulation and fibrinolysis, its effects have consequences with the function of these other proteins. For instance, Geiger and Binder (1986) reported that the functional changes that took place to glycated plasminogen were similar to that of plasminogen obtained from diabetic patients. The lag-time for the generation of plasmin from plasminogen was up to three times longer for plasminogen from uncontrolled diabetic patients. They concluded that glycation of plasminogen was likely to have contributed to the impaired activation observed. Gugliucci and Ghitescu (2002) hypothesised that glycation of endothelial membrane annexin II impairs the appropriate formation of the plasminogen/t-PA/annexin II complex and by doing so disrupts a key regulatory mechanism of fibrinolytic activity.

Hyperglycaemia in the diabetic condition may, therefore, bring about changes in functionality of not only fibrinogen but other molecules involved in coagulation and fibrinolysis contributing to the adverse effects of diabetes.

2.6 Conclusion

It is evident that the metabolic abnormalities associated with *Diabetes mellitus* result in an increase in CVD risk in those affected. Although causal pathways seem complex, an imbalance between coagulation and fibrinolysis in the diabetic condition, in part, seems to contribute to the increased CVD risk experienced. Research findings seem to point towards diabetes being associated with an increase in coagulation activity on the one hand and reduced fibrinolytic activity on the other.

Elevation of levels of key coagulation factors like fibrinogen and thrombin seem to lead to not only increased coagulability, but also to changes in structure of the fibrin networks formed, making the networks more dense, less porous and more resistant to fibrinolysis.

Changes in the fibrinolytic system that lead to a decrease in plasmin generation at the fibrin surface may lead to reduced fibrinolysis. In addition, increased levels of PAI-1 may also indirectly reduce plasmin generation by inhibiting t-PA in its role in the conversion of plasminogen to plasmin.

Glycation of plasma proteins including those involved in coagulation and fibrinolysis is a possible mechanism through which some of the observed changes in fibrinogen/fibrin network structure and function may be brought about. Both coagulation factors and fibrinolytic factors such as fibrinogen and plasminogen respectively, have been shown to be glycated *in vivo* and associations have been made between the glycation and changes in functionality of the proteins.

In view of the central role played by fibrinogen and fibrin network structure in increasing CVD risk in diabetes, this project sought to determine whether:

- There would be a difference in fasting plasma fibrinogen levels between the volunteers with type 2 diabetes and those without diabetes
- There is a difference in the level of glycation of fibrinogen between uncontrolled type 2 diabetic subjects and non-diabetic subjects at baseline

- There would be differences in the selected fibrin network structure variables between the diabetic and non diabetic volunteers at baseline
- Normalization of hyperglycaemia, with insulin treatment, of uncontrolled type 2 diabetic subjects would result in a reduction in the level of glycation of fibrinogen
- There would be a change in the fibrin network structure formed as a result of the reduced level of the fibrinogen glycation.

Chapter 3: Materials and methods

3.1 Introduction

People suffering from type 2 diabetes may have an altered fibrin network structure (Dunn *et al.*, 2005). The alteration has been implicated as a contributing factor to the increased CVD risk in those suffering from diabetes when compared to the general population. This study investigated fibrinogen glycation as a possible cause of the altered fibrin network structure.

This study was, therefore, undertaken to measure the fibrinogen levels and the levels of fibrinogen glycation of black South African volunteers without and with uncontrolled type 2 diabetes and to:

- investigate whether there were differences in fibrin network structure characteristics between the two groups,
- treat the volunteers with type 2 diabetes with insulin to determine whether glycaemic control would lead to a reduction in the level of fibrinogen glycation and
- investigate whether changes in fibrinogen glycation as a result of glycaemic control would result in alterations to the fibrin network structures formed as a result of changes in the level of fibrinogen glycation.

Forty volunteers in total were used, 20 diabetic and 20 non-diabetic. The 20 diabetic patients were worked with on an out patient basis and were enrolled into the study through the Mamelodi and Kalafong diabetic clinics of Pretoria. The 20 non-diabetic volunteers were enrolled through the Mamelodi and Kalafong hypertension clinics. The diabetic volunteers were seen by a diabetes specialist physician every week. The intervention process for the volunteers with type 2 diabetes was monitored to ensure compliance to the given protocol.

This chapter describes volunteer recruitment and the study design followed. It also presents the analytical methods that were used to assess the different variables measured in the study. Chapter 4 reports the results and Chapter 5 is the discussion

of the results that were obtained. The general conclusions of the study and recommendations for possible further research are presented in Chapter 6.

3.2 Recruitment of Volunteers

All volunteers gave informed written consent for the study and the Ethics Committees of both the University of Pretoria (where the two doctors involved in the intervention are based) and the North-West University approved the study. The ethical approval number for the North-West University is 04M13 while that at the University of Pretoria is 63/2004. A non-diabetic group of volunteers with matching age, gender, BMI and antihypertensive medication use (hydrochlorothiazide, ACE-inhibitor- Perindopril and calcium antagonist-Nifedipine) was selected as a reference group.

The inclusion criteria for the diabetic volunteers were as follows:

- The volunteers had to be on maximum oral medication but failing to control the hyperglycaemia, therefore, requiring to be put on insulin treatment.
- BMI >25 kg/m^2.
- Both males and females were included in the study.
- HbA1c > 9%.
- 40-65 years of age.
- Blood pressure sufficiently controlled (< 140/90 mmHg) not to require treatment changes during the duration of the study.
- Sulphonoureas were stopped.
- The volunteers had to have access to a phone to facilitate the follow up process.

The inclusion criteria for the non-diabetic volunteers were the same as for those with diabetes, except that they had to have normal glucose tolerance as confirmed by an oral glucose tolerance test and HbA1c value within normal range (HbA1c<6.5 %).
The following were common exclusion criteria for both groups:

- Having had major surgery in the preceding 6 months.
- The presence of macrovascular complications.

- The presence of any disease that would affect haemostasis.
- The presence of proteinurea on dipstick (> 300 mg/day).
- The presence of any acute infections.
- Those individuals who were on any of the following treatments: asprin, warfrin, steroids, hormone replacement therapy and non steroidal anti-inflammatory drugs

These exclusion criteria were decided upon in an attempt to exclude any conditions that could have haemostatic effects and possibly confound the results that would be obtained.

3.3 Study design

This was a parallel, controlled intervention study. It involved 40 black South African volunteers, 20 with type 2 diabetes and 20 without. All the volunteers belonged to the same socio-economic background and the 20 non-diabetic volunteers were included as a reference group in order to control for variation over time. The 20 diabetic volunteers were on maximum oral medication but failing to control the hyperglycaemia. The diabetic volunteers were treated with insulin to bring about glycaemic control.

The study was carried out in three phases.

3.3.1 Phase 1

Before the start of the intervention, after the signing of consent forms, the volunteers were seen by a diabetes educator who provided training on proper self-glucose monitoring and proper use of the glucometers that each volunteer was provided with for the study. The volunteers were also given instructions on how to record the glucose measurements in diaries that were provided. The glucometers were electronic with memory. This allowed for the glucose values reported by the volunteers to be checked for accuracy.

The volunteers with type 2 diabetes were trained in proper insulin injection administration and coordination of insulin use with meals. They were also given training on what to do in case they developed hypoglycaemia as a result of the insulin treatment. Each subject was issued with a Glucagen hypokit for glucagon intra-muscular administration. All hypoglycaemic events were recorded. Hypoglycaemia was taken as glucose values less than 2.8 mmol/l or in case of severe hypoglycaemia, where the subject would have required the assistance of another person.

The baseline blood samples and baseline anthropometric measurements were taken at the beginning of this phase. During this first phase the fasting capillary glucose was measured daily for 7 days to establish the baseline level of glycaemic control prior to the intervention treatment.

3.3.2 Phase 2

Fasting glucose was controlled through the use of basal analogue insulin 10 IU glargine (Lantus, Sanofi-Aventis Pharmaceuticals, Paris, France), daily at 22:00 hours. Patients continued with maximum oral dosage medication of Metformin throughout the entire intervention period, but Sulphonylureas was stopped. The insulin dosage was adjusted according to need until 4 out of 5 normal consecutive fasting capillary values (< 7.2 mmol/l) were obtained. The volunteers visited the clinic every week and had telephonic follow up calls every 3 days. Fasting glucose and post meal glucose levels were recorded in the diaries and electronically on each subject's glucometer.

3.3.3 Phase 3

Post-prandial glucose was now also controlled by pre-meal treatment with the short-acting insulin, Aspart (Novo Nordisk, Bagsvaerd, Denmark). The insulin dosages were adjusted according to individual need until the hyperglycaemia was deemed controlled by the physician (4 out of 5 subsequent readings <10mmol/l). Once both fasting glucose and post-prandial glucose were controlled, the volunteers then had to remain controlled for an 8 day period before the end blood samples were taken.

The 8 day time span was decided upon because fibrinogen has a half life of 3-4 days. Eight days were, therefore, considered enough time for fibrinogen turnover, such that fibrinogen glycation measurements would reflect the true situation prevailing after glycaemic control.

The blood samples from non-diabetic volunteers for reference were drawn within one week of blood sampling of their matched diabetic volunteers for both baseline and end samples. Figure 3.1 is an illustration of the study design for the study.

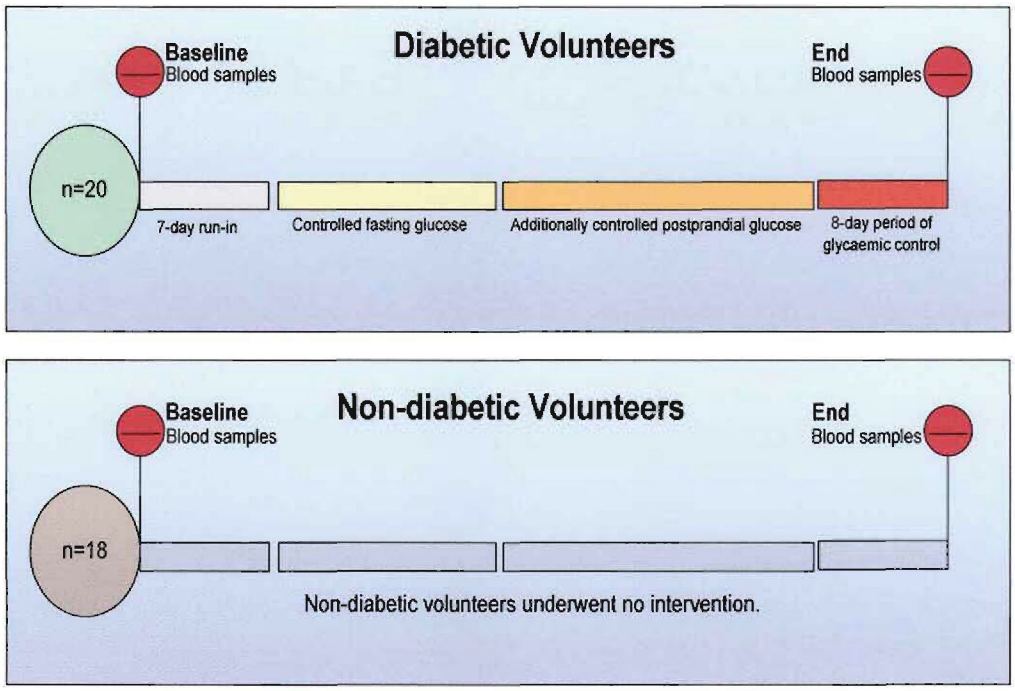

Figure 3.1. A diagram illustrating the study design followed

3.4 Blood sampling

All blood samples were collected between 07:00 and 10:00 AM to avoid effects of diurnal variation. Sixty two ml of blood was collected from each subject. Fasting venous blood was drawn with minimum stasis by a medical doctor at the beginning of phase 1 and end of phase 3 for analyses.

The blood samples were placed in different types of tubes for different analyses. For serum lipids and insulin, blood was left to clot for serum preparation. For plasma glucose, blood was collected into sodium fluoride tubes, and for fibrinogen, fibrinogen glycation and PAI into citrate tubes. The fluoride prevents the red blood cells from using glucose and the citrate in the citrated tubes binds calcium preventing clotting. These blood samples were centrifuged at 2000 g at 4 °C, for 15 minutes, within 30 minutes of collection.

The blood for HbA1c was put into EDTA tubes and was not centrifuged because the analyses require whole blood to be used.

For determination of fibrin network structure, blood was drawn into citrate tubes containing 3.8 % citrate with added 10 000 KIU/ml Trasylol® (35 μl/10ml of blood). The trasylol inhibits lysis. The blood in trasylol tubes was centrifuged twice at 3660 g for 15 minutes to yield platelet free plasma. This plasma was used for permeability, compaction and turbidimetric analyses.

All plasma and serum samples were stored at -83 °C until analysis was done.

3.5 Anthropometric measurements

Baseline weight and height measurements for the diabetic and non-diabetic volunteers were taken at the beginning of phase 1 and the end measurements were taken at the end of phase 3 of the study.

A stadiometer was used to measure the height of the volunteers. Care was taken that the volunteers stood in the correct upright position with the Frankfurt plane horizontal. A precision health scale was used for weight measurements. Both measurements were taken with the volunteers wearing light clothes and no shoes.

3.6 Fasting serum-insulin and insulin resistance

Fasting serum-insulin was measured using an enzyme-linked immunosorbent assay (ELISA) method on the Immulite 2000 analyzer (Diagnostic Products

Corporation, Los Angels, Callifornia, USA). Insulin resistance was calculated using the homeostasis model assessment (HOMA). The calculation was done using the formula,

HOMA = (fasting insulin x fasting venous glucose) / 22.5 (Katz, Nambi, Mather, Baron, Follmann, Sullivan, & Quon, 2000)

3.7 Plasma glucose, HbA1c, PAI-1_{act} and serum lipids

Plasma glucose, serum lipids and HbA1c were all measured on a Synchron LX clinical system (Beckman Coulter Inc., Fullerton, CA, USA). The low density lipoprotein cholesterol was calculated by the Friedewald formula (Friedewald, Levy, & Fredrickson, 1972). PAI-1_{act} was measured using an indirect enzymatic method (Spectrolyse pL, Biopool, Umeå, Sweden, Cat. No. 101201).

3.8 Plasma fibrinogen

Plasma fibrinogen was measured by the Clauss method using the ACL-200 automated coagulation analyzer and reagents from Instrumentation Laboratories, Milan, Italy. The between run coefficient of variation for this analysis was 3 percent.

3.9 Fibrinogen purification by IF-1 affinity chromatography

Fibrinogen was purified from the plasma of each of the volunteers separately. An IF-1 (fibrinogen monoclonal antibody, Cat No. MC-900, Kamiya Biomedical Company, Seattle, WA, USA) affinity chromatography method described by Takebe *et al.* (1995) was used. Purified fibrinogen was needed for the analysis of glycated fibrinogen and for permeability and turbidimetric analysis. A detailed description of how the method was used is given in sections 3.9.1 and 3.9.2.

3.9.1 Preparation of the chromatography column

A washing solution and 4 buffers were required for the preparation of the chromatography column. The composition of the buffers and washing solution were as follows.

Washing solution

1000 ml de-ionized distilled water, pH was adjusted to 3.0 with HCl.

Coupling buffer

This buffer was used to maintain the proper pH during the coupling reaction. The pH of 0.1 M sodium hydrogen carbonate ($NaHCO_3$) solution (MW= 84.01) was adjusted to 8.3 using 0.1 M sodium carbonate (Na_2CO_3) solution (MW= 105.99). Enough of this solution was added to 14.6 g of sodium chloride (NaCl) to make 500 ml of coupling buffer with a final NaCl concentration of 0.5M.

Blocking buffer

Distilled water was added to 3.017 ml ethanolamine (MW=61.08) to make 40ml of solution. The pH was adjusted to 8.0 with sodium hydroxide (NaOH) solution.

Washing buffer

To 6.804 g sodium acetate and 14.6 g NaCl, de-ionized distilled water was added to make 500 ml of washing buffer. The pH was adjusted to 4.0 with acetic acid

Tris buffer

100 ml of Tris buffer (pH 7.4) was made to have final concentrations of 20 mM Tris, 0.3 M NaCl, and 05 % sodium azide (NaN_3)

The chromatography column was prepared as follows;

a. The 50 ml of **Washing solution** prepared as above was added to 1.5g of cyanogen bromide (CNBr) activated Sepharose resin powder (Pharmacia, Cat No.17-0430-15 g package, Uppsala, Sweden). The resin was incubated for 10 minutes to allow it to swell.

b. The swollen resin was placed in a 30 ml sintered glass funnel and washed with 900ml of **Washing solution**. The **Washing solution** removes the preservatives and salts from the resin.

c. 1.15ml of the IF-1 antibodies (fibrinogen monoclonal antibody, Cat. No: MC-900, Kamiya Biomedical Company, Seattle, WA, USA) was placed in a 50 ml cylinder and 5ml **Coupling buffer** was added.

d. The resin in the glass funnel was washed with 60 ml of coupling buffer and immediately transferred to the 50 ml cylinder. The volume of solution in the cylinder was adjusted to 20 ml with the **Coupling buffer** after which the cylinder was covered with parafilm, to ensure that the resin would not dry out. After this the cylinder was then rotated upside down for 1 hour, at room temperature.

e. After the hour, 20 ml of **Blocking buffer** was added to the suspension in the cylinder and the cylinder was again rotated for 1 hour, at room temperature.

f. The resin suspension was then placed into a 30 ml sintered funnel and washed alternately with **Coupling buffer** and **Washing buffer**, starting with **Coupling buffer**. Finally the resin was washed with 100 ml of **Tris buffer** and transferred to a 50 ml tube and stored in the refrigerator.

3.9.2 Purification of the plasma fibrinogen

Several buffers were used in the purification process and their compositions are given here. All concentrations given in this list refer to the final concentrations of the respective items in solution.

Equilibration buffer

0.02 M Tris
0.3 M NaCl
1 mM calcium chloride ($CaCl_2$)
0.02 % NaN_3
pH 7.4 adjusted with 5 M hydrochloric acid (HCl)

Elution buffer

0.02 M Tris
0.3 M NaCl
5 mM EDTA
pH 7.4 adjusted with 5 M HCl

Dilution buffer

50 mM tris
100 mM NaCl
pH 7.4

Dialysis buffer I

0.05 M Tris
0.1 M NaCl
1 mM $CaCl_2$
pH 7.4 adjusted with 5 M HCl

Wash buffer I

0.02 M Tris
1 M NaCl
1 mM $CaCl_2$
pH 7.4 adjusted with 5M HCl

Dialysis buffer II

0.05 M Tris
0.1 M NaCl
pH 7.4 adjusted with 5 M HCl

Wash buffer II

0.05 M sodium acetate
0.3 M NaCl
1 mM $CaCl_2$
pH 6 adjusted with 5 M NaOH

The purification of the fibrinogen process was as follows:

a. Two ml citrated plasma was thawed in a water bath at 37 °C until it was completely thawed. Then 1 U/ml Heparin, 5 mM Benzadine and 20 mM $CaCl_2$ were added to the plasma. All the concentrations given are the final concentrations in solution. The $CaCl_2$ was added last. This plasma solution was then diluted to 5 ml with the **Dilution buffer** and filtered through a 2 μm syringe filter.

b. The filtered plasma was loaded onto the IF-1 chromatography column equilibrated with **Equilibration buffer**. The column was washed with 6 column volumes of **Wash buffer I** and then 6 column volumes of **Wash buffer II**. After this the fibrinogen was eluted with 3 column volumes elution buffer and approximately 18 ml of fibrinogen solution was collected. The column was equilibrated with 5 column volumes of **Equilibration buffer**. The collected fibrinogen solution was concentrated using an Amicon Centriplus®centrifugal filter device according to the manufacturer's instructions. The fibrinogen sample was concentrated at 25 °C to a volume of approximately 2 ml. This concentrated sample was dialysed twice with **Dialysis buffer I** and then 3 times with **Dialysis buffer II**. The dialyzed sample was stored at -80 °C until required for analysis.

3.9.3 Confirmation of fibrinogen purity and absence of degradation

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to confirm the purity of the extracted purified fibrinogen, and to ensure that there was no degradation of the purified fibrinogen samples. Individual samples from all volunteers were run on a 10 % SDS-PAGE gel. The composition of the running and stacking gels are given in Tables 3.1 and 3.2, respectively.

Table 3.1. The composition of the running gel

Substance	Volume in ml
Acrylamide 40 %	3.00
1.5 M Tris pH 8.8	3.75
SDS 10 %	0.15
De-ionized distilled water	8.10
10 % Ammonium persulfate (APS)	0.05 (APS stock is stored at – 20°C)
Tetramethylethylene di-amine (TEMED)	0.01
Total	**15.06 ml**

The APS and TEMED were both added just before the gel was thrown because they activate clotting of the gel.

Table 3.2. The composition of the stacking gel

Substance	Volume in ml
Acrylamide 40 %	0.49
0.5 M Tris pH 6.8	1.25
SDS 10 %	0.05
De-ionized distilled water	3.15
10 % APS	0.025 (APS stock is stored at – 20°C)
TEMED	0.005
Total	**4.97 ml**

Six mg samples of fibrinogen extract obtained from the process described in section 3.9.2 were loaded onto the gel. Once the electropheretic run was completed, the gel

was stained with Coomasie-blue in order to visualize the movement pattern of the purified samples. All the samples were run to confirm purity and lack of degradation of the fibrinogen.

3.10 Fibrinogen glycation

Fibrinogen glycation was measured to establish the level of glycation before and after the intervention period. Fibrinogen glycation was measured with a two-reagent enzymatic assay (GLYPro® assay, Genzyme Diagnostics, Cambridge, MA, USA). The first reagent (Reagent 1) contained proteinase K, which digested the purified fibrinogen sample into glycated fibrinogen fragments. The second reagent (Reagent 2) contained ketoamine oxidase. The ketoamine oxidase facilitated the oxidation of ketoamine bonds in the fibrinogen fragments resulting in production of hydrogen peroxide. The amount of hydrogen peroxide produced was then colorimetrically determined and is proportional to the amount of ketoamine bonds that were present in the sample.

The analysis was carried out in three steps:

a. 250 µl of Reagent 1 was added to 20 µl of test plasma sample and left to incubate for 5 minutes, at 37 °C. The absorbance of the resulting solution was read at 550 nm and recorded as absorbance A1.

b. 50 µl of reagent 2 was then added to the fragment solution from above and left to incubate for 3-5 minutes at 37 °C. This reaction is accompanied by colour formation. The absorbance of the resulting solution was read at 550 nm and recorded as absorbance A2.

c. The change in absorbance (ΔA sample), was calculated by subtracting absorbance A1 from absorbance A2. The amount of glycated fibrinogen in the sample was then calculated using the formula,

$$\text{Glycated fibrinogen (µmol/l)} = \frac{\Delta A \text{ sample}}{\Delta A \text{ calibrator}} \times \text{Calibrator value (µmol/l)}$$

The GLYPro® calibrator value used in the calculation was obtained from a calibrator sample that was purchased for that purpose.

3.11 Permeability of fibrin networks

Permeability of fibrin networks was determined as permeation coefficients (Ks) which represent the surface area of the fibrin network that allows flow of fluid through the fibrin network and is an indication of pore size throughout the network. The higher the value the more porous the fibrin networks formed are. A modified method of Fatah *et al.* (1996) was used. Permeation coefficients were measured for fibrin networks prepared from plasma samples before and after the intervention and also for fibrin networks prepared from fibrinogen purified from plasma samples, before and after the intervention.

3.11.1 Permeability of fibrin networks prepared from plasma

1ml Stripette disposable serological pipettes (Corning Incorporated, Corning, New York) were cut to make 30 mm permeability tubes. The permeability tubes were mechanically etched with a wire brush on the inside up to a height of approximately 12 mm to provide a rough surface for adherence of fibrin networks. The bottom etched side of the tubes was sealed with Parafilm. Then 93.4 μl of plasma was placed into the pipette tubes and clotting was initiated by adding 6.6 μl of thrombin/calcium chloride reagent to provide final concentrations of 0.5 U/ml thrombin and 25 mM calcium chloride in 100 μl. The samples were allowed to clot for 60 minutes. Plastic tubing was then connected to the pipette tubes through which 1 M Tris-imidazole buffer of pH 7.5 (0.02 M Tris base, 0.02 M Imidazole and 0.1 M NaCl) flowed from a height of 21 cm. The time taken for an accurately measured volume of buffer to flow through was recorded. The volume of buffer, the time and height were used to calculate the permeability coefficient (Ks/cm^2). Three samples were run per subject and an average of the three Ks values was calculated.

The calculation of Ks was done using the formula,

$$Ks\ (cm^2) = \frac{Q \times L \times \mu}{T \times A \times P}$$

(Fatah *et al.*, 1992)

Where Q = the volume of liquid collected in cm^3 with viscosity μ, in poise ($dyne/cm^2$)

L = the length of the fibrin network, in cm, in the permeability tube

T = the time, in seconds, taken to collect Q

A = the area of the permeability tube, in cm^2, that is in contact with the fibrin network

P = the pressure exerted by the column of buffer over the fibrin network in $dyne/ cm^2$.

The between day coefficient of variation for the plasma permeability analysis was 15.5 percent.

3.11.2 Permeability of fibrin networks prepared from purified fibrinogen

The same procedure as for the fibrin networks prepared from plasma was followed except that the final concentrations of fibrinogen, thrombin and calcium chloride in the clotting mixture was different. In addition, the height from where the buffer flowed was also reduced to 6cm to prevent the networks from collapsing under the higher pressure.

A standard purified fibrinogen concentration of 1mg/ml was used for each subject. The purified fibrinogen clots were prepared in triplicate and clotted with the addition of 1U/ml thrombin and 5mM $CaCl_2$. The Tris buffer was permeated from a height of 6cm and Ks was determined as indicated above. Ks values were calculated as described in section 3.11.1. The between day coefficient of variation for the purified fibrinogen permeability analysis was 7.05 percent.

3.12 Compaction analysis of fibrin networks

Compaction was measured using a modified method of Dhall *et al.* (1976). 1ml micro-centrifuge tubes were coated with a lecithin based aerosol (Spray and Cook®) in order to prevent the clots from adhering to the sides of the micro-centrifuge tubes. Then 925 µl plasma was pipeted into the tubes. To initiate the clotting, 75 µl of thrombin/calcium chloride reagent was added to provide final concentrations of 25 mM calcium chloride and 1U/ml thrombin. The clots were left overnight and then centrifuged at 8000 g for 45 minutes with a micro-centrifuge (Eppendorf model I5415C, West Germany). The expelled supernatant was reported as a percentage of the total volume. The volume of the supernatant represents the volume of fluid associated with the fibrin networks and a more porous less rigid structure expels more fluid upon compaction and is an indication of the extent to which a clot would compact under stress. Five replicates were run for each volunteer and an average of the repeats was calculated for each subject and the between day coefficient of variation for the compaction analysis was 7.31 percent.

The compaction analysis was done only on plasma samples because the volumes required were too large to have the analysis done with purified fibrinogen as well.

3.13 Turbidimetric analysis of fibrin networks

The turbidimetric analysis was done on both undiluted plasma samples and the purified fibrinogen for each subject. For the purified fibrinogen, a standard fibrinogen concentration of 1 mg/ml was used. The lag-time to clotting, the slope of the turbidity curves and maximum absorbance were determined using turbidity curves generated on a Labsystems Multiscan Ascent (Research Technologies Division, Helsinki, Finland). The lag-time represents the amount of time it takes for proto-fibrils to reach a sufficient length for lateral aggregation to take place and thus can also be an indication of the rate of proto-fibril formation. The slope is an indication of lateral aggregation and indicates the rate of lateral aggregation and the maximum absorbance is and indication of fibre size. Coefficients of variation were not calculated for the turbidimetric measurements because all the analyses were done in duplicate.

3.13.1 Turbidimetric analysis for plasma samples

Of each subject's plasma or fibrinogen solution, 132 μl was added to micro-wells in duplicate. Then 18 μl of thrombin/calcium chloride reagent was added to initiate the clotting. The thrombin/calcium reagent provided a final concentration of 0.5 U/ml thrombin and 20 mM calcium chloride in a final volume of 150 μl.

All absorbance readings were taken at 405 nm. Absorbance measurements were initially taken every 7 seconds for 2 minutes, then every 15 seconds for the next 30 minutes and finally, every 5 minutes for the rest of the time remaining for a total of 60 minutes. The lag-time was measured at the time taken for the absorbance to increase by 0.015 from baseline and the slope of the turbidity curves was calculated at half the maximum absorbance reached, while the maximum absorbance was taken to be the absorbance at 60 minutes minus the baseline absorbance.

3.13.2 Turbidimetric analysis for purified fibrinogen samples

In duplicate, 132 μl of purified fibrinogen solution (1 mg/ml) was added to micro-wells. Then 18 μl of thrombin/calcium chloride reagent was added to initiate the clotting. The thrombin/calcium reagent provided a final concentration of 1 U/ml thrombin and 5 mM calcium chloride in a final volume of 150 μl.

The rest of the process was done as described above for the plasma samples.

3.14 Statistical analyses

The computer package, Statistica (Statsoft Inc., Tulsa, Oklahoma, USA) was used for all statistical analysis. A power calculation was done using 1 standard deviation as a clinical significant difference, as a defined clinical significant difference is not known for the main outcome variable, fibrinogen glycation. To achieve a difference of 1 standard deviation at 80 % power, 5 % significance, each group should consist of at least 16 Individuals. Data were tested for normality. Normally distributed data are presented as the mean (95% confidence interval). Non-normally distributed data are presented as median (25^{th}, 75^{th} percentile). Differences in baseline characteristics as well as differences in changes during the intervention between the

two groups were determined using the t-test for independent samples for parametric data. For non-parametric data, the Mann-Whitney U test was used. Differences from baseline to end within each group were determined using the t- test for dependent samples for parametric data and Wilcoxon matched pairs for non-parametric data. Volunteers were divided into three baseline fibrinogen glycation categories and three baseline fibrinogen concentration categories, in order to asses the effect of glycation and fibrinogen concentration levels on fibrin network structure variables. For parametric data, ANOVA was used to measure differences in fibrin network structure variables for these subdivisions with Tukey's Honest significant difference test for post-hoc comparisons. For non parametric data, the Kruskal-Wallis ANOVA was done with Mann-Whitney U-test with Bonferroni adjustments for post-hoc test comparisons. Spearman rank order correlation coefficients were calculated.

3.15 Conclusion

The results obtained from the different analyses were used to address the study objectives and assess whether the intervention treatment resulted in changes to the level of fibrinogen glycation and whether this in turn resulted in any changes to the fibrin network structure variables of the type 2 diabetic volunteers. The results are reported in Chapter 4 and discussed in Chapter 5.

Chapter 4: Results

4.1 Introduction

This intervention study was undertaken to measure the fibrinogen levels as well as the levels of fibrinogen glycation that prevailed in a group of type 2 black South African diabetic volunteers and compare these to the levels of a reference group of non-diabetic volunteers. Investigations were carried out to determine whether there were differences in fibrin network structure characteristics of the non-diabetic compared to the diabetic volunteers. Whether normalization of hyperglycaemia of the volunteers with type 2 diabetes with insulin treatment would result in changes to the fibrinogen glycation as well as alterations to the selected fibrin network structure variables was also investigated.

Two non-diabetic volunteers did not return at the end of the intervention period when end blood samples were drawn and were, therefore, not included in the statistical analysis of results.

This chapter describes the results that were obtained from the intervention study after statistical analysis. Chapter 5 will discuss the results which will be presented here and also present the conclusions that can be drawn, based on the results that were obtained, as well as give some suggestions for further research. The Annexure has copies of two articles that have been published based on the results of the work done on this study.

The tables and figures in this chapter are not numbered in the order in which they are cited in the text. They are presented in section 4.12 of this chapter, starting on page 61. This arrangement was opted for in order to have the tables and figures presented in a format that allows for easier reading, due to the nature of the data presented.

4.2 Baseline characteristics of the study population

The baseline characteristics of the two groups are presented in Table 4.1. There were no differences observed between the diabetic and non-diabetic volunteers for BMI (kg/m^2), fasting serum-insulin (mU/l), plasma fibrinogen (g/l) and $PAI\text{-}1_{act}$ (U/ml). The diabetic volunteers, on the other hand, had significantly higher levels of venous glucose (mmol/l), triglycerides (mmol/l), insulin resistance (HOMA) and HbA1c as expected for this group. Both groups had serum-insulin levels within normal range (2-20 μU/ml) (Kasper, Braunwald, Fauci, Hauser, Longo, & Jameson, 2005). In addition, both groups were obese, with mean BMI values of 30.8 and 31.8 kg/m^2 for the diabetic and the non-diabetic volunteers, respectively. There was a significant increase in BMI ($p=0.001$) in the diabetic volunteers as a result of the intervention.

4.3 Fibrinogen concentration

There was no statistically significant difference in the fibrinogen concentration of the diabetic volunteers and the non-diabetic volunteers, both before and after the intervention (Table 4.2). There was also no significant change in the fibrinogen concentration as a result of the intervention for the diabetic volunteers.

When the whole group was taken into consideration, fibrinogen concentration at baseline had a significant negative correlation with permeability ($r = -0.41$, $p = 0.01$), a positive correlation with the slope of turbidity curves ($r = 0.37$, $p = 0.02$) and a positive correlation with the maximum absorbance ($r = 0.46$, $p = 0.003$) (Table 4.4), when these variables were measured from plasma samples. These correlations indicate an association between fibrinogen concentration and the structural characteristics of the fibrin networks formed. There was no need to correlate the fibrin network structure variables obtained from purified fibrinogen with fibrinogen concentration since the fibrinogen concentration was standardized to 1.0 mg/ml for these analyses.

4.4 Fibrinogen purification

The purification of fibrinogen was done successfully without any apparent damage to the molecule. The SDS-PAGE analysis (Fig.4.1) shows that there was no damage to the Aα, Bβ and γ fibrinopeptide components of the purified fibrinogen. The analysis also shows that there was no contamination of the purified samples.

4.5 Fibrinogen glycation

The diabetic volunteers had a significantly higher level of fibrinogen glycation than the non-diabetic volunteers (Table 4.2) ($p = 0.0002$). The diabetic volunteers had a mean level of glycation of 7.84 mol glucose/mol of fibrinogen while the non-diabetic volunteers had a mean of only 3.89mol glucose /mol fibrinogen. There was a significant decrease in the level of fibrinogen glycation in the diabetic volunteers as a result of the intervention, from 7.84 to 5.24 mol glucose/mol fibrinogen ($p = 0.0007$). The level of fibrinogen glycation for the diabetic volunteers after the intervention was, however, still higher than that for the non diabetic volunteers (5.24 vs 3.75 mol glucose/mol fibrinogen) ($p=0.001$).

At baseline there was a significant correlation between fibrinogen glycation and levels of glycaemic control like HbA1c and fasting venous glucose ($r=0.69$ and 0.80 for fasting venous glucose and HbA1c, respectively; $p<0.0001$ for both variables) (Table 4.4).

There was no significant correlation between fibrinogen glycation and any of the fibrin network structure variables such as permeability, compaction, lag-time, slope or maximum absorbance when these variables were measured using plasma samples.

When purified fibrinogen was used on the other hand, a significant correlation was observed between the level of glycation and the slope of the curves ($r=0.47$; $p=0.01$)(Table 4.5). The slope, using purified fibrinogen, also correlated with fasting venous glucose ($r=0.51$; $p=0.005$) and HbA1c ($r=0.59$; $p=0.001$).

4.6 Compaction of fibrin networks

Compaction values represent the volume of supernatant fluid released from the fibrin network upon compaction and is a measure of the extent to which the fibrin networks become compacted under stress. Compaction also gives an indication of clot rigidity. Compaction measurements were only done with plasma samples and not with purified fibrinogen because of the large volumes needed for compaction analysis. There was no significant difference in compaction values for the diabetic volunteers when compared to the non diabetic volunteers (Table 4.2), but there was a significant decrease in compaction for the diabetic volunteers after the intervention ($p=0.013$). There was a positive correlation between compaction and permeability ($r = 0.53$, $p < 0.001$).

4.7 Permeability of fibrin networks

Permeation coefficients are an indication of the porosity of the fibrin network structure. When determined from plasma samples there were no significant differences observed in permeation between the diabetic volunteers and the non diabetic volunteers or for the diabetic volunteers, before and after intervention. Permeation was negatively correlated to fibrinogen concentration and positively correlated to compaction as already indicated above. There was also a positive correlation between permeation and high density lipoprotein concentration ($r = 0.48$, $p = 0.003$), but no correlations observed with either total cholesterol or low density lipoprotein (Table 4.4).

When permeation was measured using purified fibrinogen, there was no significant difference between the diabetic volunteers and the non-diabetic volunteers at baseline (Table 4.3), but there was a significant increase in permeation for the diabetic volunteers as a result of the intervention ($p = 0.02$) that was not seen in the plasma model. The permeation coefficient increased from 2.45×10^{-8} to 2.85×10^{-8} cm^2. Though this increase is small in magnitude, it was consistent across the group. The permeation value determined using purified fibrinogen did not correlate with any of the other fibrin network variables used (Table 4.5).

4.8 Turbidimetric analysis

Turbidimetric analyses were performed on both plasma samples and purified fibrinogen and the lag-time, slope and maximum absorbance of the turbidity curves were measured at 405 nm. The lag-time represents the time required for the fibrin proto-fibrils to reach a sufficient length for lateral aggregation to take place and also gives an indication of the rate of proto-fibril formation. The slope of the curves represents the rate of increase of turbidity during the clotting process and is also an indication of the rate of proto-fibril aggregation while the maximum absorbance is an indication of fibre size.

For the fibrin networks prepared from plasma there was no significant difference between the lag-time of the diabetic and non-diabetic volunteers at baseline (Table 4.2). There was, however, a significant increase in the lag-time for the diabetic volunteers as a result of the intervention ($p=0.03$). There were no significant changes seen in the slope or the maximum absorbance of the turbidity curves. The plasma determined lag-time correlated negatively with the slope of the curves ($r = -0.43$, $p = 0.007$) and there was a positive correlation between the slope and maximum absorbance of the curves ($r = 0.66$, $p < 0.0001$).

For the fibrin networks prepared from purified fibrinogen there were no significant differences observed for the lag-time and maximum absorbance of the diabetic and non-diabetic volunteers, at baseline, nor for changes in these variables in the diabetic volunteers as a result of the intervention (Table 4.3). There was, however, a difference in the slope between the diabetic and the non-diabetic volunteers at baseline (5.86 vs 3.95; $p<0.001$). There were positive correlations between the slope of the turbidity curves and the level of glycation ($r=0.47$; $p=0.01$), lag-time ($r=-0.43$; $p=0.02$), as well as the level of glycaemic control as determined by both HbA1c ($r=0.59$; $p=0.001$), and venous glucose ($r=0.51$; $p=0.005$).

4.9 Correlations between changes from baseline to end of the fibrin network structure variables

The correlation analysis of the changes in fibrin network structure variables from plasma gave no significant correlations between the variables (Table 4.6) although the correlation between maximum absorbance and slope reached near significance (r=0.31; p=0.06).

When the analysis was done with variables from purified fibrinogen (Table 4.7), the correlation between maximum absorbance and slope reached significance (r=0.66; p=0.0001).

4.10 Comparison of fibrin network structure variables across three categories of fibrinogen glycation

The total group was divided into three levels of glycation categories (Table 4.8 and 4.9). The glycation category ranges were *category 1*: 2.75 to 5.65; *category 2*: 5.66 to 8.87 and *category 3*: > 8.87 mol glucose/mol fibrinogen. Since there was a significant difference in fibrinogen glycation between the volunteers with type 2 diabetes and the non-diabetic volunteers, this grouping was decided upon in order to have all the non-diabetic volunteers fall into one category since they represented a level of fibrinogen glycation unaffected by diabetes. This grouping by glycation level resulted in all the non-diabetic and 25 percent of the diabetic volunteers falling into category 1, with 35 and 40 percent of the diabetic volunteers falling into categories 2 and 3 respectively.

Analysis of variance of the fibrin network structure variables from plasma across the three levels of glycation showed no significant differences among the groups (Table 4.8).

For the variables determined from purified fibrinogen (Table 4.9), there were significant differences among the baseline slopes over the three glycation levels (p=0.01). The slope was higher with increase in fibrinogen glycation, implying a higher rate of proto-fibril aggregation with increase in fibrinogen glycation.

4.11 Comparison of fibrin network structure variables across three categories of fibrinogen concentration

The total group was divided into three fibrinogen concentration categories, (Table 4.10 and 4.11). Since there was no significant difference in fibrinogen concentration between the diabetic and non diabetic volunteers at baseline, the division was done to rationalize the fibrinogen concentrations into three categories of equal increments. The fibrinogen concentration ranges were *category 1*: 2.47 to 3.77; *category 2*: 3.78 to 5.07 and *category 3*: 5.08 to 6.37 g/l fibrinogen. This grouping by fibrinogen concentration level resulted in 42, 32 and 26 percent of the volunteers falling into categories 1, 2 and 3 of fibrinogen concentration, respectively.

Based on the analysis of variance done across the fibrinogen concentration levels for variables determined from plasma, significant differences were found among the three fibrinogen concentration levels for permeability coefficients, the change in lag-time and change in slope ($p=0.02$) as a result of the intervention (Table 4.10). The differences were such that the permeability decreased with increase in fibrinogen concentration. The change in lag-time increased with fibrinogen concentration indicating a larger lowering of lag-time or the rate of proto-fibril formation with fibrinogen concentration from baseline to end. There was a larger decrease in the change in slope with fibrinogen concentration from baseline to end. There was also a near significant increase in the baseline maximum absorbance ($p=0.06$) with fibrinogen concentration from baseline to end.

When the analysis of variance was done for the variables from purified fibrinogen only the change in lag-time from baseline to end differed significantly among the levels of fibrinogen concentration ($p=0.04$) (Table 4.11). The increase in lag-time was bigger with fibrinogen concentration from baseline to end. This is a similar trend to what was observed when the analysis was done in plasma.

4.12 Comparison of percent changes in the fibrin network structure variables from baseline to end between plasma and purified fibrinogen

Scatter plots of the percent changes in fibrin network structure variables of the diabetic and non-diabetic volunteers as a result of the intervention are presented in Figures 4.2 to 4.5. The differences in trend in the percentage change in a variable is most apparent for the percent change in slope where less variation in the results is observed at the end than at baseline. In addition less variation in the slope results is apparent when purified fibrinogen is used compared to the analyses done in plasma (Fig. 4.4).

4.13 Tables and figures for Chapter 4

All the tables and figures referred to in this chapter are presented in this section.

Table 4.1. Baseline characteristics of type 2 diabetic and non-diabetic volunteers

Variables	**Type 2 diabetic volunteers**	**Non-diabetic volunteers**
Patients (n)	20	18
Gender (men/women)	6 / 14	7 / 11
Age (years)	53.0 (49.1 ; 56.9)	52.9 (49.2 ; 56.6)
Body mass index (kg/m2)	30.8 (28.0 ; 33.7)	31.8 (28.8 ; 34.7)
Venous glucose (mmol/l)	14.6 (10.8 ; 18.4)*	5.18 (4.56 ; 5.80) *
Fasting serum-insulin (mU/l) #	11.0 [6.70 ; 15.8]	13.7 [8.90 ; 28.8]
Insulin resistance (HOMA)	5.18 (3.99 ; 6.95) †	3.11 (2.29 ; 7.42) †
HbA1c #	11.7 [9.50 ; 13.8] *	5.60 [5.30 ; 5.90] *
Fibrinogen (g/l)	4.25 (3.88 ; 4.63)	4.02 (3.59 ; 4.45)
Fibrinogen glycation (mol glucose/mol fibrinogen)	7.84 (6.59 ; 9.10) *	3.89 (3.46 ; 4.32) *
Systolic blood pressure (mmHg)	140.5 (129.6 ; 151.3)	143.3 (130.2 ; 156.4)
Diastolic blood pressure (mmHg)	86.6 (82.7 ; 90.6) †	89.8 (83.3 ; 96.3)
Total cholesterol (mmol/l)	4.84 (4.14 ; 5.53)	4.54 (4.16 ; 4.92)
Low density lipoprotein cholesterol (mmol/l)	2.86 (2.37 ; 3.34)	2.88 (2.43 ; 3.34)
High density lipoprotein cholesterol (mmol/l) #	0.9 (0.75 ; 1.3)	0.9 (0.80 ; 1.25)
Triglycerides (mmol/l) #	1.80 (1.25 ; 2.50) †	1.05 (0.80 ; 1.25) †
PAI-1act (U/ml) #	16.4 [9.34-19.6]	14.6 [13.6-20.1]
Duration of diabetes (years)	11.0 (8.00 ; 15.0)	n/a

Note: * P < 0.001; † P< 0.05; # Data not normally distributed and therefore reported as median [25 , 75 percentile]; Insu
resistance = (fasting insulin x fasting venous glucose)/22.5

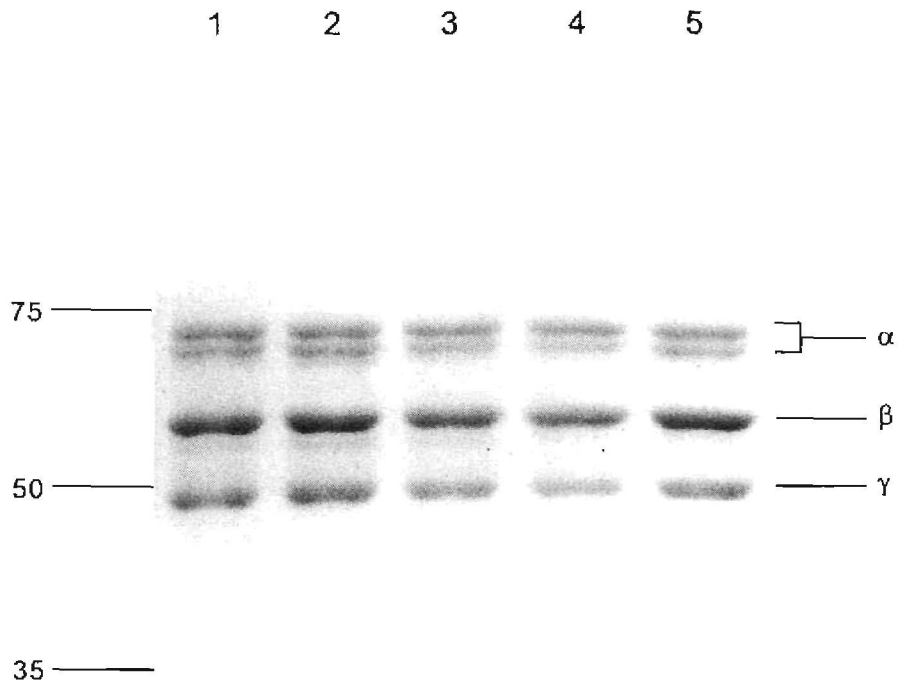

Figure 4.1 A representative SDS-PAGE analysis of purified fibrinogen samples to show that the fibrinogen purification process did not cause any damage to fibrinogen. Lane 1: a commercial human fibrinogen (MP Biologicals cat no. 151123) in comparison to Lanes 2 and 3: fibrinogen isolated from a diabetic volunteer before and after glucose control intervention, respectively, and Lanes 4 and 5: fibrinogen purified from a non-diabetic volunteer before and after the intervention period, respectively. The positions of molecular weight markers (x 10^3) of the respective peptides, Aα, Bβ, and γ are also indicated.

Table 4.2. The effect of glycaemic control on BMI, PAI-1_{act}, fasting glucose, fibrinogen, fibrinogen glycation and selected fibrin network structure variables from plasma of the type 2 diabetic and non-diabetic volunteers, before and after the intervention period

Variable	Type diabetic volunteers (n=20)				Non-diabetic volunteers (n=18)				Changes between groups (deltas)
	Baseline	End	Change	p	Baseline	End	Change	P	p
BMI (kg m^2)	30.8 (28.0; 33.7)	32.2 (28.9; 35.5)	1.79 (0.79; 2.79)	0.001	31.8 (28.8; 34.7)	32.0 (28.9; 35.1)	0.30 (-0.11; 0.72)	0.14	0.009
PAI-1_{act} (U/ml) #	16.4 [9.34;19.6]	14.8 [8.69; 17.8]	-1.16 [-7.14; 1.92]	0.41	14.6 [13.6; 20.1]	15.4 [11.3; 21.8]	-1.39 [-3.43; 2.60]	0.61	0.65
Fasting glucose (mmol/l)	14.6 * (10.8; 18.4)	6.72 (5.51; 7.92)	-7.88 (-12.4; 3.72)	0.0008	5.18 * (4.56; 5.80)	5.63 (4.95; 6.31)	0.46 (0.00; 0.91)	0.05	0.0003
Fibrinogen (g/l)	4.25 (3.88; 4.63)	4.36 (3.99; 4.73)	0.11 (-0.34; 0.55)	0.62	4.02 (3.59; 4.45)	3.85 (3.43; 4.28)	-0.16 (-0.52; 0.19)	0.35	0.33
Fibrinogen glycation (mol glucose / mol fibrinogen)	7.84 ‡ (6.59; 9.10)	5.24 † (4.47; 6.01)	2.60 (-3.82; -0.39)	0.0002	3.89 ‡ (3.46; 4.32)	3.75 † (3.43; 4.07)	-0.19 (-0.55; 0.17)	0.27	0.0007
Permeation coefficient ($x10^{-9}$ /cm^2) #	8.67 [7.60; 11.5]	9.6 [7.75; 11.7]	-0.87¥ [-3.17; 1.42]	0.33	9.57 [7.84; 13.3]	10.8 [8.3; 13.1]	0.89 [-0.79; 3.05]	0.23	0.10
Compaction (%)	39.6 (36.8; 42.3)	37.1 (33.8; 40.4)	-2.43 (-4.29; -0.58)	0.013	37.7 (34.2; 41.2)	36.5 (32.6; 40.3)	-1.84 (-5.47; 1.78)	0.30	0.72
Lag time (s)	27.8 (26.0; 29.5)	29.7 (27.2; 32.1)	1.89 (0.17; 3.61)	0.03	27.0 (25.1; 28.8)	28.1 (26.3; 30.0)	1.14 -0.46; 2.75)	0.15	0.51
Slope	23.6 (20.3; 26.2)	22.9 (19.0; 26.7)	-0.41 (-4.2; 3.39)	0.82	26.0 (21.4; 30.5)	28.4 (24.0; 32.8)	2.43 (-1.20; 6.07)	0.18	0.27
Max absorbance	1.18 (1.09; 1.26)	1.22 (1.10; 1.33)	0.04 (-0.08; 0.16)	0.53	1.22 (1.12; 1.33)	1.22 (1.10; 1.34)	0.00 (-0.09; 0.08)	0.91	0.56

* Significant difference in fasting glucose between type 2 diabetic volunteers and non-diabetic volunteers at baseline ($p < 0.001$).
‡ Significant difference in fibrinogen glycation between the type 2 diabetic volunteers and non-diabetic volunteers ($p < 0.001$) at baseline.
† Significant difference in fibrinogen glycation between the type 2 diabetic volunteers and non-diabetic volunteers ($p = 0.001$) at the end of the intervention.
# Ks and PAI-1 data not normally distributed therefore reported as median [25-75 percentile].
¥ The delta value is negative despite a higher end value because more volunteers (12 out of 20) had decreased permeation coefficients. The eight who showed an increase, however, had a larger average increase.

Table 4.3 The effect of glycaemic control on fibrin network structure variables from purified fibrinogen of type 2 diabetic and non-diabetic volunteers, before and after the intervention period

Variable	Type 2 diabetic volunteers (n=20)				Non- diabetic volunteers (n=18)				Changes between groups (deltas)
	Baseline	End	Change	p	Baseline	End	Change	p	p
Fasting glucose (mmol/l)	14.6 * [10.8; 18.4]	6.72 [5.51; 7.92]	-7.88 [-12.04; 3.72]	0.0008	5.18 *[4.56; 5.80]	5.63 [4.95; 6.31]	0.46 [0.00; 0.9]	0.05	0.0003
Fibrinogen glycation (mol glucose / mol fibrinogen)	6.81 * [5.64; 10.7]	5.02 [4.19; 5.86]	-2.49 [-3.70; -0.67]	0.0002	3.84 * [3.27; 4.20]	3.69 [3.27; 4.34]	-0.2 [-0.44; 0.26]	0.36	0.0007
Ks ($x10^{-8}$ /cm^2	2.45 [1,95; 2.65]	2.85 [2.24; 3,37]	0.45 [0.00; 1.48]	0.02	2.54 [2.0; 3.15]	2.8 [2.43; 3.59]	0.06 [-0.45; 3.6]	0.7	0.16
Lag time (s)	36.5 [34.5; 38.4]	37.6 [33.2; 40.4]	0.16 [-3.28; 2.59]	0.86	38.7 [34.6; 40.9]	38.9 [33.3; 42.9]	-0.12 [-2.02; 2.41]	0.53	0.95
Slope	5.86 * [4.78; 6.33]	5.04 [4.25; 5.81]	-0.69 [-1.02;0.69]	0.21	3.95 * [3.53;4.64]	3.69 [3.32; 4.42]	0.11 [-0.31; 0.25]	0.69	0.29
Max absorbance	0.67 [0.63; 0.78]	0.65 [0.58; 0.71]	-0.03 [-0.12; 0.06]	0.19	0.70 [0.57; 0.73]	0.64 [0.56; 0.71]	-0.03 [-0.12; 0.10]	0.76	0.67

All data reported as median [25-75 percentile].
* Significant difference between type 2 diabetic and non-diabetic volunteers at baseline ($p<0.001$)

Table 4.4 Correlations between fibrin network structure variables from plasma and fibrinogen glycation with other variables associated with diabetes for the total group at baseline.

	Permeability (cm^2x10^{-9})		Compaction		Lag time (s)		Slope		Max Absorbance		Fibrinogen glycation (mol glucose/mol fibrinogen)	
	r	p	r	p	r	p	r	p	r	p	r	p
Fasting Glucose (mmol/l)	-0.13	0.43	0.20	0.24	0.18	0.28	-0.19	0.26	-0.21	0.21	0.69	<0.0001
HbA1c	-0.24	0.15	0.17	0.33	0.16	0.36	-0.14	0.41	-0.22	0.19	0.80	<0.0001
Insulin resistance (HOMA)	-0.20	0.25	-0.13	0.47	-0.03	0.86	0.01	0.94	-0.06	0.74	0.14	0.42
BMI (kg/m^2)	-0.25	0.16	-0.17	0.32	-0.17	0.31	0.11	0.51	0.06	0.7	0.02	0.90
Fibrinogen glycation (mol glucose/mol fibrinogen)	-0.14	0.39	0.16	0.36	0.22	0.19	-0.22	0.19	-0.22	0.18	---	---
Fibrinogen (g/l)	-0.41	0.01	-0.08	0.62	-0.30	0.07	0.37	0.02	0.46	0.003	0.10	0.55
$PAI\text{-}1_{act}$ (U/ml)	0.08	0.65	0.18	0.29	-0.25	0.13	0.16	0.34	-0.22	0.18	-0.06	0.73
Triglycerides (mM)	-0.28	0.10	-0.08	0.64	0.023	0.89	-0.17	0.31	-0.21	0.23	0.40	0.01
Total cholesterol (mM)	0.22	0.20	0.14	0.44	-0.074	0.67	0.19	0.26	0.07	0.68	-0.07	0.66
HDL-Cholesterol (mM)	0.48	0.003	0.29	0.09	-0.18	0.31	0.25	0.14	0.05	0.75	-0.26	0.13
LDL-Cholesterol (mM)	0.21	0.22	0.093	0.59	-0.04	0.80	0.29	0.09	0.15	0.37	-0.12	0.49
Permeability (cm^2x10^{-9})	---	---	0.53	<0.001	-0.14	0.41	0.07	0.70	0.004	0.98	-0.14	0.39
Compaction	0.53	<0.001	---	---	-0.13	0.44	0.29	0.09	0.16	0.35	0.16	0.36
Lag time (s)	-0.14	0.41	-0.13	0.44	---	---	-0.43	0.007	-0.23	0.17	0.22	0.19
Slope	-0.07	0.70	0.29	0.09	-0.43	0.007	---	---	0.66	<0.0001	-0.22	0.19
Max Absorbance	0.004	0.98	0.16	0.35	-0.23	0.17	0.66	<0.0001	---	---	-0.22	0.18

Table 4.5. Correlations between different fibrin network structure variables from purified fibrinogen, with other variables associated with diabetes for the total group at baseline

	Permeability (cm^2x10^{89})		Lag time (s)		Slope		Max Absorbance	
	r	p	r	p	r	p	r	p
Fasting Glucose (mmol/l)	-0.24	0.21	-0.02	0.91	0.51	0.005	-0.07	0.73
HbA1c %	-0.26	0.18	-0.11	0.59	0.59	0.001	-0.01	0.63
Insulin resistance (HOMA)	0.11	0.56	-0.02	0.87	0.18	0.38	-0.20	0.31
Fibrinogen glycation (mol glucose/mol fibrinogen)	-0.25	0.18	0.02	0.93	0.47	0.01	-0.02	0.93
Fibrinogen (g/l)	0.03	0.89	-0.18	0.35	0.20	0.29	-0.01	0.95
$PAI\text{-}1_{act}$ (Collier, Rumley, Paterson, Leach, Lowe, & and Small, 1992b)	-0.03	0.89	0.14	0.47	-0.12	0.54	-0.01	0.96
Triglycerides (mM)	-0.30	0.11	0.21	0.28	0.18	0.37	-0.32	0.10
Total cholesterol (mM)	0.11	0.57	0.11	0.58	0.07	0.73	-0.02	0.91
HDL-Cholesterol (mM)	0.16	0.42	0.0009	0.996	0.09	0.67	0.26	0.20
LDL-Cholesterol (mM)	0.10	0.60	-0.13	0.52	0.01	0.97	-0.02	0.94
Permeability (cm^2x10^{-8})	---	---	-0.36	0.07	-0.03	0.88	0.17	0.41
Lag time (s)	-0.36	0.07	---	---	-0.43	0.02	-0.28	0.15
Slope	-0.03	0.88	-0.43	0.02	---	---	0.17	0.38
Max Absorbance	0.17	0.41	-0.28	0.15	0.17	0.38	---	---

Figure 4.2 Percent change in permeability obtained from turbidimetric analyses of plasma and purified fribinogen from samples obtained before and after intervention.

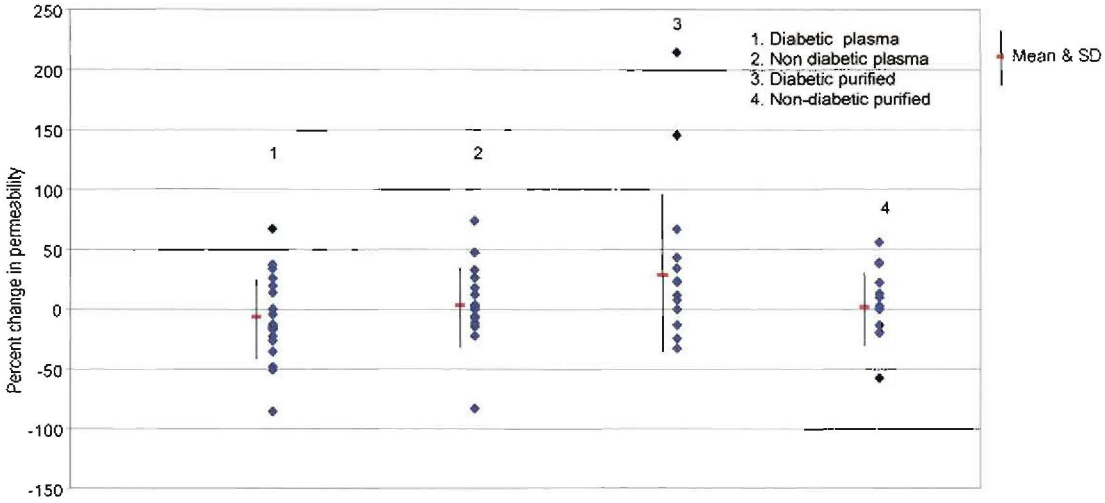

Figure 4.3. Percent change in lag-time obtained from turbidimetric analyses of plasma and purified fribinogen from samples obtained before and after intervention.

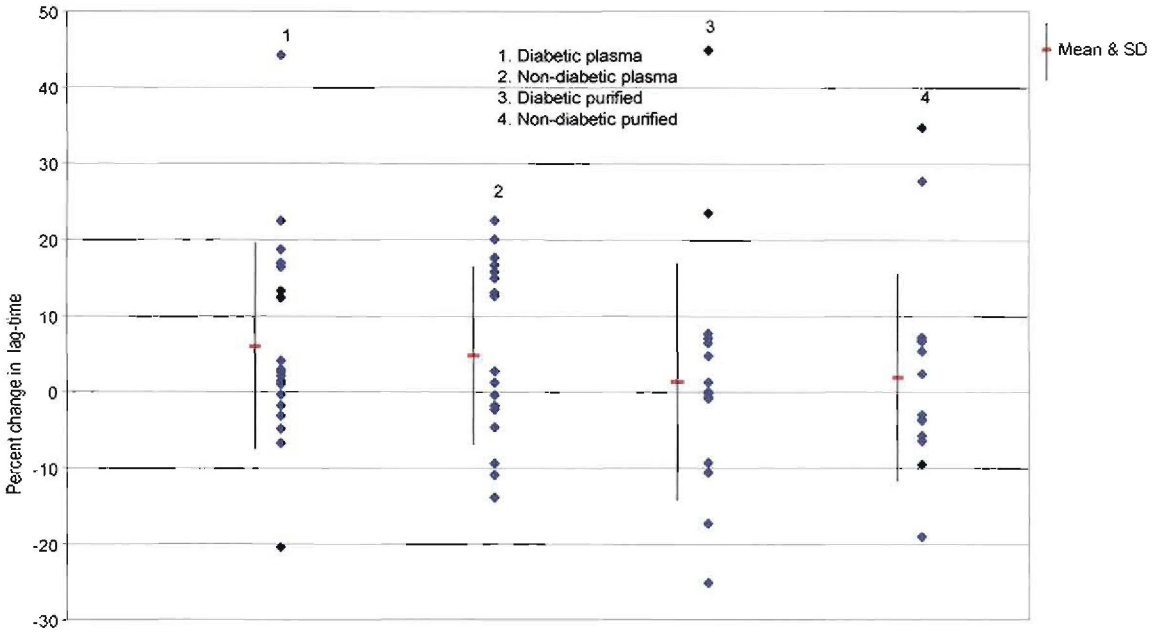

Figure 4.4 Percent change in slope obtained from turbidimetric analyses of plasma and purified fribinogen from samples obtained before and after intervention.

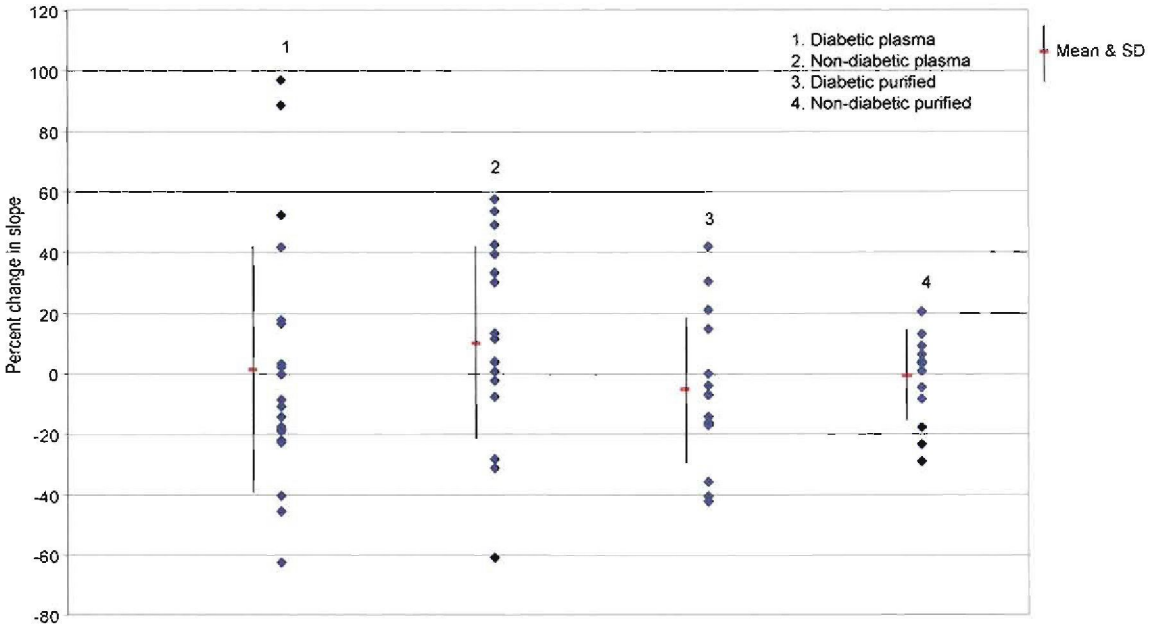

Figure 4.5 Percent change in maximum absorbance obtained from turbidimetric analyses of plasma and purified fribinogen from samples obtained before and after intervention.

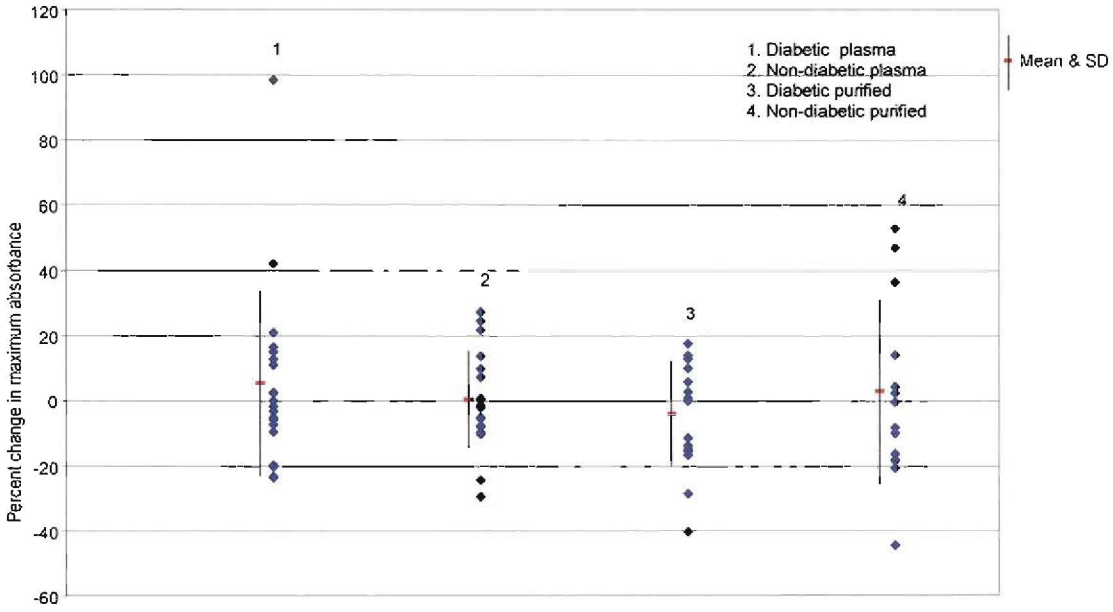

Table 4.6. Correlations between changes in fibrin network structure variables obtained from plasma from baseline to end (n=38)

	Permeability		Lag time		Slope		Max absorbance		Compaction	
	r	p	r	p	r	p	r	p		
Permeability	---	---	0.01	0.94	0.19	0.25	-0.08	0.62	0.21	0.21
Lag time	0.01	0.94	---	---	-0.07	0.65	0.005	0.98	0.10	0.55
Slope	0.19	0.25	-0.07	0.65	---	---	0.31	0.06	0.27	0.11
Max absorbance	-0.08	0.62	0.005	0.98	0.31	0.06	---	---	0.27	0.11
Compaction	0.21	0.21	0.10	0.55	0.27	0.11	0.27	0.11	---	---
Fibrinogen glycation	0.18	0.27	-0.25	0.13	0.27	0.11	0.12	0.53	0.27	0.11

Table 4.7. Correlations between changes in fibrin network structure Variables obtained from purified fibrinogen from baseline to end (n=38)

	Permeability		Lag time		Slope		Max absorbance	
	r	p	r	p	r	p	r	p
Permeability	---	---	0.10	0.62	-0.07	0.75	0.26	0.21
Lag time	0.10	0.62	---	---	-0.08	0.68	-0.02	0.94
Slope	-0.07	0.75	-0.08	0.68	---	---	0.66	0.0001
Max absorbance	0.25	0.21	-0.02	0.94	0.66	0.001	---	---
Fibrinogen glycation	-0.06	0.76	-0.11	0.59	0.25	0.19	0.21	0.28

Table 4.8. Fibrin network structure variables from plasma divided into categories according to fibrinogen glycation levels

Variable	Fibrinogen glycation categories			p
	Category 1 (n=23)	Category 2 (n=7)	Category 3 (n=8)	
Permeability at baseline (cm^2x10^{-9})#	9.88 [7.96; 13.8]	8.54 [7.28; 8.89]	9.67 [8.22; 11.5]	0.46
Delta permeability (cm^2x10^{-9})	0.13 (-9.22; 9.48)	-0.33 (-3.59; 2.93)	-0.25 (-2.35; 1.86)	0.99
Lag-time at baseline (s)	27.0 (25.4; 28.6)	27.7 (24.0; 31.3)	28.2 (24.7; 31.7)	0.73
Delta Lag-time (s)	1.23 (-0.45; 2.91)	0.81 (-1.27; 2.89)	3.05 (-0.81; 5.30)	0.37
Slope at baseline	25.5 (21.8; 29.5)	25.6 (20.7; 30.5)	20.8 (15.2; 26.3)	0.32
Delta Slope	2.61 (-0.68; 5.89)	03.25 (-9.90; 3.39)	-0.19 (-6.93; 6.55)	0.20
Max absorbance at baseline	1.20 (1.11; 1.29)	1.25 (1.19; 1.30)	1.15 (0.96; 1.33)	0.64
Delta Max absorbance	0.06 (-0.05; 0.16)	0.01 (-0.16; 0.18)	-0.08 (-0.23; 0.06)	0.33
% Compaction at baseline	38.3 (35.2; 41.5)	39.7 (34.8; 44.7)	38.9 (34.6; 43.2)	0.88
Delta % compaction	-1.32 (-4.12; 1.49)	-1.96 (-4.89; 0.98)	-4.60 (-7.90; -2.30)	0.34

Permeability data non-parametric and reported as median [25; 75 th percentile]

Table 4.9. Fibrin network structure variables obtained from purified fibrinogen divided into categories according to fibrinogen glycation levels

Variable	Fibrinogen glycation categories			p
	Category 1 (n=23)	Category 2 (n=7)	Category 3 (n=8)	
Permeability at baseline (cm^2 $x10^{-8}$) #	2.54 [2.26; 2.79]	1.98 [1.67; 2.64]	2.50 [1.47; 3.89]	0.35
Delta permeability (cm^2 $x10^{-8}$)	0.24 (-0.75; 1.24)	0.25 (-0.56; 1.06)	1.72 (-1.55; 4.98)	0.32
Lag-time at baseline (s) #	38.2 [34.6;40.9]	36.7 [32.8; 36.8]	36.4 [35.6; 36.5]	0.64
Delta Lag-time (s)	-0.21 (-2.84; 2.43)	-1.79 (-9.78; 6.21)	5.60 (-1.64; 12.8)	0.09
Slope at baseline #	4.20 [3.59; 4.97]	5.49 [4.03; 6.58]	6.05 [5.86;6.20]	0.03
Delta Slope	-0.30 (-0.78; 0.17)	0.63 (-1.13; 2.39)	-0.84 (-1.84; 0.16)	0.10
Max absorbance at baseline #	0.71 [0.57; 0.73]	0.65 [0.64; 0.68]	0.67 [0.63; 0.78]	0.89
Delta Max absorbance	-0.03 (-0.10; 0.05)	0.02 (-0.14; 0.19)	-0.07 (-0.17; 0.04)	0.62

data presented as median [25; 75 th percentile]

Table 4.10. Fibrin network structure variables obtained from plasma divided into categories according to fibrinogen concentration levels

Variable	Fibrinogen concentration categories			p
	Category 1 (n=16)	Category 2 (n=12)	Category 3 (n=10)	
Permeability at baseline cm^2x10^{-9} #	12.9 [8.72 ;16.5]	8.04 [7.60; 9.58]	8.54 [6.61; 9.88]	0.02
Delta permeability cm^2x10^{-9}	-0.24 (-3.05; 2.58)	-6.56 (-19.6; 6.45)	8.11 (-7.35; 23.6)	0.12
Lag-time at baseline (s)	28.6 (26.7; 30.5)	26.9 (25.5; 28.2)	26.1 (22.5; 29.7)	0.23
Delta Lag-time(s)	-0.22 (-1.65; 1.21)	3.22 (0.56; 5.88)	2.32 (0.66; 3.98)	0.02
Slope at baseline	22.0 (19.1; 24.9)	24.4 (19.2; 29.6)	28.8 (22.1; 35.5)	0.10
Delta Slope	4.97 (1.51; 8.43)	-1.12 (-6.56; 4.32)	-3.05 (-7.36; 1.26)	0.02
Max absorbance at baseline	1.14 (1.05; 1.24)	1.17 (1.03; 1.30)	1.32 (1.21; 1.44)	0.06
Delta Max absorbance	0.03 (-0.08; 0.13)	0.09 (-0.09; 0.28)	-0.09 (-0.17; 0.004)	0.17
% Compaction at baseline	40.5 (36.4; 44.5)	36.0 (32.6; 39.3)	39.2 (35.8; 42.7)	0.18
Delta % compaction	-2.86 (-6.56; 0.83)	-1.37 (-4.24; 1.50)	-2.01 (-5.15; 1.13)	0.78

Permeability data non-parametric and reported as median [25; 75 ^{t} percentile]

Table 4.11. Fibrin network structure variables obtained from purified fibrinogen divided into categories according to fibrinogen concentration levels

Variable	Fibrinogen concentration categories			p
	Category 1 (n=16)	Category 2 (n=12)	Category 3 (n=10)	
Permeability at baseline cm^2x10^{-8} #	2.45 [2.00; 2.64]	2.51 [1.95; 2.78]	2.49[1.67; 2.90]	0.97
Delta permeability cm^2x10^{-8}	0.45 (-0.78; 1.69)	1.33 (-0.40; 3.05)	-0.21 (-0.17; 1.32)	0.28
Lag-time at baseline #	38.3 [34.8; 40.5]	36.7 [34.5; 43.2]	36.0 [31.8; 36.8]	0.43
Delta Lag-time	-2.22 (-5.19; 0.75)	0.96 (-2.90; 4.81)	5.15 (-1.07; 11.3)	0.04
Slope at baseline #	4.49 [3.78; 5.12]	4.97 [3.53; 6.20]	5.05 [4.03; 6.05]	0.62
Delta Slope	-0.23 (-0.84; 0.38)	-0.44 (-1.21; 0.33)	-0.01 (-1.21; 1.18)	0.72
Max absorbance at baseline#	0.70 [0.65; 0.74]	0.64 [0.55; 0.72]	0.70 [0.63; 0.76]	0.64
Delta Max absorbance	-0.04 (-0.13; 0.05)	-0.01 (-0.11; 0.09)	-0.04 (-0.20; 0.13)	0.84

Data presented as median [25; 75 th percentile]

Chapter 5: Discussion

5.1 Introduction

This research was the first intervention study involving insulin treatment of out-patient, uncontrolled type 2 diabetic volunteers which has measured both the fibrinogen levels and levels of fibrinogen glycation and investigated the effects of hyperglycaemia and glycaemic control on fibrinogen glycation in order to address three main questions. One, whether there is a difference in the level of fibrinogen glycation between uncontrolled type 2 diabetic volunteers and non-diabetic volunteers; two, whether normalisation of hyperglycaemia, with insulin treatment, of out-patient, uncontrolled type 2 diabetic volunteers, would result in a reduction in levels of fibrinogen glycation and three, whether there would be alteration to the fibrin network structures formed as a result of the reduced level of fibrinogen glycation. Some of the fibrin network structure variables like permeability and turbidimetric measurements were done in both plasma and in a purified fibrinogen system to try and illustrate the role of fibrinogen glycation on its own, in the absence of other plasma constituents. Compaction analysis was done in plasma only because of the large volume of solution needed for the analysis.

5.2 Baseline characteristics of the study population

The volunteers with type 2 diabetes and the non-diabetic volunteers were very similar in terms of their baseline characteristics so inclusion and exclusion criteria succeeded very well in this regard. Smoking and alcohol consumption were not frequent in both groups. Although both groups had serum-insulin values within normal range (Kasper *et al.*, 2005) volunteers with type 2 diabetes had significantly higher levels of insulin resistance. This may be an indication that the volunteers with type 2 diabetes may have reached a stage in the progression of the diabetes where β-cell failure had commenced, as manifested by reduced insulin production. This may have resulted in the presentation of normal serum-insulin levels in spite of being insulin resistant. The Insulin Resistance

Atherosclerosis Study (Festa, D'Agastino, Wagenknecht, & Haffner, 2006), showed that the progression of type 2 diabetes involves a progression from normal glucose tolerance to impaired glucose tolerance, where increased insulin secretion compensates for insulin resistance and finally into type 2 diabetes, where β-cell failure results in reduced insulin production. The higher HbA-1c for the diabetic volunteers was expected since they were specifically selected to have uncontrolled glucose levels.

The increased BMI in the diabetic volunteers over the intervention period could be the effect of the insulin treatment. Increased BMI as a result of insulin therapy is a well established fact. Insulin therapy has been shown to increase BMI as well as hepatic insulin sensitivity (Juurinen, Tiikkainen, Hakkarainen, & Yki-Järvinen, 2007) and this may result in increased glucose and fatty-acid metabolism. Other reasons that have been observed for the increase in weight and thus BMI with insulin treatment include reduced glucose loss in urine resulting in less energy loss to the body increased fat tissue deposition due to increased fatty acid and glucose metabolism, increased appetite because of insulin mediated lowering of plasma glucose levels, as well as reduced energy expenditure resulting from a reduction in energy spent on futile recycling of fuel molecules in the hyperglycaemic condition (Boyne & Saudek, 1999).

5.3 Fibrinogen concentration

The fibrinogen concentration observed in this study was high for the diabetic as well as for the non diabetic volunteers (4.25 and 4.02 g/l, respectively). While fibrinogen levels of people with type 2 diabetes have been reported in some studies to be elevated (Asakawa, Tokunaga, & Kawakami, 2000; Avellone, Di, V, Cordova, Rotolo, Abruzzese, Raneli, De Simone, & Bompiani, 1994; Donders, Lustermans, & van Wersch, 1993; Ford *et al.*, 1991), diabetes does not explain the elevated fibrinogen in this study group since both the diabetic and non-diabetic volunteers had elevated fibrinogen levels. Black Africans have been reported to have elevated fibrinogen levels compared to Caucasians (Folsom *et al.*, 1992; Jerling, Vorster, Oosthuizen, Silvis, & Venter, 1994). James *et al.*

(2000) reported the values of 3.25 and 3.94 g/l fibrinogen adjusted for age for apparently healthy men and women, respectively, who had similar BMI (30 to 34.9 kgm^2) to our subjects. The values reported by James *et al.* (2000) were in agreement with the relatively high fibrinogen levels reported for black Africans, but were still lower than what we report in this study, indicating that other factors other than just race may have influenced the observed fibrinogen levels in this study. This may be in part due to the fact that both hypertension and obesity prevailed in both the diabetic and non-diabetic volunteers and both factors have been associated with elevated fibrinogen levels (James, Vorster, Venter, Kruger, Nell, Veldman, Ubbink, & B., 2000; Juhan-Vague, Morange, Renucci, & Alessi, 1999).

Similar levels of fibrinogen concentrations were observed between the diabetic and non-diabetic volunteers. There is lack of consensus in the literature as to what the effect of diabetes on fibrinogen concentration is (Durın & Ariens, 2004). While some researchers have reported elevated levels of fibrinogen in type 2 diabetes (Asakawa *et al.*, 2000; Ford *et al.*, 1991; Ganda & Arkin, 1992), others have reported no differences between diabetic and non-diabetic individuals (Missov *et al.*, 1996), similar to the finding of this study. Fibrinogen is an acute phase protein (Colley *et al.*, 1983; Festa *et al.*, 2002) and would, therefore, be expected to be elevated in a situation where inflammation is present. In this regard one could speculate that the inflammation status in both groups in this study were similar, which may explain the lack of difference in fibrinogen levels between them. The fact that both groups were hypertensive and obese supports this line of thinking since both obesity (Juhan-Vague *et al.*, 1999) and hypertension (Chae, Lee, Rifai, & Ridker, 2001; Schillaci, Pirro, Gemelli, Vaudo, Marchesi, Siepi, Bagaglia, & Mannarion, 2003) have been shown to be inflammatory states. High BMI (Craveri, tornaghi, Paganardi, Ranieri, Leonardi, & Di Bella, 1987), and high skin adiposity values (Carroll, Cooke, & Butterly, 2000) have both been associated with high fibrinogen levels. The elevated fibrinogen levels in this population group, however, leaves one wondering at which point it becomes a significant CVD risk factor in the population group.

Since volunteers with type 2 diabetes did not exhibit higher fibrinogen levels as a result of the diabetic condition, it is not surprising that a change was not found after intervention. Jorneskog *et al.* (2003) who treated type 1 diabetes patients with continuous sub-cutaneous insulin, did not find any differences in fibrinogen level between the patients and the non-diabetic subjects and this group too, as in this study, did not find any change in fibrinogen level as a result of intervention. D'Elia *et al.* (2001) on the other hand reported reduced fibrinogen levels for type 2 diabetic patients upon attaining glycaemic control. The study by D'Elia *et al.* (2001), however, selected participants specifically to have micro-vascular complications based on the presence of albuminuria or proteinuria as an indication of diabetic complications. The selection criteria for the volunteers with type 2 diabetes in this study group excluded those with apparent micro or macro-vascular diabetic complications and it is, therefore, possible that the diabetic condition in this study group did not significantly influence the fibrinogen level. In addition there may be no differences in fibrinogen concentration between the two groups because they had similar serum-insulin levels and fibrinogen concentration has been linked to insulin levels *per se* (Raynaud, Pérez-Martin, Brun, Aissa-Benhaddad, Fédou, & Mercier, 2000).

Since this study did not focus on inflammation, inflammation markers other than fibrinogen were not measured. This makes it difficult to assess the inflammatory status of the two groups. However, based on the results obtained, it is possible that the two groups were in a similar state of inflammation.

5.4 Fibrinogen glycation

The exposure *in vivo* to higher plasma glucose levels, as indicated by both HbA1c and fasting venous glucose, resulted in a much higher level of fibrinogen glycation in the type 2 diabetic volunteers compared to the non-diabetic volunteers. The positive correlation between both HbA1c and fasting glucose with fibrinogen glycation supports the observed level of glycation in the volunteers with type 2 diabetes. The intervention treatment significantly reduced the fibrinogen glycation (7.84 to 5.24 mol glucose /mol fibrinogen), even though at the

end of the intervention period the glycation level in the diabetic volunteers was still higher than that of the non-diabetic volunteers (3.89 mol glucose /mol fibrinogen). *In vitro* studies have shown that glycation of fibrinogen takes place when it is exposed to glucose in a dose dependent manner (Geiger & Binder, 1986; Nair *et al.*, 1991). Austin *et al.* (1987) also showed that the glycation of plasma proteins is much greater in diabetic than in non-diabetic plasma. Hammer *et al.* (1989) and Lütjens *et al.* (1985) also observed glycation of fibrinogen, *in vivo*. The results of this study further confirms not only that glycation of fibrinogen takes place *in vivo,* as a result of hyperglycaemia, but also shows that the level of glycation can be significantly reduced by glycaemic control. Of additional value from this study is the fact that it has been shown that it is possible to achieve significant reductions in fibrinogen glycation with insulin treatment, even under out-patient conditions which makes the results applicable to the general non-hospitalised diabetic population.

Other researchers have also observed higher glycation levels of fibrinogen in association with hyperglycaemia in diabetic patients (Ardawi *et al.*, 1990; Hammer *et al.*, 1989) when compared to diabetic patients with normal glycaemic levels. A direct comparison of the degree of fibrinogen glycation reduction by intervention, as was done in this study, is not possible because neither Ardawi *et al.* (1990) nor Hammer *et al.* (1989) conducted intervention studies. In addition, both Hammer *et al.* (1989) and Ardawi *et al.* (1990) reported glycation levels as percent glycated fibrinogen while we reported the number of glucose molecules attached to a fibrinogen molecule (mol glucose per mol fibrinogen). What is common to all the studies is that all reported lower glycation of fibrinogen in the presence of lower glycaemic levels.

The significant difference in fibrinogen glycation in this study that persisted between non-diabetic volunteers and type 2 diabetic volunteers after glycaemic control was achieved, is of interest, as it would imply that even non-significant differences in fasting venous glucose could result in significant levels of fibrinogen glycation. While the difference in fasting glucose between the two groups at the end of the study was not significant, the volunteers with type 2

diabetes did have slightly higher blood glucose levels. In the Hoorn study, Rijkelijkhuizen *et al.* (2007) investigated the effect of the decision made by the American Diabetes Association to lower the cut-off points for impaired fasting glucose from 6.1 to 5.6 mmol/l, a difference of only 0.5 mmol/l. The study involving 2 484 Dutch subjects reported that the subjects at impaired fasting glucose of 6.1 mmol/l had a higher risk of CVD than those with normal glucose (<5.6 mmol glucose), while for those with fasting glucose of 5.6 mmol/l, there was no difference in CVD risk compared to those with normal glucose. This would seem to indicate that even a small difference in fasting glucose of only 0.5 mmol/l can make a difference in outcomes of CVD risk. In this study, the difference in fasting venous glucose between the two groups at the end of the study was 1.09 mmol/l and this was associated with a significantly higher level of fibrinogen glycation.

5.5 Compaction of fibrin networks

There was no significant difference in compaction between the non-diabetic volunteers and those with type 2 diabetes, in this study. Compaction results are difficult to interpret because of the number of factors that influence it. Compaction gives an indication of clot rigidity and although it has been correlated to the amount of cross-linking in a fibrin network with normally cross-linked clots showing compaction values intermediate to those of totally cross-linked and non-cross-linked networks (Nair & Shats, 1997), several other factors influence clot rigidity and, therefore, compaction. Clot rigidity is influenced by factors like fibre size and branch point density and these in turn can be influenced by thrombin and fibrinogen concentration (Ryan *et al.*, 1999) as well as electrolyte balance (Di Stasio, Nagaswami, Weisel, & Di Cera, 1998). In addition, any factor in plasma that would cause conformational changes to fibrin fibres has the potential to influence clot rigidity and the final clot rigidity reached would, therefore, be determined by a variety of factors simultaneously. Since fibrinogen glycation did not correlate with any of the fibrin network structure variables measured in plasma, the lack of difference in compaction between the diabetic and non-diabetic volunteers, at baseline, in this study is not really unexpected. It does,

however, not rule out the possibility that fibrinogen glycation may have an effect on compaction. Compaction was not measured using purified fibrinogen because the large volumes of solution needed for this analysis were prohibitive. Had this been done, it would have been possible to determine the individual effect of fibrinogen glycation on compaction.

Both the γ and α chains become cross-linked upon activation of factor XIII by thrombin and calcium chloride (Lorand, 2001). Dunn *et al.* (2005) reported no difference in γ-chain cross-linking of diabetic and non-diabetic fibrin fibres, and neither did Lütjens *et al.* (1988). Although Dunn *et al.* (2005) and Lütjens *et al.* (1988) reported that the α-chain cross-linking in the diabetic groups was influenced by diabetes, experimental work has shown that γ-chain cross-linking has a much greater influence on fibrin network structure than α-chain cross-linking (Ryan *et al.*, 1999). Subsequent work done on this study also found no differences in α-chain cross-linking between diabetic and non-diabetic volunteers. In agreement with the studies done by Dunn *et al.* (2005) and Lütjens *et al.* (1988), no difference in γ-chain cross-linking was observed between the diabetic and non-diabetic volunteers as it was already completed in both groups after 10 minutes of incubation with FXIII.

The diabetic volunteers in this study showed a significant decrease in compaction at the end of the intervention period. This would seem to indicate that clot rigidity increased as a result of the intervention. Nair *et al.* (1991) on the other hand, reported compaction to decrease with increasing ambient glucose during polymerisation. However, the effect of ambient glucose is not expected to be the same as that for *in vivo* glycation of fibrinogen. Glycation of fibrinogen has been reported to take place to a much greater extent *in vitro* compared to *in vivo* (Austin, Mullins, & Morin, 1987). Because Nair *et al.* (1991) added the glucose just before the clotting was initiated, there was not enough time for fibrinogen glycation to take place and, therefore, they only measured the effect of the presence of glucose in plasma at the time of clot formation and not glycation *per se*. Austin *et al.* (1987) showed that incubation time influences the extent to which

glycation takes place. They also reported that fibrinogen was 65.1 percent glycated *in vitro,* whereas it was only 5.9 percent glycated, *in vivo*. The greater number of glucose units added to fibrinogen/fibrin *in vitro* may lead to conformational changes that may result in more significant changes in clot rigidity, but because glycation is unlikely to have been a factor when glucose is added just before clotting is initiated, the mechanism by which ambient glucose may influence fibrin network structure is not clear. Dunn *et al.* (2005) also reported that increasing concentrations of ambient glucose resulted in increased mean fibre number and mean branch point number per unit volume. Each of these factors can also influence clot rigidity, though Dunn *et al.* (2005) did not measure clot rigidity. It is, therefore, possible that a combination of factors, acting in concert, may have influenced the observed decrease in compaction and thus increase in clot rigidity, in the group with type 2 diabetes, as a result of the intervention in this study group. This possibility is supported by the fact that subsequent work done on the study group showed a small increase in the proportion of thicker fibres in the diabetic group after intervention. A higher proportion of thicker fibres can increase clot rigidity and Ryan *et al.* (1999) reported that the maximum stiffness/rigidity of fibres was found among fibres which showed intermediate branching densities and fibre diameters. It is then possible that the increase in proportion of thicker fibres observed in this study could have resulted in the increased clot rigidity with glycaemic control.

The positive correlation between compaction and permeability suggests that more permeable networks were associated with reduced tensile strength. In theory this is logical as less dense networks with lower tensile strength have been associated with more permeable networks and, therefore, a greater degree of porosity (Nair *et al.*, 1991). Unfortunately there has been no other research that has studied the relationship between compaction and glycaemic control for comparison. More work needs to be done to determine precisely how compaction is affected by the different factors that influence clot rigidity as well as their effect in combination with one another. In addition because compaction using purified fibrinogen was not measured, due to the volume constraints, it is

not possible to comment on the individual effect of fibrinogen glycation on compaction.

5.6 Permeability of fibrin networks

5.6.1 Permeability of fibrin networks prepared from plasma

There was no difference in permeation of fibrin clots prepared from plasma between the volunteers with and without type 2 diabetes, despite the fact that the fibrinogen from the volunteers with diabetes was significantly more glycated than that from non-diabetic volunteers. The fact that both groups had high plasma fibrinogen levels might have masked the effect of glycation on permeability. Kinetic factors are important in determining the structure of fibrin networks (Blomback, Carlsson, Fatah, Hessel, & Procyk, 1994) and fibrinogen, being the substrate for fibrin formation would affect the final network structure. Permeability of fibrin networks is reduced with increasing fibrinogen concentration (Blomback, Bannerjee, Carlsson, Hamasten, Hessel, Procyk, Silveira, & Zacharski, 1990). Changes in fibrinogen concentration have been reported to contribute significantly to changes in permeability measurements in type 1 diabetes (Jorneskog *et al.*, 2003). Although Jorneskog *et al.* (2003) did not observe a reduction in fibrinogen levels as a result of continuous sub-cutaneous insulin infusion of type 1 diabetic patients, they reported that among patients who showed improved HbA1c, 44 percent of the variation seen in changes to permeability, was as a result of changes in fibrinogen concentration. Ryan *et al.* (1999) also reported that fibrinogen concentration along with thrombin and calcium chloride concentrations had a significant conformational influence on fibrin network structure. It, therefore, may be that fibrinogen concentration was more important in this study group than glycation in determining permeability. This is supported by the fact that there was a significant negative correlation between permeability and fibrinogen concentration indicating that those with higher fibrinogen concentration tended to have lower permeability values. Because of the high fibrinogen concentrations that prevailed in both the groups with and without type 2 diabetes, in this study, this high substrate concentration may have resulted into a faster rate of fibrin monomer formation leading to a

tighter fibrin network (Ryan *et al.*, 1999) being formed in both groups. However, scanning electron microscopy micrographs that were subsequently taken of fibrin networks on a sub-sample of this study group showed that the diabetic volunteers had a small increase in the proportion of thicker fibres. Thus the fact that glycation may play a role in influencing fibre size and consequently the porosity of fibrin network structures in diabetes cannot be ruled out. Other researchers have indeed reported the fibrin network structures formed in association with type 2 diabetes (Dunn *et al.*, 2005), type 1 diabetes (Jorneskog *et al.*, 1996) and unspecified type of diabetes (Nair *et al.*, 1991) to be less permeable than those of healthy controls. However, the patients in the study by Jorneskog *et al.* (1996) had normal fibrinogen levels, Dunn *et al.* (2005) used a purified fibrinogen system with a standard fibrinogen concentration of 1 mg/ml and Nair *et al.* (1991) neither specified the type of diabetes nor reported the fibrinogen levels involved.

The level of glycaemic control, in this study, did not influence permeability of the networks formed in plasma, as indicated by the fact that despite obtaining significant reductions in fibrinogen glycation as a result of the intervention, this did not result in a change in permeability. This is confirmed by the lack of correlation of both HbA1c and fibrinogen glycation with permeability in this study. However, Jorneskog *et al.* (2003) reported a significant increase in permeability after continuous subcutaneous insulin infusion of type 1 diabetic patients, but as indicated earlier, the patients in the study had normal fibrinogen levels. Of interest is the fact that Jorneskog *et al.* (2003) reported that even patients who did not show an improvement in HbA1c as a result of the intervention had improved permeability, indicating that glycaemic control may not have been the only factor influencing the observed improvement in permeability. This was supported by the fact that the increase in permeability observed was mainly mediated by changes in plasma fibrinogen and lipid levels. No significant correlation between HbA1c and fibrinogen concentration was found in this study nor in the one done by Jorneskog *et al.* (1996). A comparison between the results of Jorneskog *et al.* (1996; Jorneskog *et al.*, 2003) and the results of this study is difficult to make because they worked with type 1 diabetic patients and this study worked with type 2 diabetic volunteers. In addition, Jorneskog *et al.* (2003) did not measure

the level of fibrinogen glycation in their studies. This information and the results from this study, therefore, seem to indicate that while fibrinogen glycation cannot be ruled out as a possible factor, fibrinogen concentration, at the levels that prevailed in this study, may have masked the influence of fibrinogen glycation on the fibrin network structure.

The analysis of variance of the fibrin network structure variables across fibrinogen concentration categories supports the possibility that fibrinogen concentration, in this study, may have masked the influence of fibrinogen glycation (Table 4.11). Permeability decreased with an increase in plasma fibrinogen concentration. This effect is further supported by the observed negative correlation between permeability and plasma fibrinogen concentration, while no correlation was found between fibrinogen glycation and permeability as already indicated above.

Although there was no difference in HDL-C between those with and those without type 2 diabetes, permeability from plasma correlated positively with HDL-C. The lipid profile of individuals with type 2 diabetes is associated with reduced HDL-C, high triglycerides and a tendency for normal LDL-C with a shift towards more dense LDL-C particles (Farmer, 2007). The positive correlation between HDL-C and permeability observed in this study implies that higher HDL-C levels would be associated with greater permeability and conversely this would imply that lower HDL-C levels would be associated with lower permeability. The results of this study, therefore, indirectly imply that the reduced HDL-C generally associated with type 2 diabetes could be associated with reduced permeability. Dunn *et al.* (2005) investigating type 2 diabetes only reported a near significant correlation between HDL-C and permeability ($p<0.07$), while Jorneskog *et al.* (2003) with type 1 diabetes reported that the change in LDL-C was a major determinant of fibrin network structure. The plasma triglyceride levels for the volunteers with type 2 diabetes in this study was significantly higher than for the non-diabetic volunteers, but triglycerides did not correlate with permeability or the other fibrin network structure variables measured. The effect of the different lipid fractions on fibrin network structure and permeability is thus not clear and warrants further investigation.

5.6.2 Permeability of fibrin networks prepared using purified fibrinogen

When permeability was measured using networks formed from purified fibrinogen, different trends were seen compared to what was found with networks formed from plasma samples. As was found for the plasma results, there was no difference in permeability between the volunteers with and without type 2 diabetes. However, for networks from purified fibrinogen, there was a significant improvement in permeability in the diabetic volunteers as a result of glycaemic control. It is not uncommon to find differences in results between plasma and purified samples, as there are differences in the kinetics of fibrin formation in the two systems (Shah, Nair, & Dhall, 1987). This is most likely because the formation of fibrin networks in plasma may be influenced by other plasma constituents not present when purified fibrinogen is used (Blomback *et al.*, 1990; Shah *et al.*, 1987). The fact that a change in permeability was observed when using purified fibrinogen, where the effect of fibrinogen concentration was excluded by working with a standardised fibrinogen concentration of 1 mg/ml, seems to confirm the possibility that fibrinogen concentration was a factor in masking the effect of fibrinogen glycation on fibrin network permeability in the plasma model. With standardisation of the fibrinogen concentration in the purified fibrinogen model, the only difference between the two groups would have been that for the diabetic volunteers, the fibrinogen had a higher level of glycation. There has been no other intervention study that has looked at bringing about glycaemic control in type 2 diabetes by insulin treatment and measuring changes in permeability and other fibrin network structure variables. Jorneskog *et al.* (2003) did an intervention but with type 1 diabetic patients who were given continuous subcutaneous insulin infusion. Dunn *et al.* (2005) reported a significantly lower permeability for fibrin networks prepared from fibrinogen purified from patients with type 2 diabetes compared to healthy controls without diabetes. Dunn *et al.* (2005) unfortunately did not measure fibrinogen glycation. It is, therefore, not known what the absolute level of fibrinogen glycation was in their study and how it compared to this study. Both Dunn *et al.* (2005) and this study used a purified system in which the major difference between the non-

diabetic and diabetic fibrinogen may have been the level of fibrinogen glycation. It is possible, therefore, that in these two studies, differences in the level of fibrinogen glycation may have, in part, contributed to the reduced permeability (Dunn *et al.*, 2005) or improved permeability (following glycaemic control in this study) observed for fibrin networks derived from patients with type 2 diabetes.

The difference in the observed permeability results in plasma and when purified fibrinogen was used serves to emphasise the importance of other plasma components on the final fibrin network structure produced. While in plasma a negative and positive correlation, respectively, was observed between permeability and fibrinogen and HDL-C concentrations, this was not so in the purified fibrinogen model. Plasma contains many components that can influence the final fibrin network structure (Colman *et al.*, 2001). Following the same scenario the situation *in vivo* where clots are formed in the presence of whole blood would be further complicated by the presence of yet more factors that play different roles in clot formation, including platelet (Vinik *et al.*, 2001) and endothelial functional aspects (Caballero, 2003) as well as the metabolic changes accompanying oxidative and carbonyl stress (Baynes & Thorpe, 1999), in the patient with type 2 diabetes. Since *in vivo* clots are formed in whole blood, in order to form a clearer picture of the contribution of fibrinogen glycation on clot permeability compared to all the other clotting factors, investigations using whole blood clots in type 2 diabetes would be useful.

5.7 Turbidimetric analysis

5.7.1 Lag-time of fibrin networks

At baseline, there was no difference in plasma derived lag-time between volunteers with and without type 2 diabetes. Therefore, the lag-time or rate of proto-fibril formation for the two groups in this study was similar at this stage, despite the observed differences in the level of fibrinogen glycation. Lütjens *et al.* (1988) working with type 1 diabetic patients, also reported no difference in lag-time between diabetic patients and control subjects. The increase in the plasma

derived lag-time in the diabetic volunteers, as a result of the intervention, on the other hand, indicates that a longer lag-time was required for proto-fibril formation when glycaemic control was reached compared to the situation at baseline. This indirectly implies an association between poor glycaemic control in the diabetic volunteers with shorter lag-time or faster proto-fibril formation. This does not, however, explain why no differences were observed between the diabetic and non-diabetic volunteers at baseline. The analysis of variance on plasma derived lag-time across three categories of fibrinogen glycation revealed no differences across the three categories used.

No significant differences in lag-time were found in the analysis done with purified fibrinogen both at baseline between the diabetic and non-diabetic volunteers and in the diabetic volunteers as a result of intervention. This may mean that other plasma factors may have been the cause for the increased lag-time in the diabetic volunteers in the plasma results in this study group, than the improved fibrinogen glycation. A shorter lag-time was reported by Dunn *et al.* (2005) in type 2 diabetic patients compared to controls, based on work involving purified fibrinogen. However, because Dunn *et al.* (2005) did not carry out an intervention it is not possible to shed some light on what may have happened after the diabetic patients had reached glycaemic control. In addition, because they did not measure fibrinogen glycation, it is difficult to compare their results with the results of this study.

Weisel *et al.* (1993) reported that an increased rate of proto-fibril formation was associated with shorter lag-time. This effect could also be because of increased fibrinopeptide B release that has been associated with type 2 diabetes (Dunn *et al.*, 2005). Dunn *et al.* (2005) reported an increase in the efficiency with which fibrinopeptide B cleavage took place in type 2 diabetic patients compared to non-diabetic controls. Lag-time is expected to shorten with an increase in fibrinopeptide B release, resulting in formation of shorter, thicker fibres (Dunn *et al.*, 2005). The rate of fibrinopeptide B release was not measured in this study, so it is not known whether it was affected by diabetes or glycaemic control in the study population.

When an analysis of variance of plasma derived lag-time was done across fibrinogen concentration levels, the change in lag-time from baseline to end seemed to be affected by the baseline fibrinogen concentration. The fact that this observation persisted when purified fibrinogen was used is difficult to explain since a standard concentration of fibrinogen was used. It could, however, imply that high plasma fibrinogen concentrations, *in vivo*, may influence certain reactions on fibrinogen that may later (even when purified) have conformational implications on the fibrin formation process. This would be in line with the suspicion that other plasma factors such as fibrinogen concentration, in this study group, may have played a greater role in determining lag-time than fibrinogen glycation *per se*. Increases in fibrinogen concentration could be expected to increase the rate of proto-fibril formation as a result of a substrate concentration effect on the reactions involved (Ryan *et al.*, 1999). However, this would very much depend on the balance of enzyme to substrate concentration and it would appear that, in this study group, the larger increase in lag-time from baseline to end, with increase in fibrinogen concentration may imply that the higher fibrinogen concentrations prevailing were perhaps high enough to cause a significant enzyme lowering effect (Ryan *et al.*, 1999). This may indicate possible conflicting effects of fibrinogen concentration and fibrinogen glycation on lag-time with lower lag-time being associated with higher fibrinogen glycation on the one hand and higher lag-time being associated with higher fibrinogen concentrations, on the other.

5.7.2 Slope of fibrin networks

Slope values give an indication of the rate of lateral aggregation of the proto-fibrils that are formed during the lag phase (Weisel *et al.*, 1993). Based on the plasma results, the rate of lateral aggregation for the volunteers with type 2 diabetes was not different from the rate for volunteers without diabetes. Neither was there differences observed in the volunteers with type 2 diabetes upon reaching glycaemic control. This might seem as though glycation did not influence lateral aggregation. However, it is possible that the influence of

glycation on lateral aggregation, as for permeability and lag-time, was somewhat masked by the effect of fibrinogen concentration.

The fact that the purified fibrinogen results showed a significantly higher slope for the volunteers with type 2 diabetes compared to those without diabetes, at baseline, seems to confirm that the influence of fibrinogen glycation on the rate of lateral aggregation was masked by the high fibrinogen concentrations in the plasma results. Despite the differences in lateral aggregation observed between the diabetic and non-diabetic volunteers, using purified fibrinogen, no significant change was seen after glycaemic control. It is possible that the change in fibrinogen glycation observed may not have been high enough to result in a significant change in lateral aggregation. The analysis of variance across fibrinogen glycation categories based on purified fibrinogen results, showed that lateral aggregation increased with fibrinogen glycation. Further, positive correlations were observed between slope and fibrinogen glycation as well as glycaemic control, as measured by both HbA1c and fasting venous glucose.

Conditions that favour proto-fibril growth may reduce the divergence of proto-fibrils from parent fibres due to increased non-covalent intermolecular interactions of the larger proto-fibril structures (Ryan *et al.*, 1999). This may contribute to higher rates of lateral aggregation under such conditions. It is possible that the additional glucose units added to fibrinogen with increased glycation may similarly contribute to increased non-covalent forces between proto-fibrils resulting in increased lateral aggregation in type 2 diabetes. This has further been supported by subsequent results on lateral aggregation from a sub-sample of the study group, who were especially selected based on the degree of improvement in fibrinogen glycation (Pieters, Covic, van der Westhuizen, Nagaswami, Baras, Loots, Jerling, Elgar, Edmonson, van Zyl, & Rheeder, 2008). In this sub-sample, the decrease in lateral aggregation as a result of glycaemic control reached significance. The results in terms of lateral aggregation are thus in agreement with the phenomenon described above. Dunn *et al.* (2005) did not report slope values and the only other intervention study similar to this one, done by Jorneskog *et al.* (2003), did not measure turbidity.

The timing of the cleavage of fibrinopeptide B is important and Weisel *et al.* (1993) reported that lateral aggregation was enhanced when fibrinopeptide B cleavage was done after proto-fibril formation had taken place. Whether fibrinogen glycation would influence the timing of fibrinopeptide B cleavage is not known at this stage.

The significant negative correlation between lag-time and slope regardless of whether the two were derived from plasma or purified fibrinogen, indicates that a slower rate of proto-fibril formation or longer lag-time was associated with a slower rate of lateral aggregation (lower slope) regardless of the influence from other plasma constituents. Conversely, a higher rate of proto-fibril formation or shorter lag-time would be associated with a higher rate of lateral aggregation. In agreement with this, Weisel *et al.* (1993) reported that when the rate of formation of proto-fibrils increased, the rate of lateral aggregation also increased. The fact that Weisel *et al.* (1993) did not study glycated fibrinogen, but rather simply the kinetics of fibrinogen formation, however, demands a cautious comparison with the results of this study.

5.7.3 Maximum absorbance of fibrin networks

Maximum absorbance is an indication of average fibre size of the final fibrin network structure formed (Dunn *et al.*, 2005). There was no difference in fibre size between the volunteers with and without type 2 diabetes both from plasma and purified fibrinogen.

In plasma, the analysis of variance across categories of fibrinogen glycation and fibrinogen concentration levels showed that there was no difference in fibre size across the glycation levels and only marginal differences across fibrinogen concentration levels. The marginal differences across fibrinogen concentration levels were not seen in purified fibrinogen where a standard amount of fibrinogen was used as expected. There were also no differences in fibre size across fibrinogen glycation categories when purified fibrinogen was used indicating that

fibrinogen glycation did not have an influence on fibre size in this study population. The positive correlation between maximum absorbance and slope suggests an association between higher rate of lateral aggregation and thicker fibres being formed. A higher rate of lateral aggregation has been shown to lead to the formation of thicker fibres (Weisel *et al.*, 1993). Dunn *et al.* (2005) reported that thicker fibres were formed from fibrinogen isolated from patients with type 2 diabetes. The results of this study, however, do not show any differences in fibre thickness between the two groups, despite differences in the rate of lateral aggregation. While an increased rate of lateral aggregation would be expected to result in formation of thicker fibres (Weisel *et al.*, 1993), the fact that opposing effects of fibrinogen concentration and fibrinogen glycation on the rate of proto-fibril formation were observed could, in part, have contributed to the lack of difference in fibre size between the volunteers with and without type 2 diabetes in this study. This is especially so in view of the observed effect of fibrinogen glycation on increasing the rate of lateral aggregation. Ryan *et al.* (1999) reported that networks of longer fibres tended to be made up of thicker fibres. Conversely shorter fibres formed at higher polymerisation rates in the diabetic volunteers, as might have been the case in this study, may have resulted in formation of relatively thinner fibres than expected, leading to the lack of difference in fibre thickness observed in this study. The combination of factors that influence fibre diameter in this study resulted in no differences in diameter between diabetic and non-diabetic volunteers as well as to no change in fibre diameter as a result of glycaernic control.

There would be definite interaction between the rate of proto-fibril formation and the rate of lateral aggregation on the final fibre size as determined by possible timing of fibrinopeptide cleavage (Weisel *et al.*, 1993). Weisel *et al.* (1993) showed that when both the rate of formation of proto-fibrils and the rate of lateral aggregation were increased, the fibre size was smaller, but when only either lateral aggregation or proto-fibril formation were increased, fibre size was larger. Given these effects, it is possible that the combined effect of the observed rates of proto-fibril formation and lateral aggregation in this study group did not lead to differences in fibre size.

Chapter 6: Conclusion

6.1 Introduction

This thesis investigated the effect of blood glucose control on the fibrin network characteristics of African subjects with uncontrolled type 2 diabetes using insulin treatment. This chapter will provide a summary of the main findings of the study, draw conclusions and make recommendations for future research. The main findings and conclusions drawn are especially aimed at addressing the objectives of the study. For easy referral purposes, the objectives of the study will be given below followed by the main findings and conclusion.

The objectives/research questions that were addressed by this study were:

- Will the fibrinogen levels of black South African volunteers with uncontrolled type 2 diabetes be different form those of non-diabetic volunteers?

- Is there a difference in fibrinogen glycation levels between volunteers with uncontrolled type 2 diabetes and non-diabetic volunteers?

- Will there be differences in the selected fibrin network structure variables between the volunteers with uncontrolled type 2 diabetes and non-diabetic volunteers, at baseline?

- Will insulin treatment of the volunteers with uncontrolled type 2 diabetes, on an out-patient basis, result in significant lowering of fibrinogen glycation?

- Once glycaemic control has been reached, will there be differences in the selected fibrin network structure variables, compared to the beginning of the intervention and what might these differences be?

6.2 Baseline characteristics of the study population

The volunteers with type 2 diabetes experienced an increase in BMI as a result of insulin treatment due to possible changes in energy metabolism associated with insulin treatment. In addition the volunteers with type 2 diabetes presented normal serum-insulin levels in spite of being insulin resistant. This may possibly indicate that β-cell failure had commenced resulting in reduced insulin production.

6.3 Fibrinogen concentration

The plasma fibrinogen levels of both the diabetic and non-diabetic volunteers in this study group were similar and higher than would have been expected based on being black Africans who generally exhibit higher fibrinogen levels compared to Caucasians. It is possible that being obese and hypertensive may have contributed to the higher than expected fibrinogen levels observed. No change in fibrinogen levels was observed as a result of glycaemic control.

6.4 Fibrinogen glycation

The level of fibrinogen glycation in the type 2 diabetic volunteers was higher than that of the non-diabetic volunteers. The fibrinogen glycation was reduced by glycaemic control, but not to the same level as that of the non-diabetic volunteers. Although the glycaemic level of the group with type 2 diabetes was not significantly higher than that for the non-diabetic volunteers after glycaemic control was reached, it still resulted in a significantly higher level of fibrinogen glycation. It is not known whether it would be possible to reach the same level of fibrinogen glycation in people with type 2 diabetes as in those without diabetes. It may be that longer periods of glycaemic control would be needed to reduce the fibrinogen glycation even further, as more cycles of fibrinogen production (in comparison with 2 to 3 cycles in this study) would take place in a glycaemically controlled environment. The fibrinogen glycation level of the total group correlated with glycaemic control based on both HbA1c and fasting venous glucose measurements. This study has clearly demonstrated that it is possible to reduce

fibrinogen glycation levels by insulin treatment under out-patient conditions, a situation that can easily be extrapolated to the general out-patient treatment of people with type 2 diabetes. Further research is warranted to establish whether it is possible to reduce fibrinogen glycation to the same level as for non-diabetic people by determining whether there is a glycaemic control level and duration of control that would result in similar glycation levels between non-diabetic and diabetic people.

Many of the fibrin network structure variables measured were affected by fibrinogen glycation even though in some cases this effect appears to have been masked by the effect of the high fibrinogen levels that prevailed in both the diabetic and non-diabetic groups.

6.5 Compaction of fibrin networks

There was no difference in compaction between the volunteers with and without type 2 diabetes. There was, however, a decrease in compaction in the diabetic volunteers as a result of the intervention, indicating an increase in clot rigidity with glycaemic control. Clot rigidity is influenced by several factors like fibre size, branch point density, factor XIII cross-linking and other factors that influence each one of these variables. It is possible, therefore, that the decrease in compaction or increase in clot rigidity may have been influenced by several factors acting simultaneously. One of these factors could have been the small increase in the proportion of thicker fibres in the diabetic volunteers with glycaemic control. It was unfortunately not possible to measure compaction using purified fibrinogen because the large amounts of sample required was prohibitive. It would be useful to measure compaction using purified fibrinogen to determine the individual effect of fibrinogen glycation on compaction. Further work is, therefore, warranted to determine the effects of each of the factors that influence clot rigidity both in isolation and in combination with one another. This needs to be done in plasma and purified fibrinogen.

6.6 Permeability of fibrin networks

Permeability was similar between volunteers with and without type 2 diabetes when measured from plasma. The high plasma fibrinogen level that prevailed in the study group influenced permeability such that higher fibrinogen levels were associated with lower permeability. The influence of the high fibrinogen may have indeed masked the effect of fibrinogen glycation on permeability measured from plasma. The permeability results using purified fibrinogen showed that a reduction of fibrinogen glycation by glycaemic control improved permeability in the volunteers with type 2 diabetes. This indicates that permeability is affected by fibrinogen glycation and can be improved with glycaemic control. The difference in the observed permeability results when measured from plasma and purified fibrinogen emphasises the importance of other plasma constituents in determining the final fibrin network structure and other variables that depend on this structure such as permeability.

6.7 Turbidimetric analysis of fibrin networks

There was no difference in the lag-time or rate of proto-fibril formation between the diabetic and non-diabetic volunteers when measured from plasma, but there was a significant increase in lag-time indicating a longer time for proto-fibril formation as a result of glycaemic control. No differences in lag-time were observed when it was measured using purified fibrinogen for the diabetic compared to the non-diabetic volunteers at baseline or for the volunteers with type 2 diabetes before and after glycaemic control. This may mean that plasma constituents such as fibrinogen concentration were more important in determining lag-time in this study group.

When measured from plasma, there was no difference in the rate of lateral aggregation (slope) between the volunteers with and without type 2 diabetes. Neither was there a difference found in those with type 2 diabetes before and after glycaemic control. Based on measurements from purified fibrinogen, however, the volunteers with type 2 diabetes had significantly higher rates of

lateral aggregation compared to those without diabetes at baseline, although no significant change in slope was observed with glycaemic control. It is possible that much greater reductions in fibrinogen glycation would be required in order to see a significant change in lateral aggregation with glycaemic control. There was a positive correlation between lateral aggregation and fibrinogen glycation as well as glycaemic control. An assessment of the timing of fibrinopeptide B release in association with type 2 diabetes to determine if it is affected by fibrinogen glycation is warranted. In addition, further work to determine whether lower fibrinogen glycation levels as a result of glycaemic control would further influence changes in lateral aggregation would yield useful information.

Fibre size (maximum absorbance) was not different between the two groups when measured with plasma or purified fibrinogen, despite the observed increase in lateral aggregation in the group with type 2 diabetes. There was, however, a positive association between lateral aggregation and formation of thicker fibres. The many factors that influence fibre size under different polymerisation conditions make it extremely difficult to predict an outcome when only one of these factors is modified by a specific intervention.

Finally, fibrin network structure is determined by many factors and the final clot structure formed would depend on a balance of all these factors acting together. The main factors that were found to influence fibrin network structure in this study were fibrinogen concentration and fibrinogen glycation, while the correlation between HDL-C and permeability indicates that other plasma components may also be important in influencing fibrin network structure. Permeability improved with glycaemic control and lateral aggregation increased with fibrinogen glycation. This study has shown that it is possible to reduce fibrinogen glycation significantly in type 2 diabetic patients under out-patient conditions. It has further shown that it is possible to have changes to fibrin network characteristics as a result of glycaemic control. The changes to fibrin network structure observed may contribute to reducing the CVD risk profile of people suffering from diabetes when glycaemic control is effectively managed, but to what extent this might be possible needs to be investigated.

The differences in results obtained in plasma compared to when purified fibrinogen was used, illustrate that while it is possible to design an investigation to study the effect of a single mechanism of action on fibrin network structure, the influence of an individual mechanism in the presence of other contributing mechanisms may be of much less consequence and the results should be interpreted with caution when extrapolating them to the *in vivo* situation, where there is seldom only one mechanism of action at work.

6.8 Possible new research questions emanating from this study

The results from this study have brought forth several research questions of interest that may aid in developing a better understanding of the mechanisms by which diabetes may confer increased CVD risk on people suffering from type 2 diabetes.

1. In a population such as the black South African population, where high plasma fibrinogen concentrations prevail, at what stage and under which circumstances would the fibrinogen concentration become a significant CVD risk? A risk profile study in the population group in comparison to a population with normal fibrinogen concentrations may provide some answers.

2. How would the effects of fibrinogen glycation on fibrin network structure in a population with high fibrinogen concentrations compare with its effect in a population with normal fibrinogen concentrations? A comparative study of the effect of fibrinogen glycation on fibrin network structure in a population where high fibrinogen levels prevail, and one where normal fibrinogen levels prevail may provide some answers.

3. Measuring compaction also in a purified fibrinogen model in order to determine the individual effect of fibrinogen glycation and to compare these results with results of the plasma model.

4. What is the cause/mechanism of action for the observed relationship between lipid fractions and fibrin network structure?

5. In view of all the factors that influence fibrin network structure in blood, what would be the effect of fibrinogen glycation on fibrin network structures prepared in whole blood compared to structures prepared from purified fibrinogen?

6. Under what type of physiological circumstances would African patients with type 2 diabetes exhibit even higher fibrinogen levels than the already high levels that prevail in this population group?

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Annexure

This annexure includes two papers that have been published based on the work done on this study. The first paper, published in 2006, is based on results from the plasma model, while the second paper, published in 2008, is based on results from the purified fibrinogen model. Both papers have been published in Thrombosis and Haemostasis.

The effect of glycaemic control on fibrin network structure of type 2 diabetic subjects

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Summary

Diabetic subjects have been shown to have altered fibrin network structures. One possible cause may be fibrinogen glycation resulting in altered structure/function properties. We investigated the effect of glucose control on fibrinogen glycation and fibrin network structure in type 2 diabetes. Blood samples were taken from twenty uncontrolled diabetic subjects at baseline to determine the levels of fibrinogen glycation and fibrin network structures. The subjects were then treated with insulin until blood glucose control was achieved before end blood samples were taken. Twenty age- and BMI-matched non-diabetic subjects were included as a reference group. The diabetic subjects had significantly higher mean fibrinogen glycation at baseline than the non-diabetic subjects (7.84 vs. 3.89 mol glucose / mol fibrinogen; $p < 0.001$). This was significantly reduced during the intervention (7.84 to 5.24 mol glucose / mol fibrinogen; $p < 0.0002$) in the diabetic group. Both groups had high mean fibrinogen concentrations (4.25 and 4.02 g/l, diabetic and non-diabetic subjects respectively). There was no difference in fibrinogen concentration, porosity, compaction and kinetics of clot formation between the diabetic subjects and non-diabetic subjects at baseline, nor were there any changes during the intervention despite the reduced fibrinogen glycation. Fibrin network characteristics correlated well with fibrinogen but not with any markers of glycaemic control. Improved glycaemic control resulted in decreased fibrinogen glycation but not fibrinogen concentration. It seems as though porosity, compaction and kinetics of clot formation are more related to fibrinogen concentration than fibrinogen glycation in this model.

Keywords

Diabetes, glycation, fibrinogen, fibrin network structure, glucose control

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Introduction

The major cause of mortality and morbidity amongst type 2 diabetic subjects is cardiovascular disease (CVD) (1). This is partly ascribed to a procoagulant state present in diabetic subjects. Fibrinogen contributes significantly to this procoagulant state, and a large number of studies have reported increased fibrinogen levels in diabetic patients (2). Increased fibrinogen has convincingly been demonstrated to be a powerful, independent marker for CVD (3). There are several potential pathophysiologic mechanisms by which elevated fibrinogen increases cardiovascular risk: it is a substrate for thrombin; its activation is the final step in the coagulation cascade; it is involved in platelet aggregation and endothelial function; it interacts with the binding of plasminogen with its receptor, and it is an important acute phase protein (4). Although positive correlation exists between plasma fibrinogen concentration and glycaemic control, improvement of glycaemic control in intervention trials did not consistently result in reduced fibrinogen concentrations (2).

The structure of the resultant fibrin network that forms upon the activation of fibrinogen by thrombin may also contribute to increased CVD risk. In general, fibrin clots with thinner fibres, more branch points and smaller intrinsic pores are more dense and resistant to lysis, contributing to atherothrombotic risk (5).

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Furthermore, clots formed from diabetic patients have been shown to be denser, with thinner fibres and to be less porous than those formed from control subjects (6–8).

One of the possible causes proposed for this altered fibrin network structure in diabetic patients, is glycation of fibrinogen. Fibrinogen has been shown to be glycated *in vivo* (9–11), most likely via glycation of lysine residues on the surface of fibrinogen (9, 12). As lysine is involved in cross-linking of the fibrin network by FXIII as well as the binding of tissue plasminogen activator (tPA), plasminogen and plasminogen activator inhibitor-1 (PAI-1) to fibrin, all of which are proteins involved in the lysis of the network, it is quite plausible that binding of glucose at these sites may have a significant effect on the functionality of fibrinogen specifically revealed as an altered fibrin network structure.

This is the first intervention study performed on type 2 diabetic patients investigating the effects of glycation on fibrin properties and whether decreased fibrinogen glycation, as a result of glycaemic control, will result in an improvement in the structure of the fibrin network.

Materials and methods

Study design

In this, parallel, controlled intervention, twenty black African type 2 diabetic subjects, uncontrolled on oral hypoglycaemic agents, were included and treated with insulin until glycaemic control was achieved (four out of five subsequent readings within normal glucose range). The patients then had to remain controlled for eight days before end blood samples were drawn. This eight day period was chosen in order to provide enough time for unglycated fibrinogen to be produced (half-life 3–4 days) after glycaemic control had been achieved. Twenty non-diabetic black African subjects were included as a reference group in order to control for variation over time. All subjects signed informed consent, and ethical approval was obtained from the ethics committees of both the University of Pretoria and the North-West University, South Africa.

Subjects

Type 2 diabetic subjects

Inclusion criteria: patients had to be uncontrolled (HbA1C > 9%) on maximum-dose combination oral hypoglycaemic medication; BMI > 25 kg/m^2; 40–65 years of age; blood pressure sufficiently controlled not to necessitate treatment change during intervention (< 140/90 mmHg).

Exclusion criteria: major surgery in the preceding six months; macrovascular complications; disease that can influence haemostasis (e.g. thrombocytopenia, cancer, liver disease); patients on aspirin, warfarin, steroids, hormone replacement therapy or non-steroidal anti-inflammatory drugs; proteinuria on urine dipstick (> 300 mg/day) or acute infection.

Non-diabetic controls

Non-diabetic subjects with matching anti-hypertensive drug-use (hidrochlorothiazide, ACE-inhibitor – Perindopril and nifedipine – Adalat), age, gender and BMI were recruited. The same inclusion and exclusion criteria as for the type 2 diabetic subjects were adhered to. Baseline oral glucose tolerance tests were done to rule out diabetes.

Study protocol

The intervention in the diabetic group consisted of three phases.

Phase 1

On the first visit, diabetic subjects were taught how to do self-glucose-monitoring, co-ordination of insulin use with meals, symptoms and management of hypoglycaemic events and the use of glucagon. Fasting capillary glucose was measured daily for one week.

Phase 2

Subjects received 10 IU basal analogue insulin Glargine (Lantus, Sanofi-Aventis Pharmaceuticals, Paris, France) daily at 22:00 in addition to current treatment of maximum dose oral hypoglycaemic treatment. Metformin use was unchanged from before and during the intervention. Insulin administration was adjusted individually until four out of five subsequent fasting values were less than 7.2 mM. Sulphonylureas were stopped.

Phase 3

Post-prandial glucose was now controlled with pre-meal administration of short-acting insulin Aspart (Novo Nordisk, Bagsværd, Denmark) as required to achieve post-prandial glucose values of less than 10 mM. Once both fasting and post-prandial glycaemic control was achieved, the subjects remained on treatment for eight days. Baseline blood samples and anthropometric measurements were collected at the end of phase 1 and end samples and measurements at the end of phase 3. Blood samples of non-diabetic subjects were drawn within one week of their matched diabetic subject's blood sampling.

Blood sampling

Fasting venous blood samples were drawn with minimal stasis by a medical doctor before 10 a.m. For the determination of insulin and lipids, blood was left to clot for preparation of serum. For venous glucose determination, blood was collected into sodium fluoride tubes. EDTA blood was collected for the determination of HbA1C. Citrate blood was collected for the determination of fibrinogen and fibrinogen glycation. Blood was centrifuged for 15 minutes (min) at 2,000 g at 4°C within 30 min of collection. Citrated blood (3.8% citrate) with added Trasylol® (350 U/ml blood) was collected for fibrin network determinations. This citrated blood was centrifuged twice at 3,660 g for 15 min to yield platelet free plasma. Serum and plasma were stored at –82°C until analysis.

Analytical procedures

Insulin, glucose, lipids and PAI-1_{act}

Fasting insulin was measured with an enzyme-linked immunosorbent assay (ELISA) method on the Immulite 2000 Analyzer (Diagnostic Products Corporation, Los Angeles, CA, USA). Plasma glucose, baseline HbA1C and serum lipids were measured on a Synchron LX clinical System (Beckman Coulter

Inc., Fullerton, CA, USA). Low-density lipoprotein cholesterol was calculated by using the Friedewald formula (13). Insulin resistance was calculated using the homeostasis model assessment (HOMA) as: (fasting insulin x fasting venous glucose)/22.5 (14). PAI-1 activity was measured using an indirect enzymatic method (Spectrolyse pL, Biopool, Umeå, Sweden; Cat. No. 101201).

Fibrinogen concentration, purification and glycation

Plasma fibrinogen (modified Clauss method) was measured on an Automated Coagulation Laboratory 200 (Instrumentation Laboratories, Milan, Italy) (between run CV = 3%). Fibrinogen was purified from the plasma of each subject using IF-1 affinity chromatography as described previously (15). Purified fibrinogen was run on 10% SDS PAGE gels to confirm purity and the absence of degradation of the fibrinogen preparations (Fig 1). Fibrinogen glycation was measured with a two-reagent enzymatic assay (GlyPro® assay, Genzyme Diagnostics, Cambridge, MA, USA; between run CV = 5%). This is a specific enzymatic method for the direct measurement of glycated proteins in serum or plasma. The first reagent digests the proteins and subsequently releases glycated protein fragments. Ketoamine oxidase in the second reagent facilitates the specific oxidation of the ketoamine bond of the glycated protein fragment substrate. Liberation of hydrogen peroxide allows a colorimetric determination of the amount of glycated protein in an end-point reaction. Absorbance at 550 nm is measured after the addition of reagent 1 and again after reagent 2. Results are calculated as follows: Glycated protein (μM) = ΔA sample / ΔA calibrator x calibrator value (μM).

Clot permeability

Permeability was measured essentially as described previously (7, 16) (between run CV=15%). Plasma clots of subjects were prepared in triplicate in 3-cm sections of 1-ml pipette tips. Plasma was clotted with the addition of 0.5 U/ml thrombin, 25 mM calcium chloride. Buffer was permeated through at a pressure height of 21 cm. Permeability (Ks), an indication of the intrinsic pore size of the network, was then calculated (17).

Compaction

Compaction was measured using a modified method of Dhall et al. (18) (between run CV=6%). Plasma clots were prepared in microcentrifuge tubes in quintet. The tubes were sprayed with a lecithin-based aerosol (Spray and Cook®) to render the surface non-adhering. The samples were then centrifuged for 45 seconds (sec) at 8,000 g, and the expelled supernatant was measured and expressed as % of the total volume.

Turbidity measurements

Turbidity experiments were performed with undiluted plasma, 0.5 U/ml thrombin and 20 mM Calcium chloride (final concentrations) in duplicate. Absorbance was measured at 405 nm every 7 sec for the first 2 min after addition of thrombin and calcium chloride, then every 15 sec for the next 30 min and then every 5 min up to a total time of 60 min on a Multiscan Ascent spectrophotometer (Labsystems, Helsinki, Finland). Lag phase, slope and maximum absorbance were recorded. Lag phase, represents the time required for fibrin fibres to grow sufficiently to allow lateral aggregation and was taken at the point where absorbance increased 0.015 from baseline. The slope, calculated at half maximum absorbance, represents the rate of increase of turbidity and the maximum absorbance, calculated as the absorbance after 30 min minus the baseline, is an indication of average fibre size.

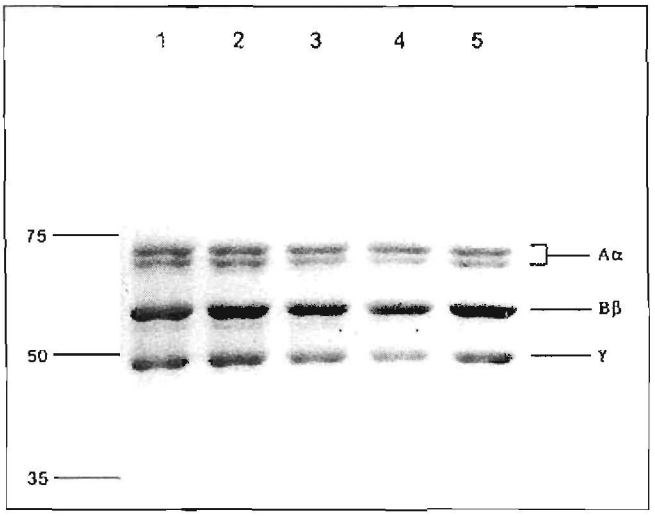

Figure 1: S-PAGE analysis of purified fibrinogen from plasma samples. Fibrinogen preparations (5 μg) from a commercial human fibrinogen (lane 1, MP Biologicals cat no. 151123) in comparison to fibrinogen isolated from a diabetic subject before (lane 2) and after (lane 3) glucose control intervention period and a non-diabetic subject before (lane 4) and after (lane 5) intervention are shown. The positions of a molecular weight marker (x 10^3) and the respective peptides, Aα, Bβ, and γ, are indicated on the left and right hand side, respectively.

Statistical methods

The computer software package, Statistica (Statsoft Inc., Tulsa, OK, USA) was used for statistical analysis. Data was tested for normality. Normally distributed data is presented as the mean (95% confidence interval) and not normally distributed data as median [25th, 75th percentile]. Differences in baseline characteristics as well as differences in changes during the intervention between the two groups were determined using the t-test for independent samples for parametric data and the Mann-Whitney U test for non-parametric data. Differences from baseline to end within each group were determined using the t-test for dependent samples. Subjects were subdivided into tertiles for baseline fibrinogen concentration, baseline fibrinogen glycation and change in fibrinogen glycation from baseline to end. ANOVA was used to measure differences in fibrin network characteristics for these subdivisions with Tukey's honest significant difference test for post-hoc comparisons. Spearman-correlation was used for all correlations.

Results

Two subjects in the reference group failed to return for end blood sampling and were hence excluded from the study. Baseline characteristics of the study population are presented in Table 1. The groups were comparable regarding age, body mass index,

Variables	Type 2 diabetic subjects	Non-diabetic subjects
Patients (n)	20	18
Sex (male/female)	6 / 14	7 / 11
Age (years)	53.0 (49.1 ; 56.9)	52.9 (49.2 ; 56.6)
Body mass index (kg/m^2)	30.8 (28.0 ; 33.7)	31.8 (28.8 ; 34.7)
Fasting Insulin (mU/l) #	11.0 [6.70 – 15.8]	13.7 [8.9 – 28.8]
Insulin resistance (HOMA)	5.18 (3.99 ; 6.95)[b]	3.11 (2.29 ; 7.42)[b]
HbA1C (%) #	11.7 [9.50 – 13.8] [a]	5.60 [5.30 – 5.90] [a]
Venous glucose (mM)	14.6 (10.8 ; 18.4)[a]	5.48 (4.56 ; 5.80) [a]
Systolic blood pressure (mmHg)	140.5 (129.6 ; 151.3)	143.3 (130.2 ; 156.4)
Diastolic blood pressure (mmHg)	86.6 (82.7 ; 90.6)	89.8 (83.3 ; 96.3)
Total cholesterol (mM)	4.84 (4.14 ; 5.53)	4.54 (4.16 ; 4.92)
Triglycerides (mM) #	1.80 [1.25 ; 2.50][b]	1.05 [0.80 ; 1.25][b]
High density lipoprotein cholesterol (mM) #	0.90 [0.75 – 1.3]	0.90 [0.8 – 1.25]
Low density lipoprotein cholesterol (mM)	2.86 (2.37 ; 3.34)	2.88 (2.43 ; 3.32)
PAI-1_{act} (U/ml) #	16.4 [9.34 – 19.6]	14.6 [13.6 – 20.1]
Duration of diabetes (years)	11.0 (8.00 ; 15.0)	

[a] P < 0.001; [b] P< 0.05; # Data not normally distributed and reported as median [25 – 75 percentile]; Insulin resistance = (fasting insulin x fasting venous glucose)/22.5.

Table 1: Baseline characteristics of subjects.

fasting insulin, blood pressure, total cholesterol, high and low density lipoprotein cholesterol and PAI-1_{act}. The diabetic subjects had significantly higher insulin resistance, HbA1C, venous glucose and triglycerides.

There was no significant difference in the baseline fibrinogen concentration, permeability, compaction, lag time, slope and maximum absorbance between the diabetic and non-diabetic group (Table 2). Both groups had relatively high fibrinogen concentrations on average (diabetic subjects: 4.25 g/l and non-diabetic subjects 4.02). The diabetic subjects had significantly higher fibrinogen glycation than the non-diabetic subjects (7.84 vs. 3.89 mol glucose / mol fibrinogen) at baseline. There was a significant decrease in venous glucose (14.6 to 6.72 mM), fibrinogen glycation (7.84 to 5.24 mol glucose / mol fibrinogen) and compaction (39.6 to 37.1 %) and a significant increase in lag time (27.8 to 29.7 s) in the diabetic group during the intervention. When compared to the changes in the non-diabetic group from baseline to end, only the decrease in venous glucose and fibrinogen glycation remained significant (p=0.0003 and 0.0007, respectively).

When the groups were subdivided into tertiles for baseline fibrinogen concentration and fibrinogen glycation, no significant differences in fibrin network structure characteristics were seen for the subdivision of fibrinogen glycation (data not shown). Maximum absorbance tended to increase with increasing fibrinogen concentration. When the groups were subdivided into tertiles for the degree of change in glycation from baseline to end, no significant differences could be found in degree of resultant change for any of the fibrin network structure characteristics between the tertiles.

All the fibrin network characteristics measured except for compaction and lag time showed significant correlations with baseline fibrinogen concentration (Table 3). No correlations were found with venous glucose, HbA1c or fibrinogen glycation and any of the fibrin network characteristics. Lag time, slope and maximum absorbance showed inter-correlations. When correlating the change in fibrin network characteristics during the intervention with the other baseline and delta (change from baseline to end) biochemical variables, the only correlations that were found were with baseline fibrinogen and change in fibrinogen (data not shown). The change in lag time and slope correlated with baseline fibrinogen (r=0.36, p=0.029 and r=-0.37, p=0.023, respectively). The change in maximum absorbance correlated with change in fibrinogen (r=0.52, p=0.0009). Correlations were also done for the diabetic and non-diabetic subjects separately, but they did not differ from the correlations found for the total group.

Discussion

Cross-sectional evidence exists for altered fibrin network structures in diabetic subjects (6, 7). It has been speculated that a possible cause for the differences seen in fibrin network structures between diabetic and healthy subjects may be glycation of fibrinogen. This is the first intervention study, however, to report whether treatment of type 2 diabetic subjects and the resultant decrease in fibrinogen glycation due to glucose control will have an effect on the fibrin network structures formed.

The diabetic subjects had relatively high fibrinogen concentration on average (4.25 g/l). This is in agreement with a large

Table 2: Differences between diabetic and non-diabetic subjects of selected variables for the intervention period.

Variable	Type 2 diabetic subjects (n = 20)				Non-diabetic controls (n = 18)				Changes between groups (deltas)
	Baseline	End	Delta	p	Baseline	End	Delta	p	p
Fasting glucose (mM)	14.6* (10.8; 18.4)	6.72 (5.51; 7.92)	–7.88 (-12.04;-3.72)	0.0008	5.18* (4.56 ; 5.80)	5.63 (4.95; 6.31)	0.46 (0.00; 0.91)	0.05	0.0003
Fibrinogen (g/l)	4.25 (3.88; 4.63)	4.36 (3.99; 4.73)	0.11 (-0.34; 0.55)	0.62	4.02 (3.59 : 4.45)	3.85 (3.43; 4.28)	–0.16 (-0.52; 0.19)	0.35	0.33
Fibrinogen glycation (mol glucose/mol fibrinogen)	7.84* (6.59; 9.10)	5.24 (4.47; 6.01)	–2.60 (-3.82;-1.39)	0.0002	3.89* (3.46 ; 4.32)	3.75 (3.43; 4.07)	–0.19 (-0.55; 0.17)	0.27	0.0007
Ks ($cm^2 \times 10^{-9}$)#	8.67 [7.60–11.5]	9.6 [7.75–11.65]	–0.87* (-3.17; 1.42)	0.33	9.57 [7.84–13.3]	10.8 [8.3–13.1]	0.89 (-0.78; 3.05)	0.23	0.10
Compaction (%)	39.6 (36.8; 42.3)	37.1 (33.8; 40.4)	–2.43 (-4.29; –0.58)	0.013	37.7 (34.2; 41.2)	36.5 (32.6; 40.3)	–1.84 (-5.47; 1.78)	0.30	0.72
Lag time (s)	27.8 (26.0; 29.5)	29.7 (27.2; 32.1)	1.89 (0.17; 3.61)	0.03	27.0 (25.1; 28.8)	28.1 (26.3; 30.0)	1.14 (-0.46; 2.75)	0.15	0.51
Slope	23.6 (20.3; 26.2)	22.9 (19.0; 26.7)	–0.41 (-4.2: 3.39)	0.82	26.0 (21.4; 30.5)	28.4 (24.0; 32.8)	2.43 (-1.20; 6.07)	0.18	0.27
Max absorbance	1.18 (1.09; 1.26)	1.22 (1.10; 1.33)	0.04 (-0.08; 0.16)	0.53	1.22 (1.12; 1.33)	1.22 (1.10; 1.34)	0.00 (-0.09; 0.08)	0.91	0.56

#Data not normally distributed and reported as median [25–75 percentile]. *Significant difference at baseline between the two groups. 'This delta value is negative even though the end Ks value is higher than the baseline. More subjects showed a decrease in permeability (12 out of 20) but the eight did show an increase showed a larger increase on average.

Table 3: Correlation between glycaemic control related markers, lipids and clot structures of diabetic and non-diabetic subjects at baseline.

Variable	Permeability		Compaction		Lag time		Slope		Max absorbance	
	r	p	r	p	r	p	r	p	r	p
Venous glucose (mM)	–0.13	(0.43)	0.20	(0.24)	0.18	(0.28)	–0.19	(0.26)	–0.21	(0.21)
Insulin (mU/l)	–0.31	(0.07)	–0.30	(0.09)	–0.09	(0.58)	0.05	(0.78)	0.13	(0.46)
Insulin resistance (HOMA)	–0.20	(0.25)	–0.13	(0.47)	–0.03	(0.86)	0.01	(0.94)	–0.06	(0.74)
HbA1C (%)	–0.24	(0.15)	0.17	(0.33)	0.16	(0.36)	–0.14	(0.41)	–0.22	(0.19)
Fibrinogen glycation (mol glucose/mol fibrinogen)	–0.14	(0.39)	0.16	(0.36)	0.22	(0.19)	–0.22	(0.19)	–0.22	(0.18)
Total cholesterol (mM)	0.22	(0.20)	0.14	(0.44)	–0.074	(0.67)	0.19	(0.26)	0.071	(0.68)
Triglycerides (mM)	–0.28	(0.097)	–0.08	(0.64)	0.023	(0.89)	–0.17	(0.31)	–0.21	(0.23)
High density lipoprotein cholesterol (mM)	0.48	(0.003)	0.29	(0.087)	–0.18	(0.31)	0.25	(0.14)	0.054	(0.75)
Low density lipoprotein cholesterol (mM)	0.21	(0.22)	0.093	(0.59)	–0.044	(0.80)	0.29	(0.087)	0.15	(0.37)
PAI-1_{act} (U/ml)	0.077	(0.65)	0.18	(0.29)	–0.25	(0.13)	0.19	(0.34)	–0.22	(0.18)
Fibrinogen (g/l)	–0.41	(0.01)	–0.08	(0.62)	–0.30	(0.07)	0.37	(0.02)	0.46	(0.003)
Permeability (cm x 10^{-9})	-	-	0.53	(<0.001)	–0.14	(0.41)	0.07	(0.70)	0.004	(0.98)
Compaction (%)	0.53	(<0.001)	-	-	–0.13	(0.44)	0.29	(0.09)	0.16	(0.35)
Lag time (s)	–0.14	(0.41)	–0.13	(0.44)	-	-	–0.43	(0.007)	–0.23	(0.17)
Slope	–0.07	(0.70)	0.29	(0.09)	–0.43	(0.007)	-	-	0.66	(<0.0001)
Max absorbance	0.004	(0.98)	0.16	(0.35)	–0.23	(0.17)	0.66	(<0.0001)	-	-

HOMA – homeostasis model assessment.

body of evidence showing high fibrinogen levels in diabetic subjects (19–23). There was no difference, however, in the fibrinogen concentration between the diabetic and non-diabetic subjects, because the non-diabetic subjects also had high fibrinogen levels. Higher fibrinogen concentration in black Africans compared to Caucasians has been reported previously (24, 25). It may also be that many of the factors responsible for the increased fibrinogen levels in diabetic subjects, for instance hypertension, obesity and age, were also present in the non-diabetic group. There is some controversy as to whether fibrinogen is increased in general in diabetic subjects or mainly in those with CVD (20, 26–28). As our subjects were specifically chosen not to have CVD, this might explain why their fibrinogen levels were not further increased.

The diabetic subjects had significantly higher fibrinogen glycation than the non-diabetic subjects. This was significantly improved after treatment, although still not to a level seen in the non-diabetic subjects. The level of glycation and how it compares with levels reported in related articles as well as its correlation with glycaemic control is discussed elsewhere (article submitted for publication).

Until now, only one group has investigated the effect of a glucose control intervention on clot structure and that was performed in type 1 diabetic subjects (29). This is the first intervention study on type 2 diabetic subjects. While a significant reduction in fibrinogen glycation was seen in the diabetic group during the intervention, this did not result in significant changes in fibrin network structure. This may in part be attributed to the fact that when using plasma clots as opposed to clots prepared from purified fibrinogen, the effect of the treatment on other plasma constituents that were not measured, as well as their mere presence, may have independently influenced the fibrin network structure, modulating the effect of the decreased fibrinogen glycation. Using plasma clots does, however, give a better representation of the process *in vivo* and it also addresses the role of fibrinogen concentration itself, while in a purified system where standardised fibrinogen concentrations are used the effect of fibrinogen concentration on fibrin network structure is excluded.

It is well known that fibrinogen concentration is one of the most important kinetic factors involved in the formation of the fibrin network and its final structure (30). Our results show that both the baseline levels as well as the changes during the intervention, of the fibrin network characteristics measured, were related to either baseline fibrinogen or the change in fibrinogen, instead of being related to any of the glucose control or lipid markers. This is somewhat in agreement with the intervention study done on type 1 diabetic subjects by Jörneskog et al. (29) who also found that the changes in fibrin gel porosity were mainly mediated by changes in fibrinogen and blood lipids and were not directly related to improvement in blood glucose control. This does, however, not explain the decreased porosity seen in type 1 diabetic subjects, in a study done by Jörneskog et al. (8) who had normal and similar fibrinogen concentration as healthy controls. It may also be that the effect of glycation may be influenced by the fibrinogen concentration itself. Although the plasma model is a standard system used for this type of study, the fact that no significant changes were seen in the fibrin network structure characteristics during the intervention does not necessarily mean that there will be no changes *in vivo* where whole blood clots are formed in the presence of platelets, with blood flow present. On the other hand, it may also be that fibrinogen glycation may not have a large influence on the fibrin network permeability, compaction or the kinetics of the network formation, but on other parameters such as lysis rate or crosslinking, as these are the processes dependent on availability of lysine groups, the suggested site for glycation. Fibrinogen glycation has been shown to impair alpha-chain crosslinking (31) *in vivo* as well as reducing the susceptibility of fibrin to degradation by plasmin *in vitro* (12). Ney et al. (32) on the other hand, found no difference in FXIII crosslinking and degradation by plasmin for glycated and control fibrinogen *in vitro*. As these experiments were performed *in vitro*, under different conditions, caution should be paid to the interpretation of the results and it should be kept in mind that extrapolation to in-vivo situations may not be possible, as there is a large difference between fibrinogen glycation *in vivo* and *in vitro* (33). Austin et al. (33) showed that fibrinogen of five diabetic subjects was on average 5.9% glycated while fibrinogen that was *in vitro* exposed to 0.5 M glucose for two days was 65.1% glycated. For this reason it is very difficult to compare the results of our study with other studies investigating the effect of glucose on fibrin network structures, where glucose was added *in vitro* to either plasma (7) or purified fibrinogen (6). Furthermore, in these studies the glucose was added directly before the experiments were started, therefore they did in fact not study the effect of glycation but the direct presence of glucose.

In conclusion, type 2 diabetic subjects had significantly higher fibrinogen glycation than non-diabetic subjects. Blood glucose control in free-living diabetic subjects led to decreased fibrinogen glycation. It had very little effect on the kinetics of fibrin formation as measured by turbidity curves, porosity and compaction of fibrin networks formed. There was also no difference in the network characteristics measured between the diabetic and non-diabetic subjects. In this plasma model, fibrinogen concentration seems to have a more dominant effect than fibrinogen glycation on fibrin network structure .

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Glycaemic control improves fibrin network characteristics in type 2 diabetes – A purified fibrinogen model

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Summary

Diabetic subjects have been shown to have altered fibrin network structures. One proposed mechanism for this is non-enzymatic glycation of fibrinogen due to high blood glucose. We investigated whether glycaemic control would result in altered fibrin network structures due to decreased fibrinogen glycation. Twenty uncontrolled type 2 diabetic subjects were treated with insulin in order to achieve glycaemic control. Twenty age- and body mass index (BMI)-matched non-diabetic subjects were included as a reference group. Purified fibrinogen, isolated from plasma samples was used for analysis. There was a significant decrease in fibrinogen glycation (6.81 to 5.02 mol glucose/mol fibrinogen) with a corresponding decrease in rate of lateral aggregation (5.86 to 4.62) and increased permeability (2.45 to 2.85 x 10^{-8} cm^2) and lysis rate (3.08 to 3.27 µm/min) in the diabetic subjects after glycaemic control. These variables correlated with markers of glycaemic control. Fibrin clots of non-diabetic subjects had a significantly higher ratio of inelastic to elastic deformation than the diabetic subjects (0.10 vs. 0.09). Although there was no difference in median fiber diameter between diabetic and non-diabetic subjects, there was a small increase in the proportion of thicker fibers in the diabetic samples after glycaemic control. Results from SDS-PAGE indicated no detectable difference in factor XIIIa-crosslinking of fibrin clots between uncontrolled and controlled diabetic samples. Diabetic subjects may have altered fibrin network formation kinetics which contributes to decreased pore size and lysis rate of fibrin clots. Achievement of glycaemic control and decreased fibrinogen glycation level improves permeability and lysis rates in a purified fibrinogen model.

Keywords

Diabetes, fibrin network, fibrinogen glycation, glycaemic control, hyperglycaemia

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Introduction

Diabetes is a powerful and independent risk factor for cardiovascular disease, with atherosclerosis among the most serious complications of type 2 diabetes (1). Since fibrin is deposited at atherosclerotic lesions, the structure of the deposited fibrin network has come under investigation as a possible contributing risk factor for increased prevalence of atherosclerosis-related cardiovascular events. Fibrin networks can vary in structure from tight networks formed with thin, highly branched fibers, to looser networks made up of larger fibers. It has been demonstrated that myocardial infarction at a young age is indeed associated with a proneness to the formation of tight and rigid fibrin gel structures (2, 3). Fibrin clots with thinner fibers, more branch points and smaller intrinsic pores are in general more dense and resistant to lysis, thereby contributing to thrombotic risk (4).

For this reason, fibrin networks of diabetic patients have received considerable attention as a possible contributor to in-

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creased cardiovascular disease risk. One of the most important plausible mechanisms for alterations to fibrin networks of diabetic patients is the non-enzymatic glycation of fibrinogen in the presence of uncontrolled blood glucose levels, which may influence the functionality of the fibrinogen. Researchers have investigated this issue by using either *in vitro* glycated fibrinogen (5–9) or collecting samples from diabetic subjects containing *in vivo* glycated fibrinogen (10–14). When using diabetic samples, a further distinction was made in the use of either plasma (11–14) or purified fibrinogen models (10, 12, 15) Apart from the differing approaches used, a vast array of variables was also measured, including both structural and functional characteristics, in order to gain a better understanding of the effects of diabetes on fibrin networks.

Interpreting results from studies where fibrinogen was glycated *in vitro* should be done with caution, as there is a significant difference between fibrinogen glycation *in vitro* and *in vivo*, making extrapolation to *in vivo* situations difficult. While using samples from diabetic patients may be more physiological, it should be kept in mind that the diabetic state has many other components that could potentially influence fibrin network structure, apart from fibrinogen glycation, such as increased lipid levels (16), increased fibrinogen concentration (17, 18) and glycation of other plasma proteins. In general, clots formed from diabetic patients were found to be denser, with thinner fibers and were less porous than those formed from healthy individuals (10, 11, 16). Pieters et al. (14) found, however, no difference in network characteristics of diabetic patients compared to healthy individuals, despite a significant difference in the level of fibrinogen glycation, when using plasma samples. In this study population, however, both the diabetic and healthy individuals had similar and raised fibrinogen levels. The fibrin network characteristics also correlated with baseline fibrinogen concentration but not with any of the markers of glycaemic control. Similar correlation patterns were observed by Jörneskog *et al.* (16) indicating the importance of plasma constituents other than fibrinogen glycation on fibrin network structure.

The ideal approach to examine the role of fibrinogen glycation on fibrin network structures of diabetic patients would be to conduct an intervention trial specifically designed to investigate the effect of glycaemic control on fibrin network structures. Most of the above mentioned studies on diabetic subjects, however, were cross-sectional by design, making the establishment of causal relationships impossible. To date, only two intervention studies have been performed on this topic, one investigating type 1 diabetic patients (16) and the other investigating type 2 diabetic subjects, performed by our group (14). The scarcity of studies such as these is not surprising since they require the unique combination of an intervention trial with patients and difficult and time-consuming structural and biophysical studies of clot networks. Two papers have been published from the study done by our group. The first describes the effect of glycaemic control on fibrinogen glycation and the association between fibrinogen glycation, fasting glucose and HbA1c (19). The second describes the effect of glycaemic control on fibrin network structure using plasma samples in order to include the possible contribution of individual fibrinogen concentrations on the fibrin networks (14). In order to determine the specific effects of fibrinogen glycation on fibrin network structures in the absence of other possibly confounding plasma proteins, we excluded the effects of other plasma components for the purpose of this paper, by purifying fibrinogen from the collected plasma samples, thereby establishing a purified fibrinogen model. The discussion will include interpretation of the results obtained from both plasma and purified fibrinogen models in order to demonstrate how results may be influenced by the model used.

Materials and methods

Study design

A minimum of 16 volunteers had to be included per group in order to achieve a difference of one standard deviation at 80% power and 5% level of significance. To accommodate possible non-compliers and drop-outs, 20 volunteers were recruited per group. A parallel, controlled intervention with blinded assessment was used. Twenty black African type 2 diabetic subjects, uncontrolled on oral hypoglycaemic agents, were included and treated additionally with insulin until glycaemic control was achieved (4 out of 5 subsequent readings within normal glucose range). Following this initial period of improving and finally achieving glycaemic control, the patients then had to remain controlled for eight days before end blood samples were drawn. This eight day period was chosen in order to provide enough time for unglycated fibrinogen to be produced (half-life 3–4 days) after glycaemic control had been achieved. Twenty non-diabetic black African subjects were included as a reference group in order to control for variation over time. All subjects signed informed consent and ethical approval was obtained form the ethics committees of both the University of Pretoria and the North-West University, South Africa.

Subjects

Type 2 diabetic subjects

Inclusion criteria: patients had to be uncontrolled (HbA1C > 9%) on maximum dose combination oral hypoglycaemic medication (Metformin and Sulphonylureas); body mass index (BMI) > 25 kg/m^2; 40–65 years of age with blood pressure sufficiently controlled (< 140/90 mmHg) not to necessitate treatment change during intervention (hidrochlorothiazide, ACE-inhibitor – Perindopril and Calcium antagonist – Nifedipine).

Exclusion criteria: major surgery in the preceding six months; macrovascular complications; diseases that may influence haemostasis (e.g. thrombocytopenia, cancer, liver disease); patients on aspirin, warfarin, steroids, hormone replacement therapy or non-steroidal anti-inflammatory drugs; proteinuria on urine dipstick (> 300 mg/day) or acute infection.

Non-diabetic controls

Non-diabetic subjects with matching anti-hypertensive drug-use, age, gender and BMI were recruited. The same inclusion and exclusion criteria as for the type 2 diabetic subjects were adhered to. Baseline oral glucose tolerance tests were done to rule out diabetes.

Study protocol

The intervention in the diabetic group consisted of three phases.

Phase 1
On the first visit, diabetic subjects were taught how to do self-glucose monitoring, co-ordination of insulin use with meals, symptoms and management of hypoglycaemic events and the use of glucagon. Fasting capillary glucose was measured daily for one week.

Phase 2
Subjects received 10 IU basal analogue insulin Glargine (Lantus, Sanofi-Aventis Pharmaceuticals, Paris, France) daily at 10 p.m. in addition to current treatment of maximum dose oral hypoglycaemic treatment. Metformin use was unchanged from before and during the intervention. Insulin administration was adjusted individually until four out of five subsequent fasting values were less than 7.2 mM. Sulphonylureas were stopped.

Phase 3
Post-prandial glucose was now controlled with pre-meal administration of short-acting insulin Aspart (Novo Nordisk, Bagsværd, Denmark) as required to achieve post-prandial glucose values of less than 10 mM. Once both fasting and post-prandial glycaemic control was achieved, the subjects remained on treatment for eight days. Baseline blood samples and anthropometric measurements were collected at the end of phase 1 and end samples and measurements at the end of phase 3. Blood samples of non-diabetic subjects were drawn within one week of blood sampling from their matched diabetic subjects.

Blood sampling
Fasting venous blood samples were drawn with minimal stasis by a physician before 10 a.m. Blood for venous glucose determination, was collected into sodium fluoride tubes. EDTA blood was collected for the determination of HbA1C. Citrate blood (60 ml) was collected for the determination of fibrinogen concentration, fibrinogen glycation and for fibrinogen purification. Blood was centrifuged for 15 minutes (min) at 2,000 g at 4°C within 30 min of collection. Serum and plasma were stored at –82°C until analysis. Baseline and end samples of an individual were analyzed in the same run for all experiments. Scanning electron microscopy (SEM), and viscoelastic measurements were performed in a different laboratory than the other analyses.

Glucose and HbA1C
Plasma glucose and baseline HbA1C were measured on a Synchron LX clinical System (Beckman Coulter Inc., Fullerton, CA, USA).

Fibrinogen concentration, purification and glycation
Plasma fibrinogen (modified Clauss method) was measured on an Automated Coagulation Laboratory 200 (Instrumentation Laboratories, Milan, Italy) (between-run CV = 3%). Fibrinogen was purified from the plasma of each subject using IF-1(fibrinogen monoclonal antibody, Kamiya Biomedical Company, Seattle, WA, USA) affinity chromatography as described previously (20). Purified fibrinogen was run on 10% SDS PAGE gels to confirm purity and the absence of degradation of the fibrinogen preparations. No bands, apart from the three intact fibrinogen chains were present on the gels. The broad-range molecular-weight standard used ranged from 35 to 250 kD. Fibrinogen glycation was measured in duplicate, with a two-reagent enzymatic assay (GlyPro® assay, Genzyme Diagnostics, Cambridge, MA, USA; between-run CV = 5%) as described previously (14).

Clot permeability
Permeability was measured essentially as described previously (11, 21). Purified fibrinogen (1 mg/ml) clots of subjects were prepared in triplicate in 3 cm sections of 1 ml pipette tips. Fibrinogen was clotted with the addition of 1 U/ml bovine thrombin, 5 mM $CaCl_2$ and incubated for 60 min at 25°C in a moist atmosphere. Buffer was permeated through at a pressure height of 6 cm (CV = 7%). These conditions were selected in order to prevent clots from collapsing. Permeability (Ks), an indication of the intrinsic pore size of the network, was then calculated (22).

Turbidity measurements
Turbidity experiments were performed in duplicate with purified fibrinogen (1 mg/ml), 1 U/ml bovine thrombin and 5 mM $CaCl_2$ (final concentrations). Absorbance was measured at 405 nm every 7 seconds (s) for the first 2 min after addition of thrombin and $CaCl_2$, then every 15 s for the next 30 min and then every 5 min up to a total time of 60 min on a Multiscan Ascent spectrophotometer (Labsystems, Helsinki, Finland). Lag phase, slope and maximum absorbance were recorded. The lag phase represents the time required for fibrin fibers to grow sufficiently to allow lateral aggregation and was taken at the point where absorbance increased 0.015 from baseline. The slope, calculated at half maximum absorbance, represents the rate of increase of turbidity, and the maximum absorbance, calculated as the absorbance after 60 min minus that at baseline, is an indication of average fiber size.

In order to determine whether fibrinogen glycation is one of the mechanisms of action through which fibrin network architecture is altered in diabetes, the experiments mentioned below were performed on subjects with striking differences in the degree of fibrinogen glycation. Due to the difficulty and time consuming nature of the experiments, a subset of 12 subjects consisting of seven diabetic subjects who showed the largest decrease in fibrinogen glycation from baseline to end and five randomly selected non-diabetic subjects were investigated for this purpose.

SEM of fibrin clots and fiber diameter measurement
SEM was used to determine the fibrin structure of clots formed from purified fibrinogen of diabetic and control subjects. Clots were formed by addition of 0.5 U/ml α-human thrombin and 3 mM $CaCl_2$ to 1 mg/ml fibrinogen in 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4. Samples were prepared as described previously (23). Clots were observed and photographed digitally in many different areas, using a scanning electron microscope (XL 20, FEI, Hillsboro, OR, USA). Fiber diameters were measured from micrographs at 10,000 x magnification using ImageJ software (National Institutes of Health, USA). The thicknesses of at least 100 different fibers were measured per micrograph, with at least six micrographs imaged for each patient.

Viscoelastic measurements of fibrin clots

A Plazek torsion pendulum was used to determine visco-elastic properties of clots, including clot stiffness (24), in triplicate, formed from purified fibrinogen from diabetic subjects and controls under the same conditions as described above. Data recorded was used to calculate G' (the dynamic storage modulus), G" (the loss modulus) and the tanδ (loss tangent), which is a function of G" over G' and gives information about irreversible deformation of the clot (24).

Lysis rate of fibrin clots measured by confocal microscopy

Fluorescein isothiocyanate (FITC) (Pierce, Rockford, IL, USA) labeled fibrin clots were prepared using 1 mg/ml purified fibrinogen, 0.15 mg/ml FITC-labeled fibrinogen, 1 U/ml bovine thrombin and 5 mM $CaCl_2$. The fibrinogen was labeled with FITC as described by Sakharov et al. (25). The clots were prepared in micro-chambers and incubated for 45 min at 37°C in a moist atmosphere before being lysed with a lysis buffer containing 1 µg/ml tissue plasminogen activator (tPA) (Actilyse from Boehringer Ingelheim, Ingelheim, Germany), 1 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, USA), 210 µg/ml human glu-plasminogen (American Diagnostica, Stamford, CT, USA), 140 mM NaCl and 50 mM Tris, pH 7.4. After a 15 min incubation at 37°C in a moist atmosphere, lysis rate was measured by following the lysis front on a PCM 2000 Nikon confocal laser scanning microscope linked to a Nikon TE300 inverted microscope equipped with an ApoPlanar 1.4NA 60 x oil immersion objective. A 5-W argon ion laser was used in combination with a 488 nm band-pass filter for excitation. Emission was monitored at 505 nm. A pinhole of 5 micron and a 10% neutral density filter were used to limit bleaching of the FITC-label. Images were obtained using a standardized format: 107 x 107 x 16 µm with four optical sections collected at z-intervals of 4 µm. Lysis front velocity was determined at three different sites along the lysis front in triplicate, in order to calculate the mean lysis rate in µm/min.

Analysis of fibrin cross-linking by SDS-PAGE

Initial SDS-PAGE results indicated that the fibrin clots made from the purified fibrinogen contained very little if any factor (F)XIII. In order to determine whether fibrinogen glycation has an effect on FXIIIa crosslinking, we performed SDS-PAGE analysis with and without added human FXIII (American Diagnostica) using an incubation time series. FXIII (22 µg/ml) was added to the purified fibrinogen (1mg/ml) and clots were formed by the addition of 1 U/ml bovine thrombin and 5 mM $CaCl_2$ (final concentrations) at 21°C in 20 µl reaction mixtures which were then incubated for 10, 45 or 90 min. Control samples without added FXIII were incubated for 90 min, in order to demonstrate the absence of FXIII in the purified fibrinogen. The ligating reaction was stopped by solubilization in 6 M urea, 40 mM dithiothreitol and 2% (w/v) SDS at 37°C for 45 min. Gel electrophoresis [8% (w/v) acrylamide] was performed by the procedure of Laemmli (26). Samples of 6 µg protein per lane were analyzed and a broad-range molecular weight standard (Amersham) was used as a reference. Coomassie brilliant blue R (Biorad, Hercules, CA, USA) was used for staining. The gel was photographed on a SynGene bio-imaging system and peaks were identified and quantified using GeneTools software version 3.06 (SynGene, Cambridge, UK).

Statistical methods

The computer software package, Statistica (Statsoft Inc., Tulsa, OK, USA) was used for statistical analysis. Data is presented as median [25th; 75th percentile]. Differences in baseline characteristics as well as differences in changes during the intervention between the two groups were determined using the Mann-Whitney U test. Differences from baseline to end within each group were determined using the Wilcoxon Matched Pairs test. Once statistical significance has been indicated, effect sizes can be used to determine whether this significance is likely to be important in practice. The effect size is independent of sample size and is an objective measure of the likelihood of a difference having a practical/clinical significance. Effect sizes were calculated according to the following formula: $r=z/\sqrt{n}$ for non-parametric data (z is the z-statistic obtained from the non parametric test and n is the number of observations). The likelihood of the found statistical significance being practically relevant, is reported as effect size (r) and can be interpreted as follows; r=0.1 is a small likelihood, r=0.3 is a medium likelihood and r=0.5 is a large likelihood for non-parametric data (27). Spearman-correlation was used for determining correlation coefficients.

Results

Two of the non-diabetic subjects failed to return for end blood sampling and were hence excluded from the study. A summary of the most relevant baseline characteristics are presented in Table 1 while the detailed baseline characteristics of the study population have been presented in detail elsewhere (14).

Variables	Type 2 diabetic subjects	Non-diabetic subjects
Patients (n)	20	18
Sex (male/female)	6 / 14	7 / 11
Age (years) #	53.0 (49.1; 56.9)	52.9 (49.2; 56.6)
HbA1c (%)	11.7 [9.50; 13.8] [a]	5.60 [5.30; 5.90] [a]
Venous glucose (mM) #	14.6 (10.8; 18.4) [a]	5.18 (4.56; 5.80) [a]
Fasting Insulin (mU/l)	11.0 [6.70; 15.8]	13.7 [8.9; 28.8]
Insulin resistance (HOMA) #	5.18 (3.99; 6.95) [b]	3.11 (2.29; 7.42) [b]
Fibrinogen (g/l) #	4.25 (3.88; 4.63)	4.02 (3.59; 4.45)
Duration of diabetes (years)	11.0 (8.00; 15.0)	

Table 1: Baseline characteristics of subjects. Adapted from (14). Note: [a] P < 0.001; [b] P< 0.05; # Data normally distributed and reported as mean (95% confidence interval); Insulin resistance = (fasting insulin x fasting venous glucose)/22.5.

Table 2: Differences between diabetic and non-diabetic subjects of selected variables for the intervention period. Data reported as median [25, 75 percentiles]. * Significant difference between groups at baseline. † Large practical relevance. ‡ p=0.06.

Variable	Type 2 diabetic subjects (n = 20)				Non-diabetic subjects (n = 18)				Changes between groups (deltas)
	Baseline	End	Delta	p	Baseline	End	Delta	p	p
Fibrinogen glycation (mol glucose/mol fibrinogen)	6.81 [5.64; 10.7] *†	5.02 [4.19; 5.86]	−2.49 [-3.70; −0.67]	<0.01 †	3.84 [3.27; 4.20] *†	3.69 [3.27; 4.34]	−0.2 [-0.44; 0.26]	0.36	<0.001 †
Ks ($cm^2 \times 10^{-8}$)	2.45 [1.95; 2.65]	2.85 [2.24; 3.37]	0.45 [0.0; 1.48]	0.02 †	2.54 [2.0; 3.15]	2.8 [2.43; 3.59]	0.06 [-0.45; 3.6]	0.7	0.16
Lag time (s)	36.5 [34.5; 38.4]	37.6 [33.2; 40.4]	0.16 [-3.28; 2.59]	0.86	38.7 [34.6; 40.9]	38.9 [33.3; 42.9]	−0.12 [-2.02; 2.41]	0.53	0.95
Slope	5.86 [4.78; 6.33] *†	5.04 [4.25; 5.81]	−0.69 [-1.02; 0.69]	0.21	3.95 [3.53; 4.64] *†	3.69 [3.32; 4.42]	0.11 [-0.31; 0.25]	0.69	0.29
Max absorbance	0.67 [0.63; 0.78]	0.65 [0.58; 0.71]	−0.03 [-0.12; 0.06]	0.19	0.70 [0.57; 0.73]	0.64 [0.56; 0.71]	−0.03 [-0.12; 0.10]	0.76	0.67
	7 Selected diabetic subjects				5 Selected non-diabetic subjects				
Fibrinogen glycation (mol glucose/mol fibrinogen)	10.7 [8.41; 11.0] *†	5.07 [4.77; 7.10]	−3.60 [-6.14; −2.53]	0.02 †	3.02 [2.93; 3.35] *†	3.15 [3.08; 3.15]	−0.12 [-0.20; 0.13]	0.89	0.004 †
Ks ($cm^2 \times 10^{-8}$)	2.24 [1.47; 2.50]	2.84 [1.81; 3.07]	0.46 [0.29; 0.86]	0.04 †	2.79 [2.67; 4.46]	3.59 [2.80; 3.74]	0.01 [-0.87; 0.71]	0.89	0.43
Lag time (s)	36.5 [36.4; 36.7]	39.1 [38.5; 41.3]	2.59 [1.75; 2.96]	0.08	31.8 [31.1; 39.3]	40.7 [31.1; 42.9]	2.20 [2.10; 8.81]	0.08	0.94
Slope	5.86 [5.49; 6.20] *†	4.62 [4.57; 5.45]	−0.51 [-0.91; −0.41]	0.04 †	3.94 [3.68; 4.24] *†	3.37 [3.01; 4.45]	0.10 [-0.31; 0.52]	0.89	0.15
Max absorbance	0.78 [0.67; 0.78]	0.66 [0.49; 0.66]	−0.12 [-0.13; −0.06]	0.08	0.73 [0.57; 0.74]	0.67 [0.56; 0.72]	−0.003 [-0.06; 0.02]	0.69	0.31
Storage modulus G' ($dyne/cm^2$)	372 [282; 451]	324 [209; 399]	−81.3 [-114; −7.30]	0.35	227 [82.1; 389]	277 [167; 455]	70.4 [-60.3; 221]	0.35	0.43
Loss modulus G'' ($dyne/cm^2$)	32.5 [24.7; 38.4]	29.9 [19.7; 32.5]	−4.3 [-7.79; −2.17]	0.18	33.4 [8.26; 44.6]	35.8 [20.0; 48.0]	11.7 [-7.23; 14.8]	0.50	0.43
Loss tangent (tanδ)	0.09 [0.08; 0.09] *†	0.09 [0.07; 0.10]	0.006 [-0.02; 0.009]	0.92	0.10 [0.10; 0.12] *†	0.11 [0.11; 0.13]	0.03 [-0.04; 0.03]	0.69	0.66
Lysis rate (μm/min)	3.08 [2.48; 3.25] ‡†	3.27 [2.92; 3.72]	0.47 [0.12; 0.62]	0.02 †	8.52 [6.18; 8.59] ‡†	8.21 [6.50; 8.64]	0.33 [-0.31; 0.94]	0.35	0.87
Fiber diameter (μm)	0.086 [0.080; 0.105]	0.092 [0.083; 0.093]	0.006 [-0.006; 0.013]	0.40	0.076 [0.069; 0.092]	0.096 [0.076; 0.099]	0.008 [-0.011; 0.008]	0.89	0.86

Fibrinogen glycation

There was a significant decrease in the level of fibrinogen glycation upon achievement of glycaemic control (6.81 vs. 5.02 mol glucose/mol fibrinogen) (Table 2) as well as a significantly higher level of fibrinogen glycation in the diabetic compared to the non-diabetic subjects (6.81 vs. 3.84 mol glucose/mol fibrinogen) at baseline. For the sub-sample, selected based on the largest decrease of fibrinogen glycation during the intervention, the level of glycation decreased from 10.7 to 5.07 mol glucose/mol fibrinogen. Effect size calculations indicated that these differences are large enough to not only have statistical significance but also have possible practical relevance.

Clot permeability

Clot permeability reflects the clot structure, specifically the average pore size. The results indicated both a statistically as well as a large likelihood of a practically relevant increase in permeability from baseline to end in the total diabetic group (2.45 to 2.85×10^{-8} cm^2). This relatively small but consistent increase was also observed in the sub-sample (2.24 to 2.84×10^{-8} cm^2) (Table 2). There was no change in permeability in the non-diabetic subjects, nor was there any difference between the diabetic and non-diabetic subjects at baseline. There was additionally a significant correlation between permeability and HbA1c at baseline ($r=-0.63$; $p=0.038$).

Turbidity measurements

Turbidity curves are used to characterize the kinetics of polymerization and clot structure. No difference in the lag time (time for fibrinopeptide cleavage and formation of oligomers) or maximum absorbance (average cross-sectional area of fibers) was observed between the uncontrolled diabetic, controlled diabetic or the non-diabetic subjects (Table 2). The slope of increase in turbidity (rate of lateral aggregation) for the diabetic subjects was, however, significantly higher than that of the non-diabetic subjects at baseline, both for the total group (5.86 vs. 3.95) as well as for the sub-sample (5.86 vs. 3.94). The slope tended to decrease in the total diabetic group, and in the sub-sample the decrease reached significance with achievement of glycaemic control (5.86 to 4.62). This decrease was consistent for each subject compared to the normal variation observed in the non-diabetic group. These effects also had a large likelihood of practical relevance. Slope correlated significantly with level of fibrinogen glycation ($r=0.65$, $p=0.04$), HbA1c ($r=0.71$, $p=0.02$), venous glucose ($r=0.77$, $p=0.009$) and negatively with lysis rate ($r=-0.77$, $r=0.009$).

Fiber diameter

Clot structure was observed directly by SEM. The median fiber diameter of the clots from diabetic and non-diabetic subjects was similar (Table 2 and Fig. 1) at baseline and no significant change in the median fiber diameter was observed after achieving glycaemic control (Table 2). There was, however, a small increase in the proportion of thicker fibers in the clots of the diabetic subjects after glycaemic control as measured from the SEM micrographs, indicating a less homogeneous clot structure (Fig. 2).

Viscoelastic properties

The mechanical properties of the clots are important for their functions. There was no significant difference in dynamic storage modulus (elastic properties) or loss modulus (viscous properties) between the uncontrolled diabetic, controlled diabetic or the non-diabetic subjects (Table 2). The results showed, however a statistically significant difference in the loss tangent (tanδ) measurement (ratio of inelastic to elastic component) between the diabetic and non-diabetic subjects (0.09 vs. 0.10) at baseline, reflecting a lower proportion of the inelastic component in the fibrin clots of diabetic patients.

Lysis rate

Fibrinolysis rates were determined by measurement of the lysis front velocity using confocal microscopy after addition of tPA to

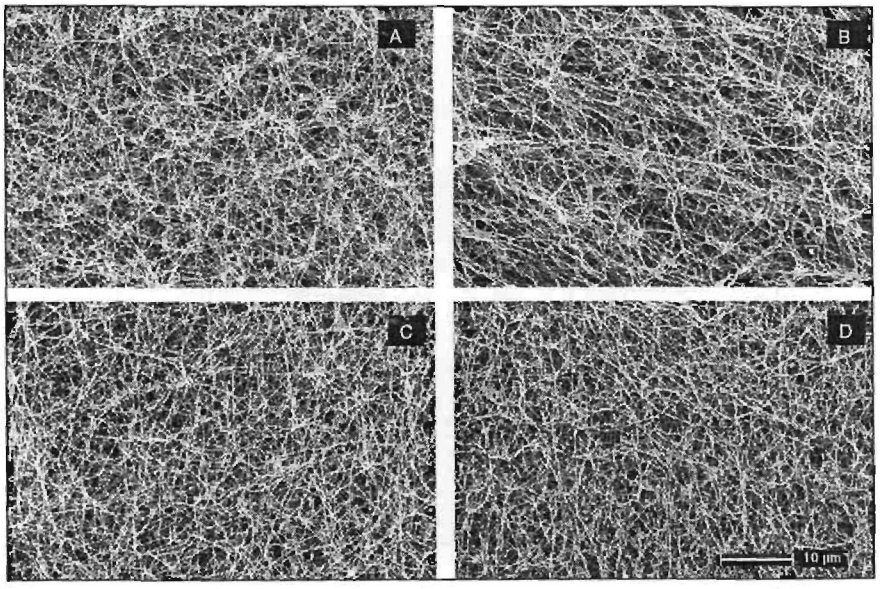

Figure 1: Representative scanning electron micrographs of fibrin clots. A) Non-diabetic subject at baseline; B) Non-diabetic subject at end measurement; C) Uncontrolled diabetic subject; D) Controlled diabetic subject.

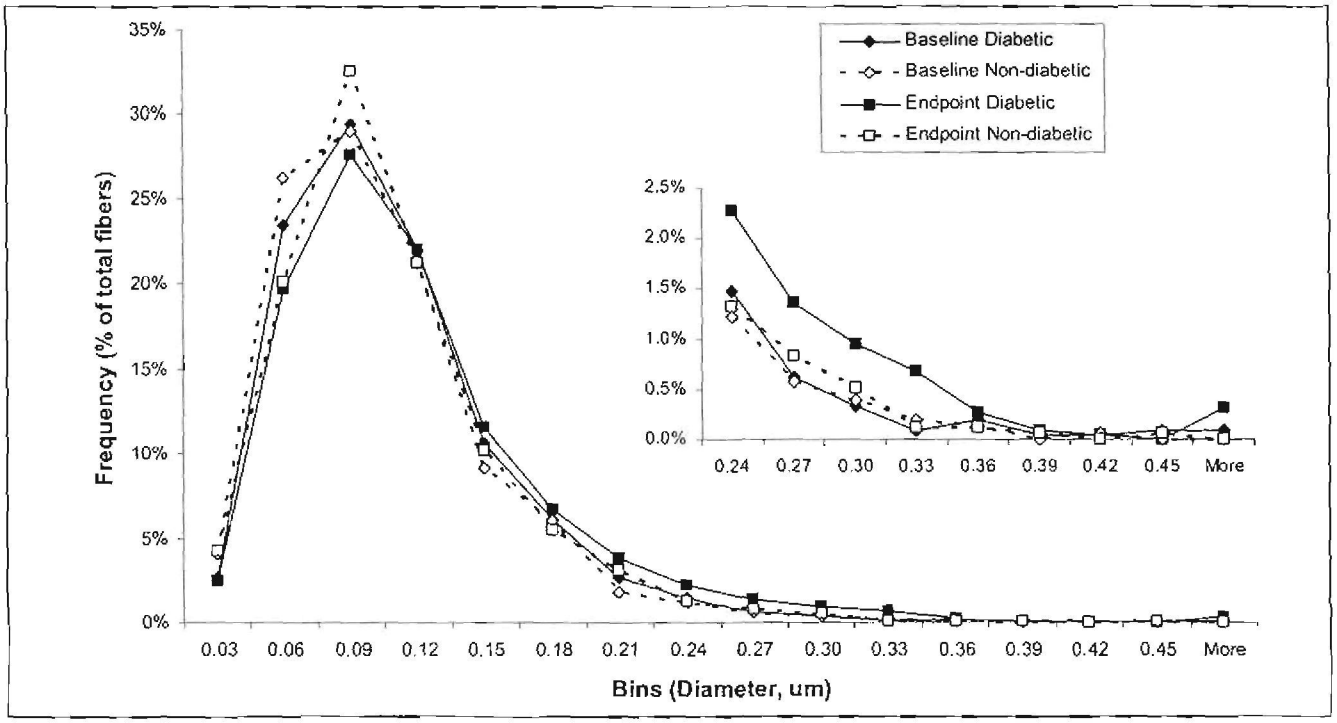

Figure 2: Histogram of fiber diameter distribution in the subsample of diabetic and non-diabetic subjects as calculated from SEM images.

the edge of a clot (Fig. 3). The diabetic subjects had a lower lysis rate than the non-diabetic subjects (3.08 vs. 8.52 μm/min). While the statistical significance was borderline (p=0.06), the likelihood of a practical relevance was considered to be large (r=0.54) (Table 2). A significant increase in lysis rate was observed with achievement of glycaemic control in the diabetic subjects (3.08 to 3.27 μm/min). Although this increase was small in comparison to the difference between the diabetic and non-diabetic subjects, it was seen consistently in each diabetic subject measured. Lysis rate showed significant negative correlations with the level of fibrinogen glycation (r=-0.63, p=0.028), HbA1c (r=-0.64, p=0.026), fasting venous glucose (r=-0.60, p=0.039) and slope (r=-0.77, p=0.009).

Effect of fibrinogen glycation on FXIIIa cross-linking

The fibrinogen isolated from subject plasma, contained very little if any FXIII (Fig. 4; lanes 7 and 8), indicating that the foregoing experiments were performed with non-crosslinked fibrin clots. When clots were crosslinked by the addition of FXIII, no difference in α-chain disappearance rate could be observed between the non-diabetic, uncontrolled diabetic, and controlled diabetic subjects, as there appeared to be no significant difference in the percentage α-chain density at any of the three time points (10 min: 19.7 ± 6.0; 22.6 ± 1.7; 21.6 ± 3.9; 45 min: 6.6 ± 4.6; 9.7 ± 3.3; 12.4 ± 3.6; and 90 min: 2.6 ± 3.6; 3.5 ± 2.0; 6.2 ± 4.1 of incubation with FXIIIa) using SDS-PAGE. Differences in γ-chain disappearance could not be measured as γ-chain crosslinking was already completed in all the subjects by 10 min (Fig. 4; lanes 1 and 2).

Discussion

This is the first intervention trial investigating the effect of glycaemic control on fibrin network structures of type 2 diabetic subjects using isolated fibrinogen. The network structures obtained from plasma clots from this same study, were reported previously (14). Using the plasma model, no difference in porosity, compaction and kinetics of clot formation between the diabetic and non-diabetic subjects at baseline was observed. Nor were any changes observed during the intervention in these variables in the diabetic group, despite a significant reduction in the level of fibrinogen glycation (14). This observed lack of effect could in part be explained by the fibrinogen concentration of the subjects. Both the non-diabetic and diabetic subjects had similar and raised fibrinogen concentrations and the results showed that the fibrin network characteristics measured were related to fibrinogen concentration and not to any of the glucose control markers. Fibrinogen concentration is acknowledged as one of the most important kinetic factors involved in the formation of the fibrin network and its final structure (28).

As described in this study, a purified fibrinogen model enables us to determine the effects of fibrinogen glycation on fibrin network structure independent of other plasma components. In contrast to that reported using plasma, there was a significant increase in permeability with achievement of glycaemic control and consequent decrease in the level of fibrinogen glycation in the diabetic subjects. A similar increase in permeability was observed in type 1 diabetic subjects on continuous subcutaneous insulin infusion, although it was not related to glycaemic control

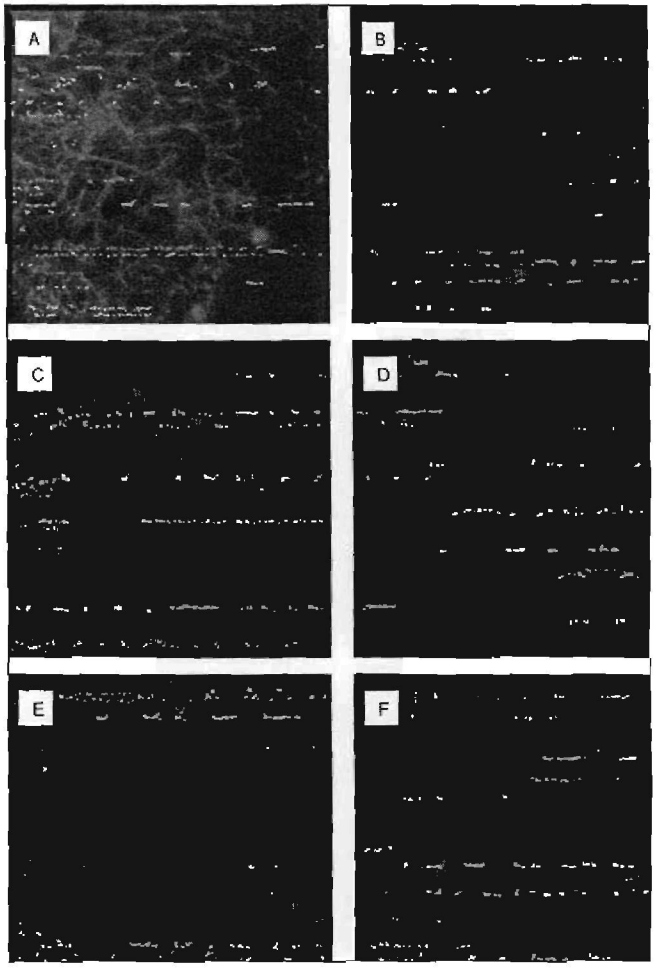

Figure 3: Representative 3D reconstructed images of purified fibrin clots from uncontrolled diabetic subjects (A, B), controlled diabetic subjects (C, D) and non-diabetic subjects (E, F) obtained by confocal microscopy (107 x 107 x 16 μm). Lysis was induced by the addition of 1 μg/ml tPA to the lysis front. Lysis times illustrated are after 0 (A, C, E) and 10 min (B, D, E).

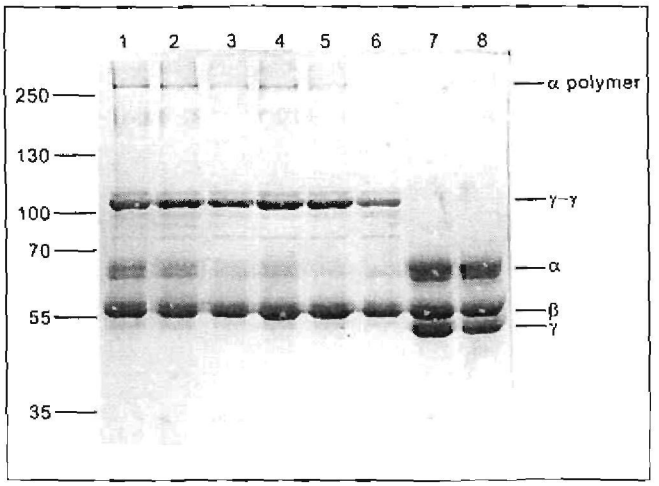

Figure 4: SDS-PAGE of FXIIIa crosslinking of fibrin clots, showing the effect of glycaemic control. Tracks 1, 3 and 5 are fibrin from uncontrolled diabetic patients after incubation with FXIIIa for 10, 45 and 90 min, respectively. Tracks 2, 4 and 6 are fibrin from controlled diabetic patients after the same incubation times. Tracks 7 and 8 are samples from uncontrolled and controlled diabetic patients respectively after 90 min of incubation without FXIII.

(16). The diabetic subjects also displayed a significantly faster rate of lateral fibrin aggregation (slope) and a slower lysis rate than the non-diabetic subjects, which decreased and increased, respectively, with glycaemic control and reduced levels of fibrinogen glycation. These changes in slope and lysis rate, although small in comparison to the differences between diabetic and non-diabetic subjects, were consistently seen in all of the selected diabetic patients in the sub-sample. Permeability, slope and lysis rate correlated significantly with markers of glycaemic control such as HbA1c, fasting venous glucose and fibrinogen glycation, confirming the observed changes were related to changes observed in glucose concentrations. This may explain why the decrease in the rate of lateral association reached significance in the subgroup, but not in the total diabetic group, as the decrease in fibrinogen glycation in the total group was on average smaller than that of the subgroup and may therefore have diluted the decrease in the slope in the total group.

The increase in permeability upon achievement of glycaemic control reflects an increased pore size of the fibrin network and may be a result of altered clot formation kinetics. The decrease in the slope of the turbidity curve upon glycaemic control indicates that the kinetics of polymerization, specifically lateral aggregation, are modified as a result of the decrease in glycation, resulting in an increased pore size. The rate of lateral aggregation may be influenced by factors such as modulation of binding affinity or possible conformational and charge changes (29), as has been documented for calcium (30) and chloride (31, 32). Similar changes may be induced by the presence of glucose on the fibrinogen molecule. Despite the increased permeability observed, there was, however, no significant difference in median fiber diameter between the uncontrolled and controlled diabetic subjects. This may be explained by the fact that the permeability measurement is a functional measurement which is also a more sensitive method than electron microscopy and is therefore able to detect smaller differences. In addition, the diabetic condition is complex, with various factors having varying degrees of influence on the different aspects of fibrin network structure, which may lead to the seemingly inconsistent results. Similar discrepancies were observed by the group of Dunn et al. (10) who found clots from diabetic patients to be more dense and less porous than that of control patients, but having increased, rather than decreased, fiber diameters.

Although this study showed no change in the mechanical properties of the clots after achievement of glycaemic control, the diabetic subjects in comparison with non-diabetic subjects, had clots with a lower proportion of the inelastic to elastic component of deformation both before and after achievement of glycaemic control. These mechanical properties of fibrin are functionally essential. Its structure has to be be strong enough to withstand the pressure of arterial blood flow and these properties

will determine whether a thrombus will deform reversibly or irreversibly, rupture or embolize, under the pressure of flowing blood (33).

Diabetes is considered a hypercoagulable state due to, amongst other reasons, a relatively inhibited fibrinolytic system (34). Ex-vivo lysis rates of type 2 diabetic subjects have been shown to be decreased when compared to non-diabetic subjects (15), using tPA as a lytic agent. Our results are in agreement with these findings. We demonstrated for the first time that lysis rate increases in type 2 diabetic subjects when glycaemic control is achieved. While lysis rate, permeability and the rate of lateral aggregation correlated significantly with markers of glycaemic control, glycation of fibrinogen may not be the only cause for the observed changes. In addition to hyperglycaemia, poor glycaemic control is also associated with oxidative and carbonyl stress (35), which in turn have been shown to alter the structure of the fibrinogen molecule (36). It is important to note that the differences in lysis rates observed in this study were obtained using non-crosslinked clots and the differences in lysis rates can therefore not be attributed to possible differences in FXIIIa-induced crosslinking. Dunn et al. (15) demonstrated that binding of both tPA and plasminogen to fibrin is impaired in diabetic subjects and that there is a corresponding decrease in plasmin generation on the clot surface, which in part explains the reduced lysis rates of diabetic subjects. These changes in enzyme kinetics may be directly related to the binding of additional molecules to fibrin as a result of hyperglycaemia or indirectly through the resultant altered fibrin network architecture (e.g. increased pore size and higher proportion of thicker fibers), which has been shown to be a modulator of fibrinolysis speed (3, 4), or most likely a combination of both.

The effect of fibrinogen glycation on FXIIIa-induced crosslinking is not clear. No differences in either γ- or α-chain crosslinking were observed when clots made from *in vitro* glycated and normal fibrinogen were compared (6–8). When diabetic and non-diabetic subjects were compared, α-chain crosslinking of diabetic subjects were previously found to be either decreased (12) or increased (10), and in our study, comparable to non-diabetic subjects. These discrepancies may be attributed to the different sample populations studied, differences in study designs and therefore different levels of fibrinogen glycation, or glycation of FXIII itself, or differences in analytical procedures. In our study there was additionally no discernable differences in FXIIIa-induced crosslinking observed between uncontrolled and controlled diabetic subjects.

In conclusion, using a purified model, we determined that achievement of glycaemic control alters the kinetics of fibrin polymerization, specifically lateral aggregation and improves permeability and lysis rate which may aid in the alleviation of the hypercoagulable state of diabetic subjects. Despite the fact that these variables were improved in all subjects, the changes observed in lateral aggregation and lysis rate were relatively small compared to the initial differences observed between the uncontrolled diabetic and non-diabetic subjects. This observation confirms that diabetes is a complex condition with many metabolic derangements having modulating effects on fibrin network structure, such as fibrinogen concentration, as was demonstrated in the plasma model (14). Therefore, the effect of individual contributing factors on fibrin network characteristics and the relative magnitude of the consequences amongst the many other causal factors *in vivo*, where whole blood clots are formed in the presence of platelets and blood flow, remain to be elucidated.

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