Fibrinogen functionality in black South Africans: The PURE study

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ABSTRACT

INTRODUCTION AND AIM

Black South Africans are experiencing an increase in the prevalence of cardiovascular disease (CVD). Fibrinogen functionality, including total and gamma prime (γ') fibrinogen concentration, as well as fibrin network structure, play an important role in CVD development and events. Several genetic and environmental factors influence fibrinogen functionality, and in turn, known CVD risk factors associated with total and γ' fibrinogen concentration have also been associated with altered fibrin clot structure. However, the main body of evidence regarding the role of fibrinogen functionality in CVD is based on studies conducted in white ethnicities and/or *in vitro*. The main aim of this study was, therefore, to determine the relationship between fibrinogen functionality and CVD in black South Africans in a plasma setting. Since there is greater genetic diversity in Africans than in non-black ethnicities, it was also our objective to investigate the influence of genetic polymorphisms in determining fibrinogen synthesis and plasma clot properties, and to determine possible gene-environment interactions altering clot properties.

PARTICIPANTS AND METHODS

The South African arm of the Prospective Urban and Rural Epidemiology (PURE) study included 2010 apparently healthy black men and women between the ages of 35 and 65 years, residing in rural or urban settlements. Blood samples were collected from the participants during a 12-week period in 2005. The following variables were analysed: total and γ' fibrinogen concentration, CVD risk factors and genetic polymorphisms in the fibrinogen and Factor XIII genes as well as turbidimetric analysis of clot formation and lysis (expressed as clot lysis time (CLT)).

RESULTS

Increased plasma levels of both total (largest contribution of 33%) and γ' fibrinogen were associated with increased fibre diameter while γ'/total fibrinogen ratio had the opposite effect. The rate of lateral aggregation of fibrin fibres (slope) increased with an increase in total fibrinogen concentration, but not fibrinogen γ'. Increasing fibrinogen γ' concentration was associated with longer CLTs and was the largest contributor to its variance (12%). Increased total and γ' fibrinogen were significantly associated with increased waist circumference, body mass index, C-reactive protein (CRP),

glycosylated haemoglobin, metabolic syndrome (MetS) and low high-density lipoprotein (HDL) cholesterol levels. Furthermore, the association of fibrinogen γ' with these CVD risk factors was independent of total fibrinogen levels. C-reactive protein was the largest contributor to variance in fibrinogen γ' levels and γ'/total fibrinogen ratio (apart from total fibrinogen). We observed significant associations between single nucleotide polymorphisms (SNPs) at rs1049636 and rs2070011 loci and increased total and γ' fibrinogen levels, respectively. Only SNP rs1800787 was associated with clot properties (increased maximum absorbance). Significant gene-environment interactions were observed between SNPs rs2227385, rs1800787, rs1800788, rs4220 and rs5985 and total and/or γ' fibrinogen levels in determining clot properties. The CVD risk factors age, MetS, CRP, HDL-cholesterol and homocysteine associated significantly with clot properties, independent of total and/or γ' fibrinogen plasma concentration.

CONCLUSION

The results of this thesis provide several novel insights relevant to this research field. Plasma γ' fibrinogen concentration and γ' ratio were found to be associated with altered clot properties in a plasma setting, and are also influenced by CVD risk factors other than fibrinogen. The associations between SNPs, total and γ' fibrinogen and clot properties differ somewhat from evidence reported in white populations. Significant gene-environment interactions between SNPs and total and γ' fibrinogen in determining clot properties existed and had opposing effects, *i.e.* both prothrombotic and antithrombotic, suggesting that the influence of genetic factors on fibrinogen should focus not only on concentration, but also on functionality. Cardiovascular disease risk factors also influence clot properties *in vivo*, through mechanisms independent of total and/or γ' fibrinogen concentration.

KEY TERMS: total fibrinogen, fibrinogen γ', genetic polymorphisms, fibrin clot properties, CVD risk factors

OPSOMMING – Fibrinogeen-funksionaliteit in swart Suid-Afrikaners: Die PURE studie

INLEIDING EN DOEL

Swart Suid-Afrikaners ondervind `n toename in die voorkoms van kardiovaskulêre siekte (KVS). Fibrinogeen-funksionaliteit, wat insluit, totale en gamma-*prime* (γ') fibrinogeen-konsentrasie, so wel as fibrien-netwerkstruktuur, speel `n belangrike rol in die ontwikkeling van KVS en -voorvalle. Verskeie genetiese- en omgewingsfaktore beïnvloed fibrinogeen-funksionaliteit en bekende KVS-risikofaktore wat verbande met totale en γ' fibrinogeen-konsentrasie het, het op hul beurt, self ook verwantskappe met veranderde fibrien-klontstruktuur. Nietemin, die oorgrote meerderheid van navorsing rakende die rol van fibrinogeen-funksionaliteit in KVS, is gebaseer op studies wat onderneem is in blankes en/of *in vitro* studies. Die hoofdoel van hierdie studie was dus om die verwantskap tussen fibrinogeen-funksionaliteit en KVS in swart Suid-Afrikaners in `n plasma-opset te bepaal. Aangesien daar groter genetiese diversiteit onder Afrikane as in nie-swart etnisiteite is, was dit ook ons doel om die invloed van genetiese polimorfismes op fibrinogeen-sintese en plasma-klonteienskappe te bepaal, en ook om moontlike geen-omgewingsinteraksies wat klonteienskappe kan beïnvloed, te bepaal.

PROEFPERSONE EN METODES

Die Suid-Afrikaanse arm van die *Prospective Urban and Rural Epidemiology* (PURE)-studie het 2010 oënskynlik gesonde swart mans en vroue, tussen die ouderdomme van 35 en 65 jaar, woonagtig in landelike of stedelike nedersettings, ingesluit. Bloed is tydens `n 12-week periode in 2005 versamel. Die volgende veranderlikes is geanaliseer: totale en γ' fibrinogeen-konsentrasie, KVS-risikofaktore, genetiese polimorfismes in die fibrinogeen- en Faktor XIII-gene asook turbiditeitsanalises van klontvorming en -lise (uitgedruk as klontlisetyd (KLT)).

RESULTATE

Verhoogde plasma-vlakke van beide totale (grootste bydrae van 33%) en γ' fibrinogeen is geassosieer met verhoogde fibrien-veseldeursnee terwyl die γ'/totale-fibrinogeen verhouding die teenoorgestelde effek gehad het. Die tempo van laterale aggregering van fibrienvesels (helling) het toegeneem soos wat totale fibrinogeen-konsentrasie toegeneem het, maar het geen verwantskap met fibrinogeen-γ' getoon nie. Toenemende fibrinogeen-γ' konsentrasie is geassosieer met langer klontlisetye (KLTe)

en was die grootste bydraer tot die variansie daarvan (12%). Verhoogde totale en γ' fibrinogeen het `n betekenisvolle verband met verhoogde middelomtrek, liggaamsmassa-indeks, C-reaktiewe proteïen (CRP), geglikosileerde hemoglobien, metaboliese sindroom (MetS) en lae hoë-digtheid lipoproteïen (HDL) cholesterol-vlakke gehad. Verder is gevind dat die verwantskap tussen fibrinogeen-γ' en hierdie KVS-risikofaktore onafhanklik van totale fibrinogeen-vlakke is. C-reaktiewe proteïen was die grootste bydraer tot die variansie in fibrinogeen-γ' vlakke en ook γ'/totale-fibrinogeen verhouding (buiten totale fibrinogeen). Betekenisvolle verwantskappe is tussen enkelnukleotied polimorfismes (SNPs) op lokus rs1049636 en rs2070011 en verhoogde totale en γ' fibrinogeen-vlakke, respektiewelik waargeneem. Enkelnukleotied polimorfisme rs1800787, was die enigste SNP geassosieer met klonteienskappe (verhoogde maksimum absorbansie). Betekenisvolle geen-omgewingsinteraksies is waargeneem tussen SNPs rs2227385, rs1800787, rs1800788, rs4220 en rs5985 met totale en/of γ' fibrinogeen-vlakke in die bepaling van klonteienskappe. Die KVS-risikofaktore ouderdom, MetS, CRP, HDL-cholesterol en homosisteïen, het verwantskappe, onafhanklik van totale en/of γ' fibrinogeen plasma-konsentrasie, met klonteienskappe gehad.

GEVOLGTREKKING

Die resultate van hierdie proefskrif verskaf nuwe insigte relevant tot hierdie navorsingsveld. Daar is gevind dat plasma fibrinogeen-γ' konsentrasie en γ'-verhouding verband hou het met veranderde klonteienskappe in `n plasma-opset, en dat dit ook beïnvloed word deur KVS-risikofaktore anders as fibrinogeen. Die verwantskappe tussen SNPs, totale en γ' fibrinogeen en klonteienskappe, het effens verskil van resultate soos geraporteer in blanke populasies. Betekenisvolle geen-omgewingsinteraksies tussen SNPs en totale en γ' fibrinogeen in bepaling van klonteienskappe het voorgekom en het uiteenlopende effekte gehad, m.a.w. beide pro-tromboties en anti-tromboties. Dit dui daarop dat die invloed van genetiese faktore op fibrinogeen nie alleenlik op konsentrasie moet fokus nie, maar ook op funksionaliteit. Verder het ons ook gevind dat KVS-risikofaktore klonteienskappe *in vivo* kan beïnvloed, deur meganismes wat onafhanklik is van totale en/of γ' fibrinogeen-konsentrasie.

SLEUTELTERME: totale fibrinogeen, fibrinogeen-γ', genetiese polimorfismes, fibrien-klonteienskappe, KVS-risikofaktore

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LIST OF ABBREVIATIONS

A	Adenine
Å	Angstrom
ADP	Adenosine diphosphate
AFM	Atomic force microscope
AIDS	Acquired immunodeficiency syndrome
Ala	Alanine
ANCOVA	Analysis of co-variance
ANOVA	Analysis of variance
Arg	Arginine
Arg-to-Lys	Arginine-to-lysine replacement
Asp	Aspartic acid
au	Absorbance units
au/s	Absorbance units per second
Aα	A alpha
α	Alpha
αC	Alpha C
BMI	Body mass index
BSA	Bovine serum albumin
Bβ	B beta
β	Beta
C	Cytosine
C/EBP	CCAATbox/enhancer-binding protein
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CHD	Coronory heart disease
CI	Confidence interval
CLT	Clot lysis time
cm	Centimetre
CRP	C-reactive protein
CstF	Cleavage stimulation factor
C-to-T	Cytosine-to-thymine replacement
CV	Coefficient variation
CVD	Cardiovascular disease

ºC	Degrees Celsius
D	Deletion
D’	Standardised disequilibrium
DE	Dynamic elastography
DNA	Deoxyribonucleic acid
DSE	Downstream sequence element
DVT	Deep vein thrombosis
EA	European American
EC	Endothelial cell
ECTIM	Etude Cas-Temoins sur l'Infarctus du Myocarde study
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
F	Forward
F13A1	Factor XIII subunit A gene
F13B	Factor XIII subunit B gene
Factor IXa	Activated Factor IX
Factor Va	Activated Factor V
Factor VIIa	Activated Factor VII
Factor VIIIa	Activated Factor VIII
Factor Xa	Activated Factor X
Factor XIa	Activated Factor XI
Factor XIIa	Activated Factor XII
Factor XIIIa	Activated Factor XIII
Fbg	Fibrinogen
FGA	Fibrinogen α
FGB	Fibrinogen β
FGG	Fibrinogen γ
FNS	Fibrin network structure
FPA	Fibrinopeptide A
FPB	Fibrinopeptide B
FTMS	Fourier transform mechanical spectroscope
FXIII	Factor XIII
γ	Gamma
γ’	Gamma prime

γA	Gamma A
g per day	Gram per day
g	Gram
G	Guanine
g/L	Gram per litre
GI	Glycaemic index
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
G-to-A	Guanine-to-adenine replacement
G-to-T	Guanine-to-thymine replacement
GW	Genome-wide
GWA	Genome-wide association
GWAS	Genome-wide association study
HbA1c	Glycosylated haemoglobin
Hcy	Homocysteine
HDL	High-density lipoprotein
HDL-cholesterol	High-density lipoprotein cholesterol
His	Histidine
His-to-Arg	Histidine-to-arginine replacement
HIV	Human immunodeficiency virus
HNF-3	Hepatocyte nuclear factor-3
Hs-CRP	High-sensitivity CRP
HSF	Hepatocyte-stimulating factor
HTL	Homocysteine thiolactone
HW	Hardy-Weinberg
I	Insertion
ICAM-1	Intercellular adhesion molecule-1
ICH	Intracerebral haemorrhage
IHD	Ischaemic heart disease
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IS	Ischaemic stroke
kB	Kilobase
kDa	KiloDalton

kg/m^2	Weight by height squared
K_s	Darcy constant
L	Litre
LD	Linkage disequilibrium
LDL	Low-density lipoprotein
LDL-cholesterol	Low-density lipoprotein cholesterol
Leu	Leucine
Lys	Lysine
μ	Fibre mass-length ratio
M	Molar mass
MAF	Minor allele frequency
Max abs	Maximum absorbance
MetS	Metabolic syndrome
mg/dL	Milligram per decilitre
mg/L	Milligram per litre
mg/mL	Milligram per millilitre
MI	Myocardial infarction
min	Minutes
ml	Millilitre
mM	Millimolar
mmHg	Millimetre of mercury
mmol/L	Millimol per litre
MONICA	Monitoring Trends and Determinants in Cardiovascular Disease study
mRNA	Messenger ribonucleic acid
μl	Micro litre
μM	Micromolar
μmol/L	Micromol per litre
n	Population size
NF-$_k$B	Nuclear factor-kappa B
ng/mL	Nanogram per millilitre
nm	Nanometer
pA1	Polyadenylation site 1
pA2	Polyadenylation site 2
PAI	Plasminogen activator inhibitor

PAI-1	Plasminogen activator inhibitor-1
$PAI\text{-}1_{act}$	Plasminogen activator inhibitor-1 activity
PAI-2	Plasminogen activator inhibitor-2
PCR	Polymerase chain reaction
PE	Pulmonary embolism
pg/ml	Picogram per millilitre
pM	Picomolar
PPAR-α	Peroxisome proliferator-activated receptor-α
PUFA	Polyunsaturated fatty acid
PURE	Prospective Urban and Rural Epidemiology
PVT	Portal vein thrombosis
r	Correlation coefficient
R	Reverse
r^2	Correlation coefficient squared
RBC	Red blood cell
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROTEM	Rotational thromboelastography
rs	Reference SNP
rsID	Reference SNP identifier
SCARF	Stockholm Coronary Artery Risk Factor study
SCFA	Short chain fatty acid
SD	Standard deviation
SEM	Scanning electron microscopy
SF2/ASF	Splicing factor 2/alternative splicing factor
SMILE	Study of Myocardial Infarctions Leiden
SNP	Single nucleotide polymorphism
T	Thymine
TAFI	Thrombin-activatable fibrinolysis inhibitor
TB	Tuberculosis
TC	Total cholesterol
TEA	Triethanolamine
TEG	Thromboelastography
TEM	Transmission electron microscopy
TF	Tissue factor

TG	Triglycerides
Thr	Threonine
Thr-to-Ala	Threonine-to-alanine replacement
TIA	Transient ischaemic attack
TIRFM	Total internal reflection fluoresence microscopy
TNF-α	Tumour necrosis factor-α
tPA	Tissue plasminogen activator
TSM	Thickness-shear mode
U/ml	Units per millilitre
UTR	Untranslated region
Val	Valine
Val-to-Leu	Valine-to-leucine replacement
Vs	Versus
VT	Venous thrombosis
VTE	Venous thromboembolism
vWF	Von Willebrand factor
WC	Waist circumference

CHAPTER 1:
Introduction

1.1 BACKGROUND

Cardiovascular disease (CVD) is an emerging global burden and it seems especially to afflict developing (low- and middle-income) countries (Yusuf *et al*., 2001) undergoing rapid industrialisation and urbanisation (Teo *et al*., 2009), including South Africa (Mbewu, 2009). Although the prevalence of CVD is lower among black South Africans than Caucasians, it is on the increase among black South Africans (Mbewu, 2009). The development of CVD involves complex interactions between risk factors and inflammatory and haemostatic systems that can eventually result in fibrin blood clot formation, which, depending on its final structure, can cause thrombotic occlusion (Scott *et al*., 2004; Standeven *et al*., 2005), embolise or lyse completely (Weisel, 2004; Weisel, 2007). The haemostatic system involves the glycoprotein, fibrinogen, which plays an important role in the final stage of blood coagulation, which includes fibrin clot formation and structure (Pulanić & Rudan, 2005). Fibrinogen is a known predictor of CVD, and its role in fibrin clot formation and/or clot structure, particularly, is of importance in CVD development. Other mechanisms by which fibrinogen may influence CVD include its involvement in platelet aggregation, plasma viscosity, vascular/endothelial function and the inflammatory process (Ernst & Resch, 1993; Stec *et al*., 2000; Pulanić & Rudan, 2005; De Moerloose *et al*., 2010; Lominadze *et al*., 2010; Papageorgiou *et al*., 2010). Fibrinogen concentration, however, can be influenced by several genetic and environmental factors. The contribution of environmental factors (*e.g*. age, sex, cohort, blood pressure, body mass index (BMI), fasting glucose, triglycerides, low-density lipoprotein (LDL)- and high-density lipoprotein (HDL) cholesterol levels) to variance in fibrinogen levels has been reported to be around 20%, while the contribution of genetic factors was found to be 30-40% (Freeman *et al*., 2002; Best *et al*., 2004). Moreover, several of the above-mentioned CVD risk factors have also been associated with altered fibrin network structure (Dunn *et al*., 2005; Sjøland *et al*., 2007b; Bhasin *et al*., 2008; Pretorius *et al*., 2010a; Alzahrani *et al*., 2012; De Lange *et al*., 2012). It is not yet clear, however, whether such associations are modulated by the fibrinogen concentration *per se* or are a result of other independent mechanisms.

The fibrinogen molecule consists of A alpha (Aα), B beta (Bβ), and gamma (γ) chains, of which the γ chain contains a gamma prime (γ') variant, which arises from alternative messenger ribonucleic acid (mRNA) splicing (Lovely *et al*., 2002; Weisel, 2005; Mannila, 2006a). The fibrinogen γ'-chain variant consists of 20 amino acid residues that have functional implications for the fibrinogen molecule, including its affinity for platelet aggregation and thrombin and Factor XIII binding (Mannila, 2006a; Standeven *et al*., 2005). The fibrinogen γ' variant contributes to approximately 8-15% of the total fibrinogen molecule (Mosesson, 2003) and has recently also been shown to play a role in CVD (Uitte de Willige *et al*., 2005; Mosesson *et al*., 2007; Mannila *et al*., 2007b; Cheung *et al*., 2008b). Moreover, it has become clear that is not only the absolute amount of fibrinogen γ', but also its relative amount, the γ'/total fibrinogen ratio, that is associated with CVD (Uitte de Willige *et al*., 2005; Cheung *et al*., 2008b; Cheung *et al*., 2009; Van den Herik *et al*., 2011). Like total fibrinogen, fibrinogen γ' also influences fibrin network structure, which is considered to be one mechanism by which fibrinogen γ' influences CVD development (Uitte de Willige *et al*., 2009b). In addition, other mechanisms by which fibrinogen γ' may influence CVD include its involvement in thrombin, platelet, and Factor XIII activities and the inflammatory process (Uitte de Willige *et al*., 2009b; Alexander *et al*., 2011; Farrell, 2012). Although only limited evidence is available, CVD risk factors, *e.g*. diabetes, age, gender, BMI, smoking, C-reactive protein (CRP) and triglycerides (TG), have been associated with fibrinogen γ' concentration (Mannila *et al*., 2007b; Cheung *et al*., 2008b; Lovely *et al*., 2010). Furthermore, recent evidence has shown that genetic factors contribute to approximately 54% of the variance in fibrinogen γ' concentration (Ozel *et al*., 2011). Most studies investigating the role of fibrinogen functionality (total and γ' fibrinogen concentration and fibrin clot properties) in CVD have been conducted in Caucasians and/or *in vitro* while limited evidence is available regarding the role of plasma concentrations *in vivo*. There is also very little known about its role in CVD in black ethnicities.

The final fibrin clot structure plays an important role in CVD and a structure consisting of thin, tightly packed fibres is associated with CVD events (Fatah *et al*., 1996; Carter *et al*., 2007; Undas *et al*., 2008; Undas *et al*., 2009a). Fibrin clot structure is influenced by several factors, including total and γ' fibrinogen concentration, as mentioned above (Ariëns, 2013). Moreover, fibrin clot structure is largely kinetically controlled and, in

order to understand its structural properties, information related to the process of clot formation is of immense value (Weisel & Nagaswami, 1992; Chernysh & Weisel, 2008). Kinetics of clot formation can be measured by using various methods, including direct measures such as rheometry and wave propagation techniques (Evans *et al*., 2006; Evans *et al*., 2008), as well as indirect measures such as spectrophotometry or turbidity and photometric techniques (Weisel & Nagaswami, 1992; Morais *et al*., 2006; Carter *et al*., 2007; Sjøland, 2007a; Pieters *et al*., 2008b; Chernysh *et al*., 2011). For the purpose of this study we will be using the turbidity method because of its high-throughput design, which makes it particularly useful in the case of large data sets (Carter *et al*., 2007). Turbidity curves, as obtained from the turbidity analysis, measure lag time (time for fibrin fibres to grow sufficiently to allow lateral aggregation), slope (rate of lateral aggregation) and maximum absorbance (average fibre size) (Wolberg, 2007), which provide information on clot properties. The main body of evidence on the role of fibrin network structure in CVD, as well as on the relationship between fibrinogen concentration, fibrin network properties and CVD, is based on Caucasians, while there is a lack of sufficient data on the characteristics of fibrin structure and the possible role it may play in the development of CVD in African populations. In this study, therefore, we will investigate the role of total and γ' fibrinogen concentration in CVD risk in an African population by determining the relation of γ' fibrinogen concentration to kinetics of clot formation and to CVD risk factors, as well as the association between total and γ' fibrinogen concentration and CVD risk factors in determining clot properties, using a plasma system.

Because the large genetic diversity in African populations in comparison with Caucasians may have a possible influence on the role of fibrinogen functionality in CVD in black ethnicities, we will also investigate the influence of known genetic polymorphisms in the fibrinogen and Factor XIII genes on total and γ' fibrinogen concentration, clot formation and lysis. We aim, in addition, to investigate whether possible interactions exist between the genetic polymorphisms and total and γ' fibrinogen concentration in determining fibrin clot properties.

1.2 AIM AND OBJECTIVES

Aim:

The main aim of this study is to determine the relationship between fibrinogen functionality (total and γ' fibrinogen concentrations and fibrin clot properties) and CVD risk in the black South African PURE population in a plasma setting.

Objectives:

- To determine the relationship between plasma total fibrinogen, fibrinogen γ' concentration, γ'/total fibrinogen ratio and fibrin network properties, including clot lysis time (CLT), using the turbidimetric analysis method of Lisman *et al*. (2005).
- To determine the association of known CVD risk factors with plasma total and γ' fibrinogen concentrations and the γ'/total fibrinogen ratio.
- To investigate the influence of genetic polymorphisms (identified from the literature), located in both the fibrinogen and Factor XIII genes, on fibrinogen synthesis and fibrin network properties, as well as possible gene-environment interactions in determining clot properties.
- To investigate the association between known CVD risk factors and fibrin network properties.

1.3 HYPOTHESES

We hypothesise that:

- Total and γ' fibrinogen plasma levels influence fibrin network properties.
- Not all, but certain CVD risk factors are associated with total and γ' fibrinogen plasma levels.
- Some genetic polymorphisms in the fibrinogen and Factor XIII genes influence fibrinogen synthesis and fibrin network properties, while others may have no effect.
- Fibrin network properties may be directly related to certain CVD risk factors independent from total and γ' fibrinogen plasma levels.

1.4 STRUCTURE OF THIS THESIS

This thesis is presented in article format and the technical aspects, required by the North-West University were applied. The document was edited by a competent language editor. A list of references for Chapters 1, 2 and 6 follows at the end of this thesis and references for Chapters 3, 4 and 5, which consist of individual manuscripts,

are provided at the end of each chapter. Chapter 1 provides background information to this study and includes the motivation for the study. This chapter also describes the aim, objectives and hypotheses of this study, lists members of the research team and their contributions, and outlines the structure of this thesis.

In Chapter 2, a literature review on fibrinogen functionality is presented, which firstly gives background information on fibrinogen, including its biochemistry and its role in the process of clot formation, determinants of fibrinogen concentration (environmental and genetics) and fibrin network structure, and then describes methods used to measure fibrin network structure. The chapter goes on to review fibrinogen γ', including its biochemistry, determinants of its concentration and the effect of fibrinogen γ' on fibrin network structure. Lastly, Chapter 2 provides background information on CVD in the black South African population and on the role that fibrinogen and fibrinogen γ' play in CVD development and events.

Chapter 3 consists of a published article (2013) titled "Evidence that fibrinogen γ' regulates plasma clot structure and lysis and relationship to cardiovascular risk factors in black Africans." (*Blood*, vol. 121(16):3254-3260). This article followed the technical style stipulated in the instructions to authors in the journal *Blood*, and investigated the relationship between plasma total and γ' fibrinogen concentration and plasma clot properties, as well as the relationship between plasma total and γ' fibrinogen concentration and CVD risk factors.

Chapter 4 consists of an article accepted for publication in the *British Journal of Haematology* (July 2014), titled "Genetic polymorphisms influencing total and γ' fibrinogen levels and fibrin clot properties in Africans." This article followed the technical style recommended in the instructions to authors in the *British Journal of Haematology* and investigated the association between genetic polymorphisms and total and γ' fibrinogen levels and fibrin clot properties.

Chapter 5 presents an article accepted for publication in *Thrombosis Research* (Aug 2014) titled "CVD risk factors are related to plasma fibrin clot properties independent of total and or γ' fibrinogen concentration" and followed the technical style stipulated in the instructions to authors in *Thrombosis Research*. The article investigated whether the

association between CVD risk factors and clot properties are mediated by total and/or γ' fibrinogen concentration.

Chapter 6 summarises the findings of this study and highlights the relevance and contribution of these findings to broaden scientific knowledge. This chapter draws conclusions from the findings of this study and provides recommendations for future research.

1.5. RESEARCH TEAM AND CONTRIBUTIONS

Table 1.1: Members of the research team and their contributions to articles presented as part of this thesis

Initials, surname and signature*	Affiliation	Role in this study
Mrs C.M. Kotzé	Centre of Excellence for Nutrition, North-West University, Potchefstroom Campus, South Africa	Full-time Ph.D student, protocol writing, laboratory analysis of haemostatic variables, data processing, statistical analysis, interpretation of results and writing up of the literature and data (co-author of Chapter 3, first author of Chapters 4 and 5).
Prof. M. Pieters	Centre of Excellence for Nutrition, North-West University, Potchefstroom Campus, South Africa	Promoter of Ph.D thesis, guidance regarding protocol writing, interpretation of results and co-author of Chapters 3, 4 and 5.
Prof. J.C. Jerling	Centre of Excellence for Nutrition, North-West University, Potchefstroom Campus, South Africa	Co-promoter of Ph.D thesis and co-author of Chapter 3.
Dr C. Nienaber-Rousseau	Centre of Excellence for Nutrition, North-West University, Potchefstroom Campus, South Africa	Assistant promoter of Ph.D thesis, guidance in writing of genetic aspects and co-author of Chapter 4.
Dr Z. de Lange-Loots	Centre of Excellence for Nutrition, North-West University, Potchefstroom Campus, South Africa	Co-author of Chapters 4 and 5; laboratory analysis of the clot lysis assay.
Dr T. Hoekstra	Department of Clinical Epidemiology, Leiden University Medical Centre, Leiden, The Netherlands	Co-author of Chapter 4 and laboratory analysis of some of the polymorphisms.
Dr M.P.M. de Maat	Department of Hematology, Erasmus University Medical Centre, Rotterdam, the Netherlands	Critical reading of final manuscript (Chapter 4).

Prof. R.A.S. Ariëns 	Theme Thrombosis, Division of Cardiovascular and Diabetes Research, Multidisciplinary Cardiovascular Research Centre and Leeds Institute for Genetics, Health and Therapeutics, School of Medicine, University of Leeds, UK	Critical reading of final manuscript (Chapters 3 and 5).
*With my signature I declare that I approved the above-mentioned articles, that my role in the study, as indicated above, is representative of my actual contribution and that I hereby consent that it may be published as part of the thesis of Retha C.M. Kotzé		

CHAPTER 2:

Fibrinogen functionality in black South Africans

2.1 INTRODUCTION

Cardiovascular disease (CVD) is a world-wide epidemic and a major cause of morbidity and mortality. Although CVD initially affected developed (high-income) countries, it has lately also begun to affect developing (low- and middle-income) countries (Teo *et al*., 2009), including South Africa, to such an extent that the burden of CVD in these countries already contributes to 80% of global CVD (Yusuf *et al*., 2004) and it is estimated that this contribution will exceed 80% by 2020. It is suggested that developing countries, particularly those exposed to transitions such as rapid industralisation and urbanisation, carry the heaviest burden (Teo *et al*., 2009).

Cardiovascular disease has been associated with various proven causally linked risk factors, such as smoking, hypertension, obesity, physical inactivity, dyslipidaemia and diet, as well as risk markers for which the association of cause and effect has still to be proved, such as increased homocysteine (Hcy) levels, low socioeconomic status, inflammatory markers and increased fibrinogen levels (Yusuf *et al*., 2001). Fibrinogen *per se* is a known predictor of CVD and plays an important role in the development of CVD. Elevated fibrinogen levels have been associated with increased platelet aggregation, plasma viscosity and red blood cell (RBC) aggregation, which all contribute to thrombus formation (Ernst & Resch, 1993; Pulanić & Rudan, 2005; De Moerloose *et al*., 2010). Furthermore, elevated fibrinogen levels have been shown to impair vascular and endothelial function, and to lead to a proinflammatory state (Lominadze *et al*., 2010; Davalos & Akassoglou, 2012). Fibrinogen is also known as an acute-phase reactant that, along with other inflammatory markers, enhances the process of atherosclerosis (Pulanić & Rudan, 2005; Bot *et al*., 2008; Davalos & Akassoglou, 2012). Lastly, it is known to alter fibrin network structure, resulting in network alterations that have been associated with various CVD events (Ajjan & Grant, 2005; Ariëns, 2011). Fibrinogen levels, however, are influenced by various factors, including genetic and environmental factors. Regarding the role of genetics, various studies, both candidate-gene and genome-wide association studies (GWASs), have investigated the effect of genetic factors on fibrinogen concentration and fibrin network structure (Yang *et al*., 2003; Soria *et al*., 2005; Yang *et al*., 2007; Danik *et al*., 2009; Dehghan *et al*., 2009). However,

genetic data obtained from Caucasian populations cannot simply be extrapolated to African populations, owing to the larger genetic variability in Africans (Chen *et al*., 1995; Schuster *et al.,* 2010). Genome-wide association (GWA) data for black South Africans are also still lacking, necessitating genetic studies in Africans.

Fibrinogen's alternatively spliced fibrinogen gamma (γ) chain variant, gamma prime (γ'), has also recently been shown to influence fibrinogen functionality and so play a role in the development of CVD (Uitte de Willige *et al*., 2009b). Fibrinogen γ' is described as an emerging new risk marker for CVD (Farrell, 2012) and seems to have different roles in arterial and venous disease states. It has been shown to modulate various factors and processes in the pathology of thrombosis that can result in either a prothrombotic or an antithrombotic effect. The relationship between total fibrinogen, fibrinogen γ', γ'/total fibrinogen ratio and CVD, however, is not yet clear. Although fibrinogen γ' is a fibrinogen isoform, it has also demonstrated associations with CVD events, independent of total fibrinogen concentration (Mannilla *et al*., 2007b; Van den Herik *et al*., 2012). In comparison with fibrinogen, determinants of fibrinogen γ' levels, and the role of fibrinogen γ' in CVD are just beginning to emerge, and only limited data are currently available.

The majority of studies investigating the association of total fibrinogen, fibrinogen γ' and the γ'/total fibrinogen ratio with CVD and its determinants have been conducted in Caucasians. Additionally, studies investigating fibrinogen γ', the γ'/total fibrinogen ratio and its determinants make use mainly of a purified system, but information regarding its functionality in plasma or *in vivo* is largely unknown. In comparison with Caucasians, the African population presents with higher fibrinogen levels despite a lower, albeit increasing, CVD prevalence. However, the role of fibrinogen in CVD in the African population is still not fully understood and data on the association between fibrinogen γ', the γ'/total fibrinogen ratio and CVD risk or its determinants in Africans are currently lacking. Therefore the question arises: to what extent do the typically higher fibrinogen levels found in Africans, as well as fibrinogen γ' and the γ'/total fibrinogen ratio, relate to genetics, fibrin network structure and environmental factors shown to play a role in CVD in Caucasians?

The outline and focus of the following literature review chapter includes: an overview of the biochemistry of fibrinogen and the process of clot formation; the genetic, demographic and environmental determinants (tabulated) of fibrinogen levels and fibrin network structure; and the methods used to measure clot structure. A discussion is also provided on the biochemistry of fibrinogen γ', determinants of its plasma concentration and the effect of fibrinogen γ' on fibrin network structure. Lastly, an overview is given on CVD in black South Africans and the relationship of both fibrinogen and fibrinogen γ' with CVD.

2.2 OVERVIEW: FIBRINOGEN AND CLOT FORMATION / FIBRINOGEN FUNCTIONALITY

The overview of fibrinogen and clot formation / fibrinogen functionality includes: the biochemistry of fibrinogen (section 2.2.1) which describes the biochemistry, molecular structure, gene regulation and expression of fibrinogen; clot formation (section 2.2.2), which describes the process of clot formation and stabilisation upon vessel injury and factors affecting fibrinogen concentration; fibrin network structure (section 2.2.3), which summarises the influences of genetic, environmental, biological and demographic factors on fibrinogen concentration and fibrin network structure; and lastly, methods of measuring clot structure (section 2.2.4), which encompass the methods of measuring the structural and mechanical properties as well as the kinetics of clot structure and formation.

2.2.1 Biochemistry of fibrinogen

Fibrinogen is a large, soluble, fibrous glycoprotein with a molecular weight of 340 kiloDalton (kDa) (Mosesson, 2005; Weisel, 2005). It is synthesised mainly in the liver at a rate of 1.7–5.0 g per day and although most (80–90%) of it circulates in the plasma, fibrinogen is also present in platelets, lymph nodes and interstitial fluid (El-Sayed *et al*., 2004; Weisel, 2005). The normal circulating plasma concentration of fibrinogen varies between 1.5 and 4.5 g/L while only 0.5 to 1.0 g/L is required for haemostasis (Kamath & Lip, 2003). Fibrinogen has a half-life of approximately three to five days (Kamath & Lip, 2003; Weisel, 2005), but the plasma protein pathways are unknown. In healthy individuals a small percentage (2–3%) of fibrinogen is lost *via* coagulation and fibrinolysis while the fibrinogen degradation products seem to contribute to regulating fibrinogen turnover (Weisel, 2005).

The fibrinogen molecular structure is a dimer and consists of two sets of three polypeptide chains: A alpha (Aα), B beta (Bβ), and γ chains with molecular weights of 66.5, 52.0 and 46.5 kDa, respectively (Figure 2.1). The fibrinogen molecule is 45 to 46 nm in length (Blombäck, 1996; Weisel, 2005; Lim *et al*., 2008) and has a width of 9 nm (Blombäck, 1996). It consists of two domains/regions, designated as the E region, which forms the centre (location of the N-terminus of the molecule) and the D regions, located at the end of the molecule. The three polypeptides protrude from the E region to form α-helical coiled-coil rods until they reach the D regions at the end of the molecule. The D regions consist also of the C-terminal ends of the Bβ, and γ chains, as well as part of the Aα chain. The C-terminal ends of the Aα chains emerge from the D region and interact with each other and move back to the E region, where they remain until fibrinogen is converted to fibrin (Blombäck, 1996; Mosesson, 2005; Standeven *et al*., 2005; Weisel, 2005). The E region contains, in addition, the N-terminus of the fibrinogen molecule, as previously mentioned, which plays an important role in the process of fibrin clot formation (which will be further discussed in section 2.2.2). To initiate the process of fibrin polymerisation or clot formation, thrombin must first cleave the N-terminal regions of both the Aα- and Bβ-chain polypeptides of fibrinogen, after which the polypeptides are known as fibrinopeptide A (FPA) and fibrinopeptide B (FPB), respectively (McDowall, 2006). The N-termini of the Aα and Bβ chains thus host FPA and FPB, respectively (Mosesson, 2005; Weisel, 2005).

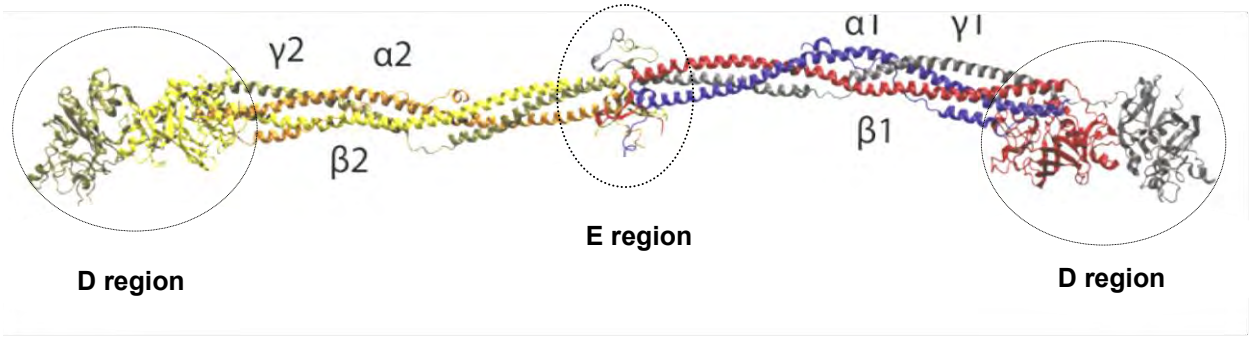

Figure 2.1: Fibrinogen structure [Adapted from Lim *et al*. (2008)]

The Aα, Bβ, and γ chains contain 610, 461 and 411 amino acid residues, respectively, which are linked together by five symmetrical disulphide bridges within the N-terminal E region (Mosesson, 2003). In addition, there are four biantennary-type carbohydrate chains that are linked to the Bβ and γ chains. The Aα chains do not contain carbohydrates. The carbohydrate chains are heterogeneous and consist of mannose,

galactose, glucosamine and sialic acid (Blombäck, 1996; Weisel, 2005). These carbohydrate chains play an important role in fibrin polymerisation and clot structure. Fibrinogen with high levels of sialylation of its carbohydrates (as in patients with liver cirrhosis and other liver diseases), for example, produces clots with thinner fibres and many branch points. The removal of sialic acid (desialylation) from the carbohydrate of fibrinogen, however, produces clots consisting of thicker fibres (Weisel, 2005). Fibrinogen also contains calcium binding sites (weak and strong) that play a role in the stability of fibrinogen's structure and function. It has further been suggested that both calcium-binding sites and the carbohydrate chains of fibrinogen modulate lateral aggregation (Weisel, 2005).

The fibrinogen molecule is synthesised during a stepwise assembly of the Aα, Bβ, and γ chains. These three chains are encoded by three independent genes (Huang *et al*., 1993; Huang *et al*., 1996; Weisel, 2005), fibrinogen Aα, Bβ and γ, which are clustered in the distal third (in a 50-kilobase region) of chromosome 4, bands q23–q32. The genes are transcribed and translated separately in a highly coordinated manner (Kant *et al*., 1985; Weisel, 2005; Mannila, 2006a; De Moerloose *et al*., 2010). The Aα-chain gene is positioned in the middle of the cluster with the Bβ-chain gene on the one side (downstream), and γ chain on the other side. The Aα- and γ-chain genes are behind each other and are transcribed towards the Bβ-chain gene; the Bβ chain is transcribed in the opposite direction (towards the Aα- and γ-chain genes) (Kant *et al*., 1985).

Transcription results in alternatively spliced variants or isoforms of the fibrinogen Aα, Bβ and γ chains (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/). There are two isoforms of the Aα chain that arise as a result of alternative splicing, designated the Aα chain and the Aα–E chain. The Aα chain is encoded by exons one to five which code for 625 amino acid residues, of which 15 (611–625) are removed posttranslationally. The Aα–E chain is encoded by exons one to six and is an extended Aα-chain subunit containing 847 amino acids (Fu & Grieninger, 1994; Weisel, 2005). This subunit further contains an additional Bβ- and γ-chain-like C-terminus, which leads to a molecular weight of approximately 110 kDa, amounting in total to a 420-kDa fibrinogen molecule. The Aα–E chain is present in only one to two percent of adult fibrinogen molecules, but seems to be less susceptible to proteolytic degradation than the Aα chain (Fu & Grieninger, 1994).

Formation of the Bβ chain is a rate-limiting step in fibrinogen molecule assembly (Zito, *et al*., 1997). The Bβ-chain gene consists of eight exons (Weisel, 2005). A strong splicing factor 2/alternative splicing factor (SF2/ASF) binding site or enhancer sequence within exon seven of the Bβ-chain gene has been identified. This, according to Spena *et al*. (2006), suggests the existence of a splicing-regulatory network, which is normally silent in the natural splicing environment of the Bβ chain.

The γ-chain gene consists of ten exons and alternative processing of the γ-chain messenger ribonucleic acid (mRNA) transcript also gives rise to two types of γ-chain variants: the γ' and gamma A (γA) chain (Weisel, 2005; De Moerloose *et al*., 2010). The γ' and γA chains contain 427 and 411 amino acid residues, respectively (Mannila, 2006a). The γA chain arises from splicing of intron nine, resulting in the translation of exon nine, followed by exon ten up to the stop codon after the fourth amino acid residue (Cooper *et al*., 2003; Standeven *et al*., 2005; De Moerloose *et al*., 2010). The γ'-chain variant results from alternative processing at exon nine and exon ten boundaries and translation is from exon nine into intron nine up to the stop codon present after 20 amino acid residues (Cooper *et al*., 2003). It is thus an extension of the γA chain by 16 amino acid residues (Standeven *et al*., 2005), and the four codons of exon ten are replaced with 20 alternative codons, leading to a more acidic chain (Blombäck, 1996; Standeven *et al*., 2005). Such a replacement at the C-terminal amino acid sequence of the γA chain has functional implications for the fibrinogen molecule (Cooper *et al*., 2003; Standeven *et al*., 2005). The γ' chain, for example, has lower affinity for platelet aggregation, but has a high affinity for thrombin binding and Factor XIII binding (Mannila, 2006a; Standeven *et al*., 2005). Presence of the γ' chain can also alter clot structure and function (Falls & Farrell, 1997; Cooper *et al*., 2003). The γ'-chain variant contributes to about eight percent of the total fibrinogen γ-chain population (Mosesson, 2003). Approximately 85% of the fibrinogen molecule is homodimeric, meaning that both halves of the molecule contain γA chains (γA/γA), while approximately 8–15% of the molecule is heterodimeric, which means that each half contains one γ' chain and one γA chain (γ'/γA) (Cooper *et al.*, 2003; Mannila, 2006a). Less than one percent of the circulating molecule is homodimeric, which means that each half contains two γ' chains (γ'/γ') (Mannila, 2006a). Fibrinogen γ' and its role in clot structure and CVD will be discussed in more detail in sections 2.3.3 and 2.4.3.

The Aα, Bβ, and γ chains are secreted in the parenchymal cells of the liver (Huang *et al*., 1993). The individual chains are translated, processed and assembled in the rough endoplasmic reticulum and are then secreted into the circulation as mature fibrinogen (Huang *et al*., 1996; Weisel, 2005; Mannila, 2006a). Intracellular chain assembly occurs through specific intermediates and seems to follow a stepwise progression from a single chain to two-chain to three-chain complexes that dimerise to finally form a fibrinogen molecule (Huang *et al*., 1993; Weisel, 2005). Huang *et al*. (1996) suggested from their own laboratory data that initial steps of fibrinogen assembly involve formation of Aαγ and Bβγ dimers linked by disulphide bonds. A third chain is then added to each of the dimers to eventually form trimeric (Aα Bβ γ) half molecules which dimerise and become linked by five disulphide bonds to form a fibrinogen molecule.

Fibrinogen synthesis is controlled at transcription level (De Moerloose *et al*., 2010) and can be regulated by hormones such as glucocorticoid dexamethasone, hepatocyte-stimulating factor (HSF) (Otto *et al*., 1987), oestrogen estadiol-17 (Weisel, 2005) and thyroid hormones, as well as by interleukin-6 (IL-6) and transcription factors such as peroxisome proliferator-activated receptor (PPAR)-α (Mannila, 2006a). Because fibrinogen is an acute-phase reactant protein, its synthesis can be upregulated during various physiological and inflammatory conditions (De Moerloose *et al*., 2010). Factors affecting fibrinogen concentrations will be discussed in more detail in section 2.2.3. Fibrinogen is the major coagulation protein in blood and plays an important role in the process of haemostasis, wound healing and inflammation. Although fibrinogen is a soluble molecule, it forms an insoluble gel or clot when converted to fibrin by the action of thrombin (Weisel, 2005), as will be discussed in the following section. Fibrinogen is an important determinant in blood viscosity and is necessary for platelet aggregation (Weisel, 2005; De Moerloose *et al*., 2010).

2.2.2 Clot formation

Upon blood vessel injury the process of blood coagulation is activated in order to form a fibrin clot which has to be dissolved again through the process of fibrinolysis once the vessel wall injury has been healed. Reactions during blood coagulation involving coagulation factors seem to be confined to the surfaces of specific cells, leading to the cell-based model, according to which the process of haemostasis occurs in three

separate, but overlapping steps, namely, 1) initiation, 2) amplification and 3) propagation (Monroe & Hoffman, 2006).

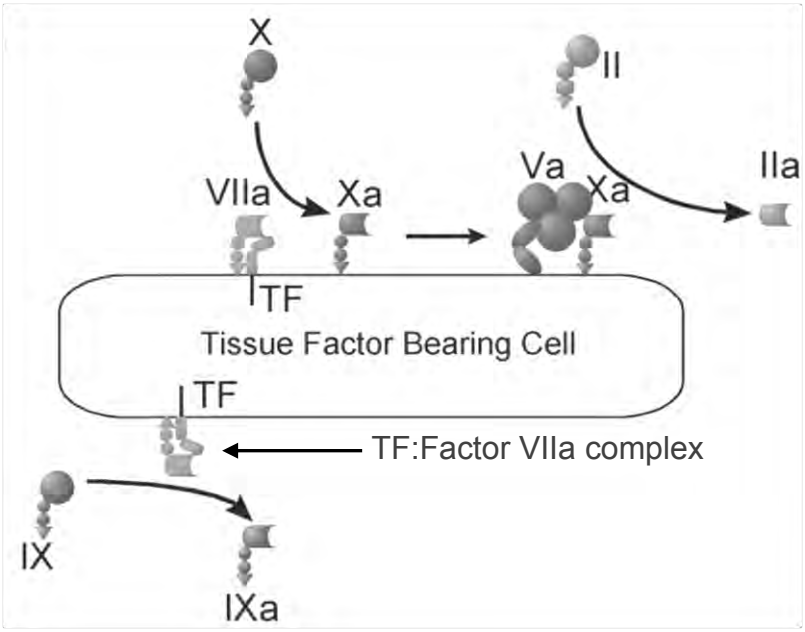

Figure 2.2: Initiation phase [Adapted from Monroe and Hoffman (2006)]
TF: tissue factor; II: prothrombin; IIa: thrombin

1) Initiation phase: (Figure 2.2). During blood vessel injury, blood is exposed to tissue factor (TF)-bearing cells that are situated in the extra-vascular space. Tissue factor is the membrane receptor of Factor VII (Colman *et al*., 2000:17) and contact with TF results in the binding of Factor VII to TF, which results in the formation of enzymatically active TF/Factor VIIa complexes (Colman *et al*., 2000:17). These complexes activate Factor IX to Factor IXa and Factor X to Factor Xa. Factor V is then activated by Factor Xa or non-coagulation proteases to Factor Va. Factor Va and Factor Xa together form prothrombinase complexes, which convert prothrombin to limited amounts of thrombin (Colman *et al*., 2000:18; Monroe & Hoffman, 2006).

2) Amplification phase: (Figure 2.3). This phase takes place on the platelet surface and, although limited, the amount of thrombin generated is vital to the activation of platelets. Addition of thrombin to the partially activated platelets through adherence at the site of injury results in a much higher level of procoagulant activity than the adhesion alone (Monroe & Hoffman, 2006). Platelets degranulate and some contents of the α-granules, such as fibrinogen, von Willebrand Factor (vWF) and Factor V, are released into the extracellular space (Mannila, 2006a). Further

functions of thrombin are to activate Factor V, Factor VIII and Factor XI on the activated platelet surface. By the end of the amplification phase, conditions have been set for large-scale thrombin generation during the propagation phase (Monroe & Hoffman, 2006).

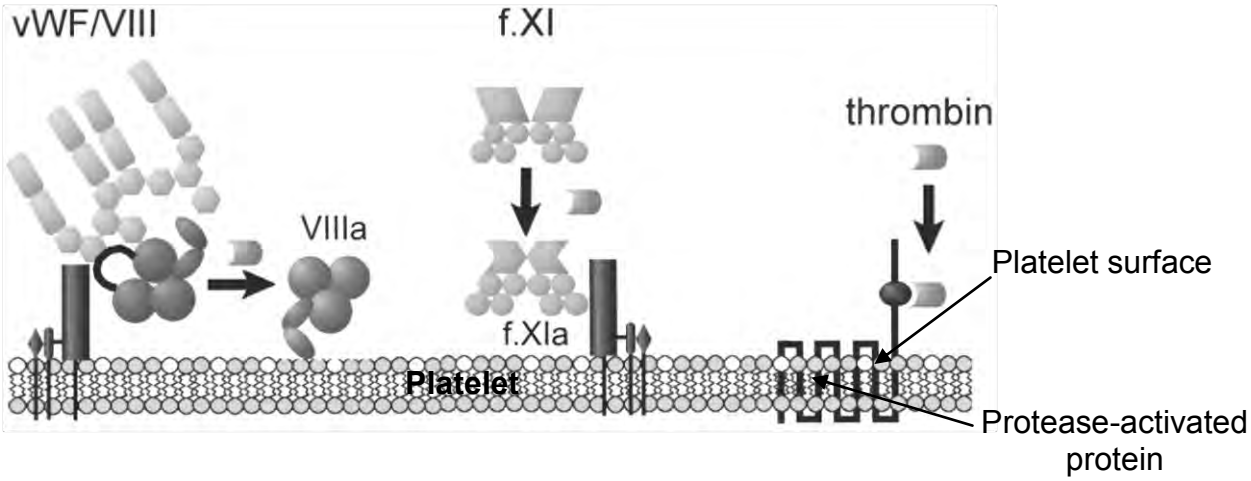

Figure 2.3: Amplification phase [Adapted from Monroe and Hoffman (2006)]
f.XI: factor XI; f.XIa: factor Xa; vWF: von Willebrand Factor

3) Propagation phase: (Figure 2.4). The propagation phase occurs on the surface of the activated platelets. During this phase Factor IXa which has been activated during the initiation phase, binds to Factor VIIIa on the platelet surface and forms the Factor IXa/Factor VIIIa complex (tenase complex) (Mannila, 2006a; Monroe & Hoffman, 2006). This step is followed by activation of Factor X to Factor Xa (Mannila, 2006a). Factor Xa cannot effectively move from the TF-bearing cell to the platelet surface and additional Factor IXa is, therefore, supplied by platelet-bound Factor XIa in order for the Factor IXa/Factor VIIIa complex to provide Factor Xa directly onto the platelet surface (Mannila, 2006a; Monroe & Hoffman, 2006). Factor Xa then rapidly forms a complex with platelet surface Factor Va (the prothrombinase complex) to produce sufficient thrombin generation to activate fibrinogen to form the fibrin network (Mannila, 2006a; Monroe & Hoffman, 2006).

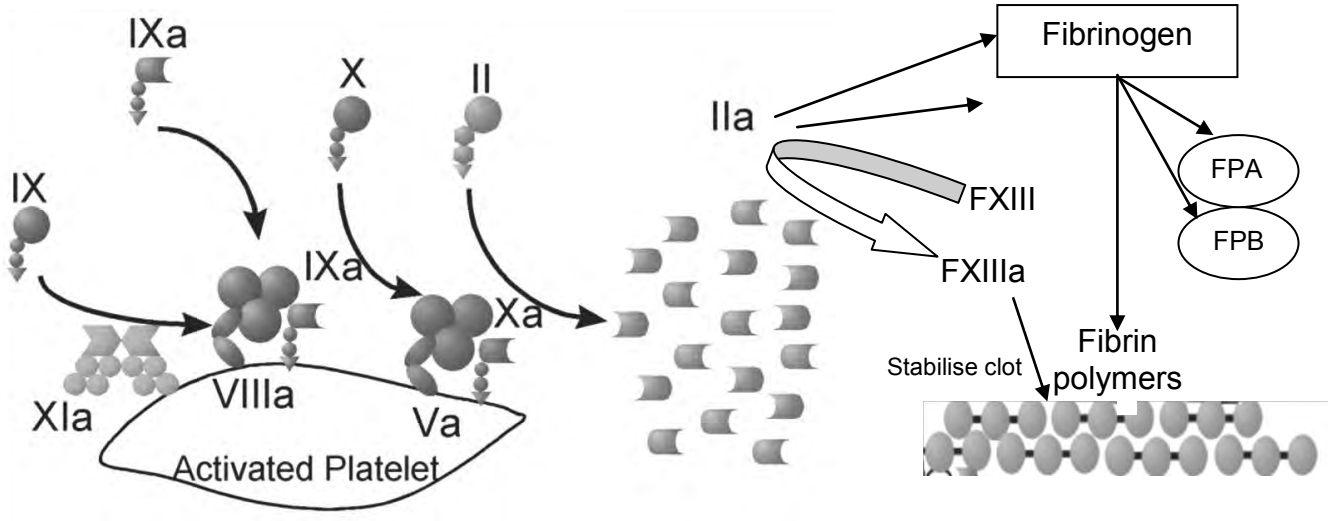

Figure 2.4: Propagation phase [Adapted from Monroe and Hoffman (2006)]
FXIII: Factor XIII; FPA: fibrinopeptide A; FPB: fibrinopeptide B; II: prothrombin; IIa: thrombin

Fibrin clot formation is the final step in blood coagulation. Clot formation is initiated by thrombin-catalysed cleavage of FPA and FPB from the N-termini of the fibrinogen Aα and Bβ chains, respectively (Standeven *et al*., 2005, Mannila, 2006a) to convert fibrinogen (soluble) to fibrin monomers, which aggregate into insoluble polymers (Weisel, 2005) (Figure 2.5). Cleavage of FPA and FPB by thrombin results in the exposure of specific binding or polymerisation sites ("knobs") in the E region that interact with complementary "holes" exposed at the D regions of other fibrinogen molecules (Weisel, 2005). Fibrinopeptide A is the first to be cleaved (Evans *et al*., 2006); it is cleaved between the Arganine (Arg) 16 and Glycine (Gly) 17 residues (Binnie & Lord, 1993; Litvinov *et al*., 2007) and exposes polymerisation sites in the central E region, the E_A sites, which interact with complementary "holes" in the D region, the D_a sites (Mannila, 2006a). These E_A:D_a ("knob-hole") polymerisation site interactions lead to aggregation of fibrin monomers in a half-staggered overlapping end-to-middle binding, *i.e*. the E region of the molecule with the complementary D region of the adjacent molecule, to form oligomers and two-stranded protofibrils (structures containing more than eight monomers) (Weisel, 2005; Evans *et al*., 2006; Chernysh *et al*., 2011).

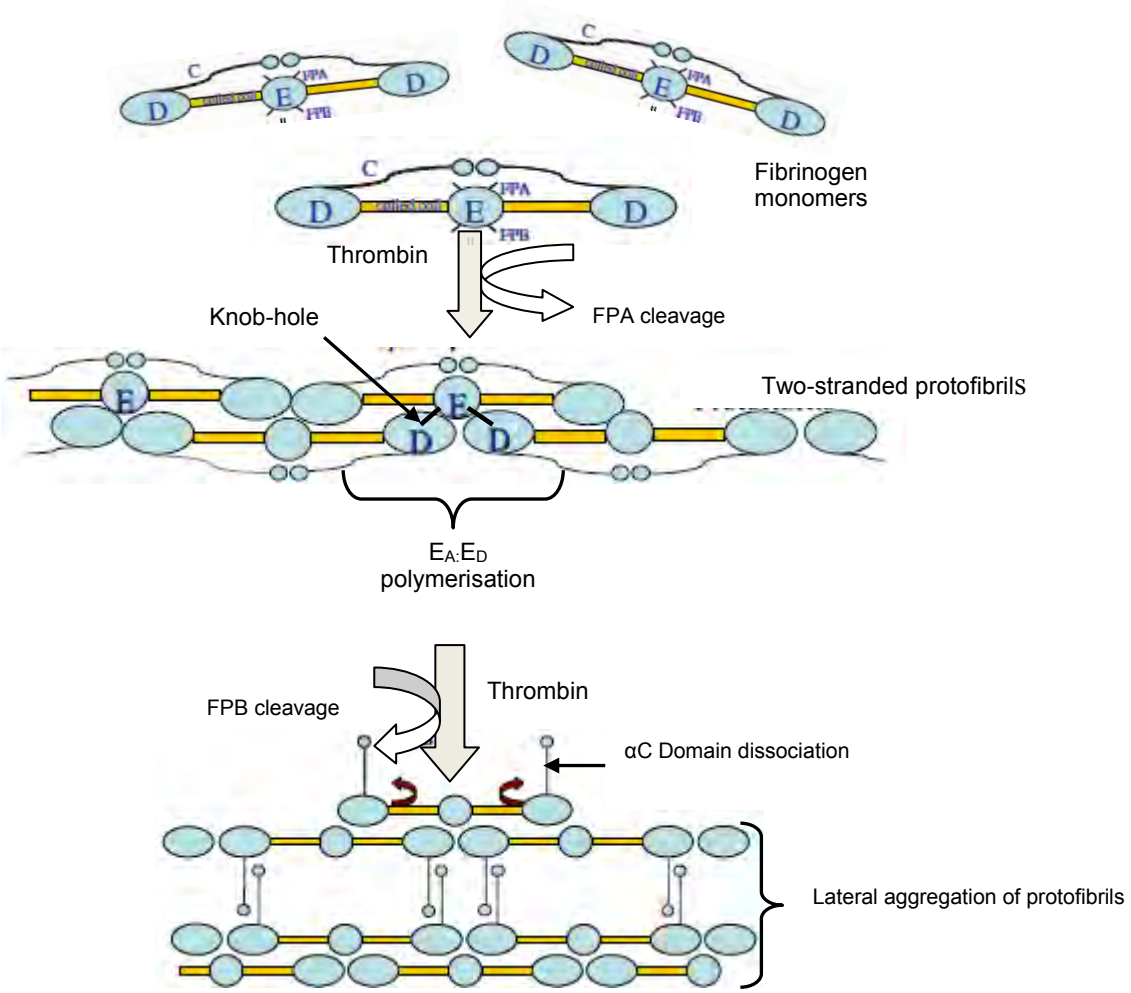

Figure 2.5: Diagram of fibrin polymerisation [Adapted from Standeven *et al*. (2005) and Weisel (2005)]

C: C-terminus; D: D region; E: E region; FPA: fibrinopeptide A; FPB: fibrinopeptide B

Thrombin also cleaves FPB from the N-terminus of the Bβ chain between Arg14 and Gly15 (Binnie & Lord, 1993; Litvinov *et al*., 2007); this cleavage occurs at a slower rate than the cleavage of FPA. Cleavage of FPB results in the exposure of polymerisation site E_B, which interacts with complementary D_b sites located in the C-terminus of the Bβ chain (Mosesson *et al*., 2001; Mannila, 2006a). These interactions lead to the formation of intermolecular contacts between the C-termini of the Bβ chain (β_C:β_C) (Mannila, 2006a). Following cleavage of FPB there is a conformational change resulting in the dissociation of the alpha C (αC) domain of the Aα chain from the E region of the fibrinogen molecule. The αC domain then further interacts with other αC domains (αC:αC) and the αC:αC interactions change from intramolecular to intermolecular and promote lateral aggregation of fibrin protofibrils (Standeven *et al*., 2005; Weisel, 2005; Mannila, 2006a).

When fibrin protofibrils reached a specific length of approximately 600–800 nm, the process of lateral aggregation takes place to form a three-dimensional mesh of fibrin fibres or fibre bundles (Weisel, 2005; Sjøland, 2007a). Fibrin protofibrils making up fibres are twisted around each other; this, therefore limits, the lateral growth of fibres (as explained below). When several protofibrils undergo lateral aggregation, the intermolecular interactions that occur are specific so that the fibres that are formed maintain a periodicity or repeat of 22.5 nm (almost half the length of a fibrinogen molecule) and is reflected by a distinctive band pattern (Weisel, 2005). As the path length increases with fibre diameter (when protofibrils are added to growing fibres), protofibrils must undergo stretching, which can then determine the fibre's final thickness or diameter (Standeven *et al*., 2005; Weisel, 2005). Increases in fibre length are usually associated with increases in fibre diameter (Weisel, 2007). The addition of protofibrils to growing fibres will stop when the energy needed for stretching exceeds the energy needed for bonding of protofibrils (Standeven *et al*., 2005; Weisel, 2005). Lateral aggregation, therefore, controls fibre diameter (Lord, 2011). Fibres are, furthermore, paracrystalline structures, in which molecules are precisely aligned, mainly in the longitudinal direction and partly in the lateral direction.

The opposite of lateral growth of fibres is branching, which is also important as it produces a space-filling gel (Weisel, 2005).

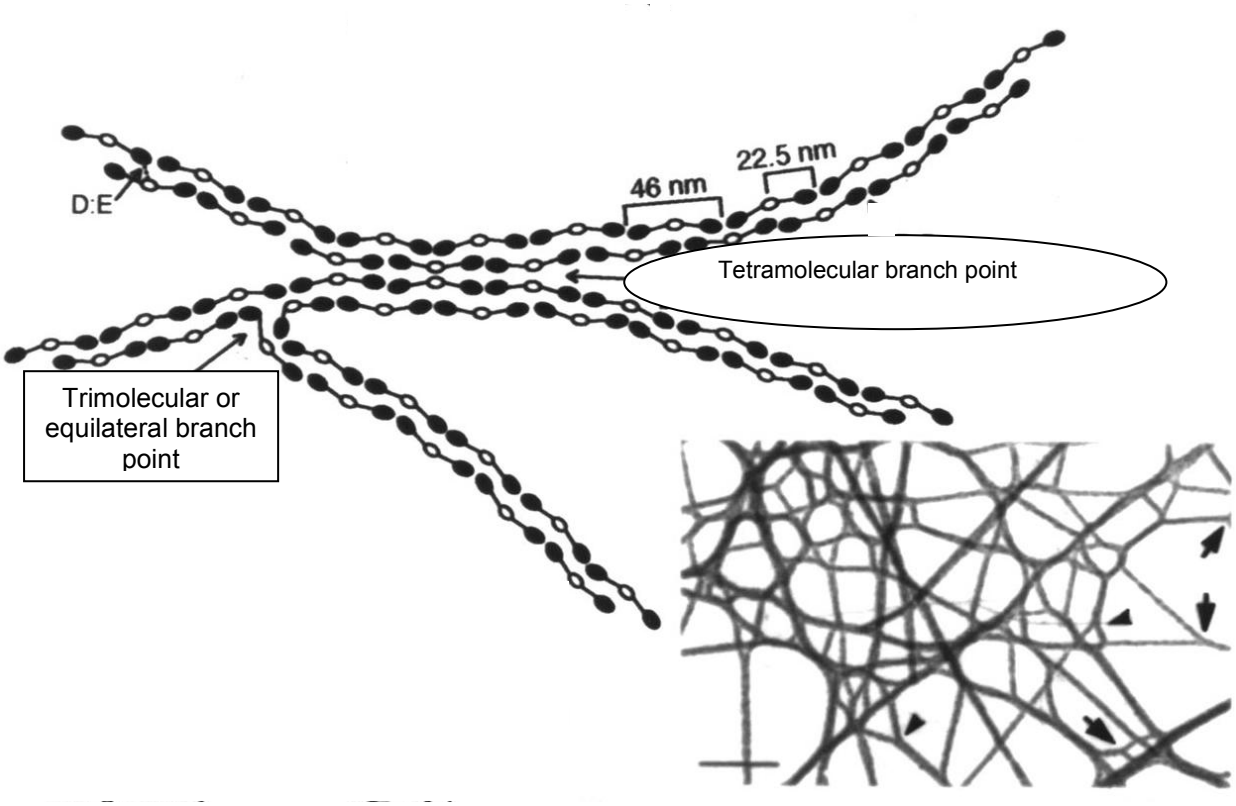

Figure 2.6: Schematic diagram of fibre branching [Adapted from Mosesson *et al*. (2001)]

D: D region; E: E region

Branching is basically the divergence of protofibrils that interact with each other to form fibres. Often the branch points in clots are made up of three fibres at a junction (Figure 2.6). In cases where more branching occurs, thinner fibres are produced while thicker fibres yield less branching (Weisel, 2007). Two types of branching that affects fibrin clot structure occur: 1) tetra-molecular or bilateral branch points form when two-stranded fibrin protofibrils line up in a side-to-side position to form a four-stranded protofibril, a type of branching that provides strength and stability to the clot; 2) trimolecular or equilateral branch points form when a fibrin monomer binds to the D region of another monomer and then deviates to bind to a second fibrin monomer at its E region. Such interactions then form three two-stranded fibrin protofibrils of the same width (Mosesson *et al*., 2001; Standeven *et al*., 2005; Weisel, 2005; Mannila, 2006a). This type of branching seems to occur when the rate of fibrinopeptide cleavage is slow (Standeven *et al*., 2005). These conditions favour a more branched fibrin network compared with conditions when the thrombin levels are high (Mosesson, 2003).

Once a fibrin network has formed, it is not yet stable. Cross-linking by activated Factor XIII plays an important role in stabilising the fibrin clot, forming a rigid, elastic clot which is resistant to mechanical and proteolytic disruption (such as cleavage by plasmin) to prevent bleeding (Standeven *et al*., 2005; Weisel, 2005; Mannila; 2006a). Factor XIII is a transglutaminase with a tetrameric structure, consisting of two A and two B chains (A_2B_2 zymogen form). The activation of Factor XIII occurs *via* the action of thrombin in the presence of calcium and fibrin. Thrombin cleaves off a 37-amino acid activation peptide between the Arg37 and Gly38 residues from the N-terminus of the A_2 chain (Standeven *et al*., 2005; Weisel, 2005; Mannila, 2006a). Following the cleavage of the peptide bond, the A_2 chain dissociates from the B_2 chain in the presence of calcium and the A_2 chain is then activated (Standeven *et al*., 2005; Mannila, 2006a). The dissociation of the B_2 chain is necessary to expose the active-site cysteine of plasma Factor XIII (Weisel, 2005). Fibrinogen or fibrin γ chains have a single cross-linking site in their C-terminal region where activated Factor XIII (Factor XIIIa), in the presence of calcium, forms cross-links between the C-termini of γ chains of associated fibrin monomers to produce γ dimers (Mosesson, 2003; Sjøland, 2007a). The γ chain is the first to be cross-linked by Factor XIIIa after protofibril formation (Standeven *et al*., 2005; Weisel, 2005). The cross-linking occurs through the incorporation of ε-(γ-glutamyl) lysine (Lys) bridges between a Lys residue at γ406 of one γ chain and a glutamine (Gln) residue at γ-398/399 of the other γ chain (Mosesson, 2003) (Figure 2.7). Cross-linking of the C-terminus of γ chains is followed by the cross-linking of the C-termini of the Aα chains (Weisel, 2005). Cross-linking of the Aα chain thus occurs more slowly than that of the γ chain (Standeven *et al*., 2007). The Aα chain contains multiple sites of cross-linking, for example, between α-glutamyl Lys residues αLys208, 219, 224, 418, 427, 429, 446, 448, 508, 539, 556, 580, 601 and 606 and αGln221, 237, 328 and 366 (Lorand, 2001).

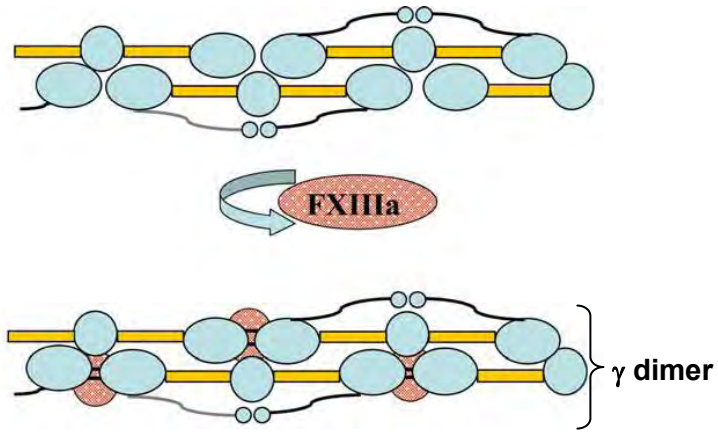

Figure 2.7: Schematic diagram of fibrin γ-chain cross-linking [Adapted from Standeven *et al*. (2005)]
FXIIIa: activated Factor XIII

Cross-linking can occur between Aα chains (which stabilise the fibrin network) (Standeven *et al*., 2005) and, to a lesser extent between Aα and γ chains. Apart from the formation of γ dimers upon cross-linking, smaller amounts of higher-order forms of cross-linked γ chains, such as γ trimers and γ tetramers, can also form (Mosesson *et al*., 2001). Other proteins, including α2-antiplasmin, plasminogen activator inhibitor (PAI)-2, fibronectin (Weisel, 2005), thrombin-activatable fibrinolysis inhibitor (TAFI), vWF (Standeven *et al*., 2005) and trombospondin (Mannila, 2006a), are also cross-linked to fibrin by Factor XIIIa. Unlike the Aα and γ chains, the Bβ chains do not have Factor XIIIa cross-linking sites (Ariëns *et al*., 2012). Cross-linking plays an important role in fibrin clot structure by enhancing clot rigidity, decreasing inelastic deformation (*via* stabilising interactions between assembled protofibrils) (Weisel, 2007) and making clots more resistant to lysis (Collet *et al*., 2005a).

2.2.3 Factors affecting fibrinogen concentration and fibrin network structure

Both fibrinogen concentration and fibrin network structure have been associated with CVD risk (De Moerloose *et al*., 2010; Undas & Ariëns, 2011b). Circulating fibrinogen concentrations are highly variable within a population and are affected by various genetic, environmental and biological influences (Standeven *et al*., 2009), which may affect the final fibrin network or clot structure, either directly or through their effect on fibrinogen concentration. Fibrinogen concentration is a major kinetic factor that significantly affects fibrin network structure (Ariëns, 2011) and therefore, factors that affect fibrinogen concentration can ultimately alter fibrin network structure. Fibrin

network or clot structure has two extreme forms, namely fine and coarse networks (Mosesson *et al*., 1993). Coarse fibrin networks are looser or less rigid and are non-thrombogenic or more susceptible to lysis. Fine fibrin networks, on the other hand, are tight or rigid networks with small pores that are considered thrombogenic or less prone to lysis (Blombäck, 1996; Wolberg, 2010). Increased fibrinogen concentration, for example, has been shown to result in tight, rigid fibrin networks (Scott *et al*., 2004; De Moerloose *et al*., 2010). The following section will discuss genetic, demographic and environmental factors that can influence fibrinogen concentration.

2.2.3.1 Genetics

Introduction to fibrinogen genetics

The presence of elevated fibrinogen levels is a known independent predictor of CVD in Caucasians (El-Tarras *et al*., 2012) and the contribution of genetics to the differences in individual fibrinogen concentration is estimated to be approximately 20 to 50 percent (Mannila, 2006a; Standeven *et al*., 2009). It is important, therefore, to understand the genetic architecture that determines the distribution of CVD risk traits, such as fibrinogen, in a population (Sing & Moll, 1990). According to Sing and Moll (1990), the genetic architecture of a trait depends on 1) the number of genes involved in the regulation of metabolic processes which determine the expression of a trait, 2) the number of alleles of the genes and their frequencies, 3) how each allele affects the level and variability of the trait and 4) their effect on the relationship of a trait with other risk factors involved in CVD development. The genetic susceptibility of CVD in a population might be affected, by among other factors, the prevalence of single nucleotide polymorphisms (SNPs) (such as in the fibrinogen and Factor XIII genes), which is further determined by factors such as ethnic background, genetic makeup of a population and geographical location of a population (El-Tarras *et al*., 2012).

Genetic studies are normally used to determine the prevalence of SNPs and the association between polymorphisms in the specific genes with CVD risk factors. Such genetic studies can reveal the possible association of polymorphisms with the risk of developing vascular diseases. However, this does not necessarily imply that a specific polymorphism is the cause of the disease phenotype (De Moerloose *et al*., 2010), since genetically susceptible individuals may or may not develop CVD, depending on the interaction of various genetic and environmental factors (Gelehrter *et al*., 1998).

Until recently, studies investigating genetic determinants of fibrinogen made use of the candidate-gene approach (Danik *et al*., 2009), which relies on the knowledge of underlying biological pathways associated with a specific disease (De Moerloose *et al*., 2010). These candidate-gene studies focused mainly on the fibrinogen γ (FGG), the fibrinogen α (FGA) and the fibrinogen β (FGB) genes (Danik *et al*., 2009), located on chromosome 4q28.1, 4q28.2 and 4q28.3, respectively (Undas & Ariëns, 2011b). Although some polymorphisms have been identified, specifically in the FGB promoter and gene regions, they explain only a small proportion of the variance in circulating fibrinogen concentrations (Soria *et al*., 2005; Danik *et al*., 2009). It has thus been suggested that there should be other loci contributing to the variance in fibrinogen concentrations within the genome, including loci outside of the fibrinogen gene. As a result, the latest trend is to undertake GWASs to investigate potential associations between SNPs, and plasma fibrinogen concentrations (Danik *et al*., 2009; De Moerloose *et al*., 2010). Unlike the candidate-gene studies that focus on single-gene disorders, the GWASs test or scan the whole genome across millions of SNPs or loci in search of unsuspected genetic associations with a specific phenotype (De Moerloose *et al*., 2010; Manolio, 2010). Evidence from GWASs have revealed that loci or SNPs associated with fibrinogen levels reside on chromosomes 1, 2, 3, 4, 5, 10, 12, 14 and 17 (Yang *et al*., 2003; Soria *et al*., 2005; Yang *et al*., 2007; Danik *et al*., 2009; Dehghan *et al*., 2009). A GWAS by Williams *et al*. (2009) further demonstrated quantitative trait loci for fibrin clot phenotypes on chromosomes 5, 6, 9, 16 and 17.

The contribution of genetics to fibrinogen concentrations and fibrin network structure has been investigated by numerous studies; multiple polymorphisms in and outside the fibrinogen gene that contribute to variation in fibrinogen concentrations have already been identified, as reviewed by Lane and Grant (2000), Green (2001) and Iacoviello *et al*. (2001). The aim of this chapter is not, however, to describe the effect of each SNP identified to date, but to focus on the SNPs of the fibrinogen and Factor XIII genes that have most commonly been found to significantly alter fibrinogen concentration and/or fibrin network structure. These SNPs were identified by virtue of GWASs and existing literature.

To date, most of these SNPs have been identified only in white populations. It is known that genetics differ among different ethnic groups and that SNPs of the fibrinogen gene present in Caucasians are thus not necessarily present in African populations and *vice*

versa (Cook *et al*., 2001; Reiner *et al*., 2006; Albert *et al*., 2009). An example of this is that the SNP rs1800789 identified in a GWAS as having the strongest association with fibrinogen concentration (Dehghan *et al*., 2009) was not present in our black South African study population (results will be presented in Chapter 4). To our knowledge, no studies have been conducted so far to investigate the prevalence of polymorphisms affecting fibrinogen levels and fibrin network structure in the black South African population. What makes this question particularly interesting is the fact that black Africans are known to have higher fibrinogen levels than Caucasians (Pieters & Vorster, 2008a), which may be the result of their different genetic makeup. For the purpose of this study, SNPs that have shown the most profound effect on fibrinogen levels and fibrin structure in Caucasians were selected to be investigated in a black South African population. Five novel SNPs in the promoter area of the FGB (being the rate-limiting factor) not previously described in the Seattle database of the Program for Genomic Applications of the National Heart, Lung and Blood Institute (http://pga.gs.washington.edu/) were also identified. The selected SNPs for this study included polymorphisms of the FGB gene, FGB 1038G/A (rs1800791), FGB 1643C/T (rs1800788), FGB -148C/T (rs1800787) and FGB βArg448Lys (rs4220); the FGA gene, FGA 2224G/A (rs2070011) and FGA 6534A/G or Thr312Ala (rs6050); the FGG gene, FGG 10034C/T (rs2066865) and FGG 9340T/C (rs1049636), as well as two polymorphisms of the Factor XIII gene, Factor XIII A Val34Leu (rs5985) and Factor XIII B His95Arg (rs6003). Although Factor XIII B His95Arg does not seem to affect fibrinogen levels or fibrin network structure, we included this SNP as part of the study because it is highly prevalent in Africans (Bieber *et al*., 1997; Ryan *et al*., 2009; Muszbek *et al*., 2011) and has been associated with stroke (Pruissen *et al*., 2008), which is also prevalent among Africans. In addition to the above-mentioned polymorphisms, two SNPs, *i.e*. an A/G polymorphism at Seattle position 40 (rs2227385) and an A/G polymorphism at Seattle position 749 (rs2227388), were identified in our study population in the promoter area of the FGB and were also included. The above-mentioned polymorphisms will be further discussed below. The effect of these selected SNPs on fibrinogen concentration and fibrin structure is summarised in Table 2.1.

Beta-chain synthesis is believed to be the rate-limiting step of production of the mature fibrinogen molecule and the Bβ-chain SNPs have, therefore, been the main focus of studies (Kamath & Lip, 2003; Maghzal *et al*., 2003; De Moerloose *et al*., 2010). It is further estimated that polymorphisms of the Bβ chain contribute to approximately 15

percent of the variation in fibrinogen concentrations (Mannila *et al.*, 2006a). Since polymorphisms of the β chain of fibrinogen are in linkage disequilibrium (LD), there is uncertainty about their individual effect on fibrinogen levels (Ajjan & Grant, 2005).

FGB -455G/A (rs1800790)

Polymorphism FGB -455G/A (which can be detected by *Hae*III restriction enzyme) in the non-coding region of the β gene (Ajjan & Grant, 2006) is one of the polymorphisms that has been most intensively studied (Lane & Grant, 2000). The FGB guanine-to-adenine (G-to-A) transition, located in the 5' promoter region of the β gene (-455G/A mutation), is associated with the most consistent differences in fibrinogen concentrations (Tybjaerg-Hansen *et al*., 1997; Brown & Fuller, 1998) and is associated with increased fibrinogen concentrations (Undas & Zeglin, 2006a). The prevalence of the rare A allele of FGB -455G/A was shown to be less common in black African populations (immigrants of Africa residing in the United Kingdom) than in Caucasians and South Asians (Cook *et al*., 2001). In our study, however, no variation was observed for this SNP (see Chapter 4).

FGB -854G/A (rs1800791)

Fibrinogen β-854G/A or FGB 1038G/A is a polymorphism with a G-to-A substitution at -854, and is located 5' upstream in the promoter area of the FGB gene, chromosome 4, position 155483309 (http://www.ncbi.nlmnih.gov/projects/SNP/). The minor allele frequency (MAF) in Caucasians has been reported to vary from 0.13 to 0.27 (Van't Hooft *et al*., 1999; Green, 2001; SeattleSNPs, 2001) and in a black African American population it was estimated to be 0.12 and 0.15 (Reiner *et al*., 2006). However, in sub-Saharan Africans the MAF is much lower at 0.04 (http://www.ncbi.nlmnih.gov/projects/SNP/). It seems that FGB -854G/A might affect fibrinogen levels, but although some studies show that this polymorphism has an effect on fibrinogen levels (Van't Hooft *et al*., 1999; Undas & Zeglin, 2006a), others did not support this finding (Mannila *et al*., 2004; Carty *et al*., 2008; Sun *et al*., 2008). The homozygotes for the rare -854A allele, rather than the heterozygotes, have specifically been shown to be associated with higher fibrinogen levels. It has been demonstrated, moreover, that the rare A allele contributes to 4.4% of variation in fibrinogen levels in healthy men (Van't Hooft *et al*., 1999). Fibrinogen β-854G/A has been shown to be involved with the binding of nuclear proteins and the G-to-A substitution at position -854 of the FGB gene increased the rate of transcription (Van't Hooft *et al*., 1999). These

mechanisms could possibly contribute to the effect of FGB -854G/A on fibrinogen levels. Fibrinogen β-854G/A is, moreover, in complete negative allelic LD with polymorphisms FGB -455G/A (rs1800790), FGB -148C/T (rs1800787) (Van't Hooft *et al*., 1999), FGB Arg448Lys (rs4220) and β BcⅠ (Behague *et al*., 1996), which have been associated with increased fibrinogen levels (Zito *et al*., 1997; Lane & Grant, 2000; Cook *et al*., 2001; Kain *et al*., 2002; Maghzal *et al*., 2003; Keavney *et al*., 2006; Undas & Zeglin, 2006a). The common allele, G, of FGB -854G/A always appears with the rare allele, A, of FGB -455G/A (Mannila *et al*., 2004) and *vice versa* (Van't Hooft *et al*., 1999). A tight but not complete LD with FGG 9340T/C (rs1049636) and FGA 2224G/A (rs2070011) (shown to affect fibrin clot porosity) has also been reported (Mannila *et al*., 2006b).

Evidence regarding the effect of FGB -854G/A on CVD is controversial and seems to be different in white and black ethnicities. For example, in African Americans FGB -854G/A was associated with CVD mortality while in European Americans no association was found between FGB -854G/A and myocardial infarction (MI) or CVD mortality, despite a significant association with increased risk of ischaemic stroke (IS) (Carty *et al*., 2008). Sun *et al*. (2008) could also not show a significant association between FGB -854G/A and coronary artery disease (CAD) in a Chinese population.

The possibility exists that the effect of FGB -854G/A on fibrinogen levels and also on CVD could be affected by its linkage or interaction with other polymorphisms within the fibrinogen gene. For example, polymorphism FGG 9340T/C (rs1049636) appears to interact with FGB -854G/A on MI risk. A haplotype model, including the minor allele, C, of FGB 9340T/C and the common allele, G, of FGB -854G/A, contributed to the risk of MI and was not accounted for by fibrinogen levels. However, another haplotype model, including the common T allele of FGB 9340T/C and the common G allele of β-854G/A, appeared to be protective against MI (Mannila *et al*., 2006b). Behague *et al*. (1996) further demonstrated that the association of FGB -854G/A with fibrinogen levels became significant only after adjustment for polymorphism β *Hae*III (FGB -455G/A) in a univariate analysis in smokers. Although no evidence seems to exist on the effect of FGB -854G/A and fibrin clot structure, it has been suggested that its contribution to MI risk could be *via* an indirect effect on fibrin clot structure as a result of its complete negative LD with FGB Arg44Lys (rs4220), which has been associated with fibrin clot structure (Mannila *et al*., 2006b).

FGB -249C/T (rs1800788)

Also located 5' upstream in the promoter area of the FGB gene (Behague *et al*., 1996; Reiner *et al*., 2006) is polymorphism FGB -249C/T or 1643C/T, with a cytosine-to-thymine (C-to-T) substitution on chromosome 4, position 155484015 (http://www.ncbi.nlmnih.gov/projects/SNP/). The frequency of the minor T allele in Caucasians varies from 0.18 to 0.21 (Van't Hooft *et al*., 1999; Green, 2001; SeattleSNPs, 2001; Mannila *et al*., 2004; Lovely *et al*., 2011) and was estimated in African Americans and sub-Saharan Africans to be 0.04 and 0.15, respectively (http://www.ncbi.nlmnih.gov/projects/SNP/; Reiner *et al*., 2006). Polymorphism FGB -249C/T does not seem to affect fibrinogen levels *per se* in Caucasians (Mannila *et al*., 2004; Reiner *et al*., 2006), but rather, functional fibrinogen levels, as the minor allele has been associated with decreased functional fibrinogen levels (Reiner *et al*., 2006). It also did not show an association with CAD, even after controlling for confounders (Theodoraki *et al*., 2010). Polymorphism FGB -249C/T and known polymorphisms FGB -455G/A (rs1800790) and FGB -854G/A (rs1800791) have been shown to be in complete LD by Mannila *et al*. (2005), but in contrast, Van't Hooft *et al*. (1999) demonstrated that FGB -249C/T is in complete negative LD with FGB -455G/A and FGB -148C/T (rs1800787). Behague *et al*. (1996) also demonstrated that FGB -249C/T is in negative LD with FGB -455G/A (β *Hae*III) and in complete negative LD with FGB Arg448Lys (rs4220) and β Bcl/I.

FGB -148C/T (rs1800787)

Polymorphism FGB -148C/T, also known as the *Hind*III polymorphism, arises from a C-to-T substitution at position -148 of the FGB gene (Ajjan & Grant, 2006; http://alfred.med.yale.edu/) and is also located in the 5' promoter area of the FGB gene (Verschuur *et al*., 2005) on chromosome 4, position 155484015 (http://www.ncbi.nlmnih.gov/projects/SNP/). It seems that FGB -148C/T is more common in the white than in the black or Asian populations (Cook *et al*., 2001; Wassel *et al*., 2011). The frequency of its rare allele, T, has been estimated to vary from 0.21 to 0.26 in Caucasians (Iso *et al*., 1995; Van't Hooft *et al*., 1999; Blake *et al*., 2001) and to vary from approximately 0.06 (http://www.ncbi.nlmnih.gov/projects/SNP/) to 0.10 in African Americans (Wassel *et al*., 2011). Furthermore, the minor T allele seems to occur with the minor A allele of FGB -455G/A, but in Africans the minor T allele occurs frequently with the major G allele of FGB -455G/A instead (Cook *et al*., 2001).

It has been demonstrated that FGB -148C/T affects fibrinogen levels (Cook *et al*., 2001; Liu *et al*., 2001; Keavney *et al*., 2006; Wypasek *et al*., 2012), but there are also studies that showed no such association (Schmidt *et al*., 1998; Sun *et al*., 2008). The minor T allele has been associated with higher fibrinogen levels compared with homozygotes for the C allele (Liu *et al*., 2001; Wypasek *et al*., 2012); it was also shown that the T allele in black, white and South Asian populations resulted in a 0.14 and 0.15 g/L increase in fibrinogen levels for women and men, respectively (Cook *et al*., 2001; Keavney *et al*., 2006). It has been suggested that, particularly in black women, it is the T allele of FGB -148C/T rather than the A allele of FGB -455G/A (rs1800790) that is associated with increased fibrinogen levels (Cook *et al*., 2001).

The effect of FGB -148C/T on fibrinogen levels could possibly be attributed to its being located close to an IL-6 (the main mediator of acute-phase-induced fibrinogen synthesis) responsive element, which can affect fibrinogen synthesis in response to an acute-phase reaction (Humphries *et al*., 1997; Schmidt *et al*., 1998; Verschuur *et al*., 2005), as well as to its location between binding sites for a hepatocyte nuclear factor-3, (HNF-3), which is needed for the IL-6 response of fibrinogen β-promoter, and CCAATbox/enhancer-binding protein (C/EBP) (also important to the IL-6 response) (Verschuur *et al*., 2005). It was further suggested that the rare T allele interferes with the co-operation between the HNF-3 and C/EBP binding sites and thus influences the IL-6 response (Humphries *et al*., 1997; Verschuur *et al*., 2005). Polymorphism FGB -148C/T is also regarded as a mild mutation of the HNF-3 site and acts as a functional polymorphism (Verschuur *et al*., 2005). Furthermore, polymorphism FGB -455G/A, which is associated with increased fibrinogen levels, has been shown to be in complete LD with FGB -148C/T (Liu *et al*., 2001); however, this is not the case in Africans (Cook *et al*., 2001).

Although the majority of studies demonstrate an effect of FGB -148C/T on fibrinogen levels, some studies did not observe an influence of the genotype itself on CVD (Blake *et al*., 2001; Keavney *et al*., 2006; Sun *et al*., 2011). Theodoraki *et al*. (2010), however, found an association between FGB -148C/T and a decreased risk of CAD even after adjusting for confounders whereas the T allele was associated with postoperative non-fatal IS in four patients following a coronary artery bypass grafting (CABG) procedure (Wypasek *et al*., 2012).

FGB Arg448Lys (rs4220)

An arginine-to-lysine (Arg-to-Lys) or G-to-A substitution at codon 448 in exon eight of the fibrinogen β chain gives rise to the coding polymorphism FGB Arg448Lys (Behague *et al*., 1996; Maghzal *et al*., 2003) or 8059G/A (Yamada *et al*., 2006), also known as the *Mnl*I polymorphism (http://alfred.med.yale.edu/), which is located on chromosome 4, position 155491759 (http://www.ncbi.nlmnih.gov/projects/SNP/). Polymorphism FGB Arg448Lys is specifically located 13 amino acids away from the C-terminus of the β chain (Carter *et al*., 1997), which has been said to contain the 'b' polymerisation site (Maghzal *et al*., 2003), and it is also relatively close to a β-chain interaction site for the C-terminal region of the fibrinogen α chain and a β chain calcium-binding site (Ajjan *et al*., 2008). Fibrinogen FGB Arg448Lys is a common polymorphism in Caucasians, with the MAF (Lys448) varying from 0.15 to 0.22 (Green, 2001; Ajjan *et al*., 2008), but seems less common in Africans (African Americans and sub-Saharan Africans) with the MAF varying between 0.06 and 0.12 (http://www.ncbi.nlmnih.gov/projects/SNP/). The FGB Arg448Lys polymorphism has been associated with increased fibrinogen levels in some studies (Behague *et al*., 1996; Kain *et al*., 2002) while others found no association (Lee *et al*., 1999; Lim *et al*., 2003; Ajjan *et al*., 2008; Mallik & Majumder, 2011). The association of FGB Arg448Lys with higher fibrinogen levels in a South Asian population was observed in heterozygotes only and not in homozygotes (Kain *et al*., 2002). Furthermore, *in vitro* recombinant FGB Arg448Lys has been shown to influence fibrin clot formation and structure, with the Lys448 allele showing a lower rate of fibre aggregation (reduced slope) and producing a tighter fibrin structure composed of thinner fibres compared with the Arg448 allele, which resulted in a more porous clot with thicker fibres (Ajjan *et al*., 2008); however, in a plasma-purified system, FGB Arg448Lys did not influence clot structure (Maghzal *et al*., 2003). Polymorphism FGB Arg448Lys also seems to affect clot permeability with the Lys448 allele, which resulted in lower permeability in comparison with those homozygous for the Arg448 allele (Lim *et al*., 2003; Ajjan *et al*., 2008). No association was found, however, between FGB Arg448Lys and clot permeability in post-stroke subjects (Maghzal *et al*., 2003).

The possible effect of FGB Arg448Lys on fibrinogen levels could be influenced by its strong LD with FGB -455G/A (rs1800790), but its contribution to increased fibrinogen levels seems to be smaller than that of polymorphism FGB -455G/A (Kain *et al*., 2002). FGB Arg448Lys also lies in LD with FGB -148C/T (rs1800787), which has been associated with increased fibrinogen levels (Standeven *et al*., 2009). Furthermore, the

location of FGB Arg448Lys close to the 'b' polymerisation site could potentially affect its role in clot formation and final clot structure (Maghzal *et al*., 2003; Ajjan *et al*., 2008). As a possible explanation for the association of thinner fibres with the Leu448 allele, it has been suggested by molecular modelling that the Arg-to-Lys substitution could result in conformational changes in the β chain, such as weakened or lost hydrogen bonds in an area close to this 'b' polymerisation site, which can result in altered αC-domain conformation, which impairs lateral aggregation (Ajjan *et al*., 2008). Another mechanism by which FGB Arg448Lys could possibly affect clot structure is its LD with polymorphism FGA Thr312Ala (rs6050) (Kain *et al*., 2002), which is located within the αC domain (C-terminus) of the α gene and has been associated with lateral aggregation (Standeven *et al*., 2003). The LD between FGB Arg448Lys and FGA Thr312Ala was observed only in Caucasians, however, and not in a South Asian population (Kain *et al*., 2002).

FGA 2224G/A (rs2070011)

Polymorphism FGA 2224G/A (rs2070011) with a G-to-A substitution, also known as the 1299+79T/C or -58G/A polymorphisms (Mannila *et al*., 2007a), is located in the 5' flanking and promoter region of the FGA gene on chromosome 4, position 155511897 (http://www.ncbi.nlmnih.gov/projects/SNP/). Fibrinogen FGA 2224G/A seems to be prevalent in both Caucasian and African populations, with the MAF of FGA 2224 in Caucasians varying from 0.14 (Mannila *et al*., 2007b) to 0.38 (Mannila *et al*., 2005; Reiner *et al*., 2006; Lange *et al*., 2008) and in African Americans from 0.20 to 0.32 (http://www.ncbi.nlmnih.gov/projects/SNP/; Reiner *et al*., 2006). The polymorphism FGA 2224G/A has, furthermore, been associated with fibrinogen levels (Mannila *et al*., 2006b; Mannila *et al*., 2007b; Carty *et al*., 2008; Jacquemin *et al*., 2008), but evidence from studies is conflicting. In Caucasians, both the minor A allele (in control subjects) (Mannila *et al*., 2006b) and the major G allele (in patients) (Mannila *et al*., 2007b) have been associated with higher fibrinogen levels whereas in European Americans, FGA 2224G/A was associated with lower fibrinogen levels, while no effect was found in African Americans (Carty *et al*., 2008). Mannila *et al*. (2007a), on the contrary, did not demonstrate an association between the SNP and fibrinogen levels in Caucasians. Fibrinogen FGA 2224G/A also seems to influence the relationship between clot structure and fibrinogen levels, as homozygotes for the major G allele showed the steepest decrease in clot permeability with increasing fibrinogen levels in comparison

with heterozygotes and homozygotes for the A allele. Fibrinogen FGA 2224G/A was an independent predictor of clot porosity (Mannila *et al*., 2006b).

Interaction or epistatic effects between FGA 2224G/A and other polymorphisms might also influence the possible effect exerted by FGA 2224G/A on fibrinogen levels and clot structure. For example, significant epistasis on fibrinogen levels was demonstrated between FGA 2224G/A and Factor XIII Val34Leu (rs5985), with homozygotes for both minor alleles, A and T, respectively, which were associated with the highest fibrinogen levels in controls. Furthermore, FGA 2224G/A is unlinked with FGG 9340T/C (rs1049636) (Mannila *et al*., 2006b), but together, their major alleles, G and T, respectively, were more common in patients with total fibrinogen levels above the 75th percentile compared with those whose fibrinogen γ' and total fibrinogen levels were below the 75th percentile (Mannila *et al*., 2007b). In a haplotype model the minor FGA 2224 A and FGG 9340 C alleles also seemed protective against MI independent of fibrinogen levels and explained 8.8% of the variation in clot permeability. As an explanation for the relationship between FGA 2224G/A and clot permeability, it has been suggested that FGA 2224G/A might be a proxy for a functional coding SNP that either affects fibrinogen levels or alters fibrin network structure. It is thus worth noting that FGA Thr312Ala (rs6050), which has been shown to affect fibrin clot structure, is in complete LD with FGA 2224G/A (Mannila *et al*., 2006b).

FGA Thr312Ala (rs6050)

The FGA 6534A/G polymorphism, commonly known as Thr312Ala (rs6050) - a threonine-to-alanine (Thr-to-Ala) replacement, arises from an A-to-G substitution at codon 312 within the αC domain (C-terminus) of the FGA gene (http://www.ncbi.nlmnih.gov/projects/SNP/; Standeven *et al*., 2003; Uitte de Willige *et al*., 2005). Fibrinogen Thr312Ala is located in exon five of the FGA gene on chromosome 4, position 155507590 (http://www.ncbi.nlmnih.gov/projects/SNP/). Its location within the αC domain of the FGA gene is a region important for Factor XIII cross-linking at amino acid residues Aα328 and 303 involved with fibrin α/α- chain and fibrin α-chain/α_2-antiplasmin cross-linking, respectively. Furthermore, FGA Thr312Ala is surrounded by amino acid residues Aα242 and 424, which play a role in Factor XIII activation by promoting the dissociation of the A and B subunits of Factor XIII (Standeven *et al*., 2003). The prevalence of FGA Thr312Ala seems to be more common in Africans than in Caucasians, with the MAF in Caucasians varying from 0.20

to 0.25 (Green, 2001; Reiner *et al*., 2006; Lovely *et al*., 2011) in comparison with 0.38 and 0.50 in sub-Saharan Africans and African Americans (http://www.ncbi.nlmnih.gov/projects/SNP/; Reiner *et al*., 2006).

The relationship of FGA Thr312Ala with fibrinogen levels is not clear. Although a few studies reported no effect on fibrinogen levels (Lee *et al*., 1999; Lim *et al*., 2003; Reiner *et al*., 2006), associations of minor Ala312 allele homozygotes with lower fibrinogen levels (Liu *et al*., 2001; Carty *et al*., 2008) and the major Thr312 allele homozygotes with higher fibrinogen levels were found (Siegerink *et al*., 2009). The minor allele, Ala312, was, in addition, associated with decreased functional fibrinogen levels rather than fibrinogen protein levels in European Americans (Reiner *et al*., 2006). In a Chinese population, FGA Thr312Ala contributed to 5.36% of fibrinogen variance (Liu *et al*., 2001).

In addition to the possible effect of FGA Thr312Ala on fibrinogen levels, it also seems to have an effect on clot formation and fibrin clot structure. Like FGA 2224G/A, FGA Thr312Ala appears to influence the relationship between fibrinogen levels and clot permeability. An increase in the numbers of Ala312 alleles along with increasing fibrinogen levels has been shown to increase the rate of change of permeability in comparison with homozygotes with the Thr312 allele, which was associated with decreased permeability with increasing fibrinogen levels. Thus, in the presence of high fibrinogen levels, clots produced from Ala312 were less permeable. It was also noted that fibrinogen levels of 3.40–3.81 g/L (10.0–11.2 µmol/L) and higher possibly represent a critical range at which Thr312Ala becomes prothrombotic (Lim *et al*., 2003). In *in vitro* studies, Ala312 produced stiffer clots with thicker fibres and increased α-chain cross-linking, compared with Thr312 (Standeven *et al*., 2003). Fibrinogen Thr312Ala also appears to influence clot stability and, because it has been related to pulmonary embolism (PE) and not deep vein thrombosis (DVT), it has been suggested that FGA Thr312Ala can increases a clot's susceptibility to embolisation. It was further shown that in those homozygous for Thr312 rather than Ala312, the possession of Leu34 was inversely related to PE, demonstrating an inverse association between Leu34 and Ala312 (Carter *et al*., 2000). Mills *et al*. (2002), however, did not show a clear or consistent relationship between FGA Thr312Ala and measures of fibrin clot structure. Fibrinogen Thr312Ala has also been shown to affect D dimer levels, a thrombosis-related quantitative phenotype reflecting the conversion of fibrinogen to cross-linked

fibrin and clot lysis. Additional copies of the minor allele of FGA Thr312Ala were associated with 20% higher D dimer levels in European Americans, but not in African Americans, who had the highest D dimer levels (Lange *et al*., 2008).

There are potential interactions between FGA Thr312Ala and other polymorphisms that can influence its effect on fibrinogen levels and clot structure. A potential interaction, for example, has been shown with FGB -455G/A (rs1800790) in determining fibrinogen levels, but no association was seen with CVD (Carty *et al*., 2010). As previously mentioned, polymorphism FGA Thr312Ala is, moreover, in complete LD with FGA 2224G/A (Mannila *et al*., 2006b), which is associated with fibrinogen levels and clot permeability (Mannila *et al*., 2006b; Mannila *et al*., 2007b). A strong LD also exists between FGA Thr312Ala and FGG 10034C/T (rs2066865) (Uitte de Willige *et al*., 2009a), a polymorphism that has itself been associated with decreased fibrinogen γ' levels and fibrinogen γ'/total fibrinogen ratio, suggesting that the effect of FGA Thr312Ala on clot structure could be *via* its linkage with reduced fibrinogen γ' levels (Uitte de Willige *et al*., 2009a). The possible role of FGA Thr312Ala in clot structure also seems to be associated with its interaction with Factor XIII Val34Leu, specifically by influencing Factor XIII-dependent processes such as α-fibrin/α-fibrin cross-linking and not by influencing α_2-antiplasmin/α-fibrin cross-linking or Factor XIII activity (Carter *et al*., 2000).

FGG 10034C/T (rs2066865)

The polymorphism FGG 10034C/T or *216C/T (Mannila *et al*., 2005), with a C-to-T substitution, is located in the 3' untranslated region (UTR) in intron nine of FGG (Lange *et al*., 2008) on chromosome 4, physical position 155525276 (http://www.ncbi.nlmnih.gov/projects/SNP/). This 3' region contains a GT-rich downstream sequence element (DSE) at nucleotides 10030 to 10047 which is located nine nucleotides downstream from the second polyadenylation signal (pA2), used for the formation of fibrinogen γA. Furthermore, this region showed a 78% match with the cleavage stimulation factor (CstF) binding site, which is a multi-subunit complex that is important for efficient cleavage and polyadenylation of pre-mRNAs (Uitte de Willige *et al*., 2007). The FGG 10034C/T SNP is thus located in a region involved with alternative FGG pre-mRNA processing and fibrinogen γ' splicing (Lange *et al*., 2008).

The minor T allele of FGG 10034C/T has been reported to occur more frequently in Africans than in Caucasians (Reiner *et al*., 2006). The MAF of FGG 10034C/T in Caucasians has been reported as 0.14 and 0.23 (Reiner *et al*., 2006; Lange *et al*., 2008) and varies in African Americans and sub-Saharan Africans from 0.30 to 0.33 (http://www.ncbi.nlmnih.gov/projects/SNP/; Reiner *et al*., 2006; Uitte de Willige *et al*., 2009a).

The FGG 10034C/T polymorphism seems to affect functional fibrinogen levels such as its association with reduced fibrinogen γ' levels (γA/γ' and γ'/γ'), and with reduced fibrinogen γ' to total fibrinogen ratio (γ'/γ ratio) (Reiner *et al*., 2006, Uitte de Willige *et al*., 2007; Cheung *et al*., 2008b; Smalberg, 2012) rather than fibrinogen protein levels (Reiner *et al*., 2006; Cheung *et al*., 2008b; Uitte de Willige *et al*., 2006). However, there is evidence showing an association of FGG 10034C/T with lower fibrinogen levels, where the addition of each minor allele was associated with levels that were 10 mg/dL lower (Carty *et al*., 2008). Fibrinogen 10034C/T has further been shown to affect fibrinogen levels indirectly, as fibrinogen γ' levels were strongly associated with total fibrinogen levels in its presence (Uitte de Willige *et al*., 2005).

No direct effect of FGG 10034C/T on fibrin clot structure has so far been reported, but homozygotes for the minor T allele have been reported to be a risk factor for venous thromboembolism (VTE) in Caucasians rather than African Americans (Uitte de Willige *et al*., 2009a); they were also associated with an increased risk of DVT (Uitte de Willige *et al*., 2005; Grünbacher *et al*., 2007). Moreover, the minor allele was associated with higher D dimer levels in European Americans (Lange *et al*., 2008).

The overall effect of FGG 10034C/T on thrombosis risk appears to be *via* its effects on fibrinogen γ' levels (γA/γ' and γ'/γ') and γ'/total fibrinogen ratio, which is ascribed to the location of its minor T allele in the CstF consensus 2a sequence. The T allele is associated with a stronger consensus sequence and such an improvement of the CstF site can possibly result in more frequent cleavage downstream of pA2 and thus increased splicing of intron nine, which can result in the production of more of the γA variants than of the γ' variant (Uitte de Willige *et al*., 2005). It is suggested that the reduced fibrinogen γ' levels and γ'/total fibrinogen ratio increase the risk for venous thrombosis (VT) (Uitte de Willige *et al*., 2005; Smalberg, 2012). As previously

mentioned, FGG 10034C/T is in strong but not complete LD with FGA Thr312Ala (rs6050), which itself is associated with clot structure. This linkage, however, does not appear to greatly influence the possible role of FGG 10034C/T in the risk of VT as FGA Thr312Ala could not be identified as an independent risk factor for VT. The rare allele of FGG 10034C/T is also in LD with the rare alleles of other polymorphisms, FGG 7874G/A (rs2066861) and FGG 9615C/T (rs2066864), which were associated with fibrinogen γ' levels (Lovely *et al*., 2011).

FGG 9340T/C (rs1049636)

Polymorphism FGG 9340T/C or 1299+79T/C (7792T/C) (Mannila *et al*., 2005; Cheung *et al*., 2008a) is located within intron nine (Mannila *et al*., 2005), chromosome 4, position 155525970 (http://www.ncbi.nlmnih.gov/projects/SNP/). Its location is close to the boundary between exon nine and ten and this region is involved with the splicing of fibrinogen γ' (Reiner *et al*., 2006). The MAF for the C allele seems to be lower in sub-Saharan Africans than in both African Americans and Caucasians. The MAF for African Americans varies between 0.25 and 0.27 (http://www.ncbi.nlmnih.gov/projects/SNP/; Reiner *et al*., 2006; Uitte de Willige *et al*., 2009a) and in Caucasians, between 0.22 and 0.34 (http://www.ncbi.nlmnih.gov/projects/SNP/; Mannila *et al*., 2006b; Reiner *et al*., 2006; Uitte de Willige *et al*., 2009a), while in sub-Saharan Africans the values 0.195 and 0.199 (http://www.ncbi.nlmnih.gov/projects/SNP/) have been reported.

Fibrinogen FGG 9340T/C seems to have conflicting effects on fibrinogen levels. It has been associated with increased fibrinogen levels (Reiner *et al*., 2006; Mannila *et al*., 2007b), particularly the minor C allele, in European and African Americans (Reiner *et al*., 2006), as well as with decreased levels in women, but not men (Kolz *et al*., 2009). There are, however, other studies that did not report any effect on fibrinogen levels (Uitte de Willige *et al*., 2006; Mannila *et al*., 2007a; Cheung *et al*., 2008b). The FGG 9340T/C SNP, has furthermore, been associated with fibrinogen γ' levels (Mannila *et al*., 2007b; Lovely *et al*., 2011) and has been shown to affect clot permeability (Mannila *et al*., 2006b). In haplotype models, the minor C allele of FGG 9340T/C, particularly, has been associated with a decreased risk of MI (Mannila *et al*., 2006b) and VTE in Caucasians and African Americans (Uitte de Willige *et al*., 2009a). Fibrinogen FGG 9340T/C also appeared to be protective against IS (Cheung *et al*., 2008b).

There may, however, be interactions with other polymorphisms that influence the effect of FGG 9340T/C on fibrinogen levels, clot structure and thrombotic diseases. As previously mentioned, FGG 9340T/C and FGA 2224G/A (rs2070011) are unlinked (Mannila *et al*., 2006b), but together their major alleles, T and G, respectively, were more common in patients with fibrinogen levels above the 75th percentile than in those with fibrinogen γ' and fibrinogen levels below the 75th percentile (Mannila *et al*., 2007b). Furthermore, the interaction between FGG 9340T/C and FGA 2224G/A may also influence its effect on clot porosity as FGA 2224G/A itself is an independent predictor of clot porosity (Mannila *et al*., 2006b). The SNPs FGG 9340T/C and FGA 2224G/A also appeared to interact in determining fibrinogen γ' levels (Mannila *et al*., 2007b). Epistatic effects between FGG 9340T/C and FGB 1038G/A (rs1800791) on MI risk have also been demonstrated despite a weak LD between these two SNPs (Mannila *et al*., 2006b). A tight LD, however, exists between FGG 9340T/C and FGG 10034C/T (rs2066865) (Reiner *et al*., 2006) which, as previously mentioned, affects fibrinogen γ', and may also influence the effect of FGG 9340T/C on fibrinogen γ'. Apart from the possible effect of other polymorphisms, it has been suggested that the location of FGG 9340T/C close to the splicing site within intron nine influences the splicing process and, as a result, affects the amount of fibrinogen γ', which itself can influence the final clot structure (Mannila *et al*., 2005).

FGB A/G variant at rs2227385

The FGB A/G variant at rs2227385 (Seattle position 40) is located on chromosome 4 at position 155482311 in the promoter area of the FGB gene (http://www.ncbi.nlmnih.gov/projects/SNP/). This SNP appears to be present only in African populations as to our knowledge no variation has been reported in Caucasian or Asian populations (http://www.ncbi.nlmnih.gov/projects/SNP/). The MAF of its G allele reported in African Americans and sub-Saharan Africans is 0.02 and 0.04, respectively (http://www.ncbi.nlmnih.gov/projects/SNP/). Furthermore, to our knowledge, no studies have investigated its possible association with levels of fibrinogen and fibrinogen γ' or clot structure.

FGB A/G variant at rs2227388

Also located in the promoter area of the FGB gene is an A/G variant at 2227388 (Seattle position 749) on chromosome 4 at position 155483020

(http://www.ncbi.nlmnih.gov/projects/SNP/). Like the FGB A/G variant at rs2227385, this SNP also seems to be present only in Africans and not in Caucasians. The MAF reported in African Americans is 0.02 (http://www.ncbi.nlmnih.gov/projects/SNP/). To date no studies have investigated its possible association with levels of fibrinogen and fibrinogen γ' or clot structure.

Factor XIII A Val34Leu (rs5985)

In addition to the fibrinogen gene polymorphisms, genetic polymorphisms of Factor XIII can also influence fibrinogen and fibrin network structure. The Factor XIII A Val34Leu polymorphism is located in the Factor XIII A subunit. This SNP results from a guanine-to-thymine (G-to-T) transition that codes for a valine-to-leucine (Val-to-Leu) substitution in codon 34 of exon two (Kobbervig & Williams, 2004; De Lange *et al*., 2006; Cilia La Corte *et al*., 2011) within the Factor XIII A1 gene (Muszbek *et al*., 2011). This Val-to-Leu substitution takes place in the activation peptide, three amino acids away from the thrombin cleavage site at position 37 (between Arg37 and Gly38) (Ariëns *et al*., 2002; Scott *et al*., 2004; Standeven *et al*., 2005). Polymorphism Factor XIII Val34Leu is located on chromosome 6, position 6318795 (http://www.ncbi.nlmnih.gov/projects/SNP/). It is a common polymorphism in Caucasians, and the minor Leu allele is also more common in Caucasians than in African populations. The Leu allele frequencies in Caucasians vary between 0.25 and 0.30 whereas in Africans they vary from 0.13 to 0.17 (Ariëns *et al*., 2002).

Evidence of the possible effect of Factor XIII A Val34Leu on fibrinogen levels is controversial. While some studies do not report any effect on fibrinogen levels (Lim *et al*., 2003; Mannila *et al*., 2007a), homozygotes for the minor T (Leu) allele have been associated with the highest fibrinogen levels in healthy controls (Mannila *et al*., 2006b). However, there does appear to be an interaction between Factor XIII A Val34Leu and fibrinogen levels on clot permeability and structure. In the presence of low fibrinogen levels, homozygotes for the Leu allele produce fibrin clots with lower permeability and a tighter fibrin network with thinner fibres, while at high fibrinogen levels, homozygotes for the Leu allele form clots with higher permeability and a more porous fibrin network with thicker fibres in comparison with homozygotes for the major Val allele (Lim *et al*., 2003). Such evidence points towards a protective effect of Factor XIII A Val34Leu at higher fibrinogen levels (Boekholdt *et al*., 2006; Mannila *et al*., 2006b); in particular, fibrinogen concentrations of 3.40–3.81 g/L (10.0–11.2 µmol/L) and higher suggest a critical range

of fibrinogen concentrations above which the Leu variant has a protective effect in comparison with the Val variant (Lim *et al*., 2003). Furthermore, Factor XIII A Val34Leu has also been shown to affect clot formation as homozygotes for the Leu allele were associated with a shorter lag phase (time required for protofibrils to reach sufficient length to allow lateral aggregation) and faster polymerisation than homozygotes for the Val allele. A slightly lower maximum absorbance (indicating the presence of thinner fibrin fibres) was also evident in Leu variants than in Val variants (Ariëns *et al*., 2000). However, Mills *et al*. (2002) could not demonstrate a relationship between Factor XIII A Val34Leu and fibrin clot structure.

The possible effect exerted by Factor XIII A Val34Leu on fibrinogen levels and fibrin clot structure might be influenced by interaction with other polymorphisms, such as with FGA 2224G/A (rs2070011), FGA Thr312Ala (rs6050) and His95Arg polymorphism (rs6003) and PAI-1 4G/5G polymorphism. Homozygotes for both the minor alleles of Factor XIII A Val34Leu and FGA 2224G/A were associated with the highest fibrinogen levels (Mannila *et al*., 2006b). An interaction between Factor XIII A Val34Leu and FGA Thr312Ala further indicates a possible influence of FGA Thr312Ala on Factor XIII-dependent processes such as α-fibrin/α-fibrin cross-linking and clot stabilisation. In homozygotes for the Thr312 allele, possession of the Leu allele is inversely associated with PE while such an association is lost in possession of the Ala312 allele (Carter *et al*., 2000). A possible interaction might also exist between Factor XIII Val34Leu and the Factor XIII B gene polymorphism, Factor XIII B His95Arg, in determinig MI risk. The possible protective effect against MI of the Leu variant has been shown to be boosted in the presence of the Arg95 variant (Reiner *et al*., 2003; Gemmati *et al*., 2007).

Although evidence is controversial, there appears to be a possible interaction between Factor XIII A Val34Leu and the PAI-1 4G/5G polymorphism. It has been shown that patients with the Factor XIII A Val34Leu polymorphism, particularly homozygotes for the wild-type G (Val) with a history of MI, had higher PAI-1 levels as well as increased numbers of the PAI-1 4G/4G genotype than patients without MI. It has been suggested that an increased frequency of PAI-1 4G/4G, associated with impaired fibrinolysis, can negate the possible protective effect of 34Leu (Kohler *et al*., 1998). There is opposing evidence, however, from other studies that either suggest a synergistic contribution of PAI-1 4G and 34Leu to impaired fibrinolysis (Dossenbach-Glaninger *et al*., 2003) or

report no interaction between Factor XIII A Val34Leu and the PAI-1 4G/5G polymorphism (Elbaz *et al*., 2000).

The position of Factor XIII A Val34Leu in close proximity to the thrombin cleavage site may further contribute to its effect on clot formation and structure. The position of the Leu allele, in particular, has been associated with increased Factor XIII activation peptide cleavage that results in altered clot structure (Cilia La Corte *et al*., 2011). *In vitro*, the activation peptide release of the Leu variant is similar to the cleavage rate of FPA, compared with the Val variant's activation peptide release, which is slower and occurs almost in-between the activation peptide release of FPA and FPB. The faster activation of the Leu variant has further been associated with earlier cross-linking of fibrin γ and α chains than occurs with the Val variant, which results in a tighter fibrin network with thinner fibres. However, when fully activated, Leu and Val variants have the same cross-linking activity (Ariëns *et al*., 2000).

Factor XIII B His95Arg (rs6003)

Factor XIII-B*2 or allele-2 is one of three polymorphisms or phenotypes (Factor XIII-B*1/allele-1, Factor XIII-B*2/allele-2 and Factor XIII-B*3/allele-3) identified within the Factor XIII B gene of the Factor XIII B subunit (Board, 1980), resulting in an A-to-G transversion at exon three, codon 95, which gives rise to a histidine-to-arginine (His-to-Arg) substitution at nucleotide 8259 in the second Sushi domain (Komanasin *et al*., 2005; Ryan *et al*., 2009; Cilia La Corte *et al*., 2011; Muzbek *et al*., 2011). Polymorphism Factor XIII B His95Arg (rs6003) is located on chromosome 1, position 197031021 (http://www.ncbi.nlmnih.gov/projects/SNP/). This SNP occurs with high frequency in people of African ancestry (Bieber *et al*., 1997; Ryan *et al*., 2009; Muszbek *et al*., 2011) as the suballeles were located near the ancestral node (Iwata *et al*., 2009). Although Factor XIII B His95Arg does occur in Caucasians (Komanasin, *et al*., 2005), the Arg allele is frequently found in African populations and is the major allele in this population whereas in Caucasians the Arg allele is the minor allele (Ryan *et al*., 2009). The Arg95 allele has a frequency of 0.73 in the African Yoruba population (http://www.ncbi.nlmnih.gov/projects/SNP/) compared with 0.15 (Muszbek *et al*., 2011) and 0.08 in Caucasians, whereas the His95 allele has a frequency of 0.28 in the African population and 0.93 in Caucasians (http://www.ncbi.nlmnih.gov/projects/SNP/).

No evidence seems to exist on the effect of Factor XIII B His95Arg on fibrinogen levels. However, Factor XIII B His95Arg has been associated with thrombosis; the Arg allele, in particular, has been shown to increase the risk of VT by 50% (Komanasin *et al*., 2005) and a higher rate for homozygotes of the Arg allele than for heterozygotes has been associated with an increased risk of IS (a gene-dose effect) (Pruissen *et al*., 2008). On the other hand, the Arg allele could also possibly be protective against MI (Reiner *et al*., 2003; Gemmati *et al*., 2007). This effect of Factor XIII B His95Arg on thrombosis could, however, be influenced by its interaction with polymorphism Factor XIII A Val34Leu. The presence of both the Arg95 and 34Leu alleles resulted in a reduced risk of MI (the reduced risk is lost in the absence of 34Leu) when compared with those homozygotic for both the His95 and Val34 alleles (Reiner *et al*., 2003). Together, the 34Leu and Arg95 alleles were further associated with reduced occurrence of adverse events after MI (Gemmati *et al*., 2007). Functionality studies have shown that the Arg allele is associated with increased dissociation of the Factor XIII A_2B_2 complex upon limited thrombin activation in plasma, but in a purified system the steady-state binding was unaffected (Komanasin *et al*., 2005).

Table 2.1: Effect of selected fibrinogen and Factor XIII SNPs on fibrinogen concentration and fibrin structure

SNP	rsID	Location	Linkage disequilibrium (LD)	Study population description	Effect on fibrinogen levels	Effect on fibrin network structure	Study reference
FGB 1038 / -854G/A	1800791	5' Upstream in promoter area	In negative LD with rs1800790, rs1800787 and rs4220 In tight but not complete LD with rs1049636 and rs2070011	1233 male subjects with confirmed MI and controls, (ECTIM study)	No significant association with fibrinogen levels in univariate analysis, but associated with fibrinogen levels after adjustment for FGB -455G/A in smokers.	Not reported.	Behague *et al.* (1996)
				210 healthy male subjects	↑ Fibrinogen levels.	Not reported.	Van't Hooft *et al*. (1999)
				1108 male survivors of first MI and controls	No significant effect.	Not reported.	Mannila *et al*. (2004)
				4688 subjects (European and African American) with confirmed MI and stroke (older than 65 yrs)	No association.	Not reported.	Carty *et al*. (2008)
				1254 subjects with confirmed CHD and controls	No association.	Not reported.	Sun *et al*. (2008)
FGB -249C/T	1800788	5' Upstream in promoter area	In complete LD with rs1800790 and rs1800791 (Mannila *et al*., 2005) In complete negative LD with rs1800790 and rs1800787 (Van't Hooft *et al*., 1999) and in complete negative LD with rs4220 (Behague *et al*., 1996)	3788 healthy European and African American subjects	No association with fibrinogen levels only with decreased functional fibrinogen levels.	Not reported.	Reiner *et al*. (2006)
				1108 male survivors of first MI and controls	No significant effect on fibrinogen levels.	Not reported.	Mannila *et al*. (2004)
				895 subjects, MI survivors (5 European countries)	No association with fibrinogen levels in single SNP analysis, only in haplotype model defined by itself.	Not reported.	Jacquemin *et al*. (2008)

FGB -148C/T	1800787	5' promoter area	In complete LD with rs1800790	293 healthy Caucasian and Japanese subjects	↑ Fibrinogen levels.	Not reported.	Iso *et al*. (1995)
				399 neurologically asymptomatic subjects	No association.	Not reported.	Schmidt *et al*. (1998)
				1577 healthy subjects (whites, blacks and South Asians residing in London)	0.14 g/L (women) and 0.15 g/L (men) ↑ in fibrinogen levels (all ethnic groups).	Not reported.	Cook *et al*. (2001)
				403 healthy Chinese subjects	↑ Fibrinogen levels.	Not reported.	Liu *et al*. (2001)
				8145 subjects with confirmed MI and controls	0.14 g/L ↑ in fibrinogen levels.	Not reported.	Keavney *et al*. (2006)
				1254 subjects with confirmed CHD and controls	No association.	Not reported.	Sun *et al*. (2008)
				243 consecutive white patients with stable angina undergoing CABG surgery	↑ Fibrinogen levels.	Not reported.	Wypasek *et al*. (2012)
FGB Arg448Lys	4220	Codon 448 in exon 8, close to C-terminus and poly-merisation site of β chain	In LD with rs6050	1233 male subjects with confirmed MI and controls (ECTIM study)	↑ Fibrinogen levels (only in smokers).	Not reported.	Behague *et al*. (1996)
				305 white patients with a clinical diagnosis of acute stroke and controls	↑ Fibrinogen levels (only male patients 3 months following stroke).	Not reported.	Carter e*t al*. (1997)
				149 Korean patients with CAD and controls	No association.	Not reported.	Lee *et al*. (1999)
				537 healthy white Europeans	No influence.	Not reported.	Freeman *et al*. (2002)
				200 apparently healthy subjects (white and South Asian)	↑ Fibrinogen levels.	Not reported.	Kain *et al*. (2002)
				125 white patients with clinical diagnosis of acute stroke	No association.	Associated with permeability. Lys448 allele homozygotes associated with lower permeability *vs* Arg448 allele homozygotes. Lys448 allele associated with	Lim *et al*. (2003)

						tighter FNS and thinner fibres (unpublished).	
				1108 post MI, Lipid clinic subjects and controls	Not reported.	No effect on FNS in purified system *in vitro*	Maghzal *et al*. (2003)
				In vitro, recombinant βArg448Lys and fibrinogen	No association.	Influenced clot formation and final FNS as well as fibrinolysis. Lys448 showed lower rate of fibre aggregation (reduced slope), *vs* Arg448. Lys448 clots have a tight dense FNS with thinner fibres and smaller pores *vs* Arg448 with thicker fibres and bigger pores. Lys 448 clots associated with higher clot stiffness *vs* Arg448. Lys448 had lower permeability *vs* Arg448. Lysis rate slower for Lys448 *vs* Arg448.	Ajjan *et al*. (2008)
				895 subjects, MI survivors (5 European countries)	Significant positive association with fibrinogen levels.	Not reported.	Jacquemin *et al*. (2008)
				484 subjects, first-degree relatives who had one or more of the following: (a) undergone coronary bypass surgery or angioplasty, or (b) had a MI and survived and unrelated individuals	No association.	Not reported.	Mallik and Majumder (2011)
FGA 2224G/A	2070011	5' Upstream, promoter area	FGA Thr312Ala in complete LD with FGA 2224G/A (rs2070011)	774 unselected survivors of a first MI and controls	Minor A allele associated with highest fibrinogen levels in controls.	Influenced relationship between fibrinogen and permeability (independent predictor of fibrin clot porosity). Homozygotes for G allele steepest decrease in	Mannila *et al*. (2006b)

						permeability with increasing fibrinogen levels *vs*. other alleles.	
				2774 survivors of first MI and controls	No influence on fibrinogen levels.	Not reported.	Mannila *et al*. (2007a)
				2774 survivors of first MI and controls	Major G allele associated with ↑ total fibrinogen levels in patients.	Not reported.	Mannila *et al*. (2007b)
				4688 subjects (European and African American) with confirmed MI and stroke	Minor A allele associated with ↓ levels in EA.	Not reported.	Carty *et al*. (2008)
				895 subjects, MI survivors (5 European countries)	Significant positive association with fibrinogen levels.	Not reported.	Jacquemin *et al*. (2008)
FGA Thr312Ala	6050	Codon 312 in exon 5 within the αC domain	In complete LD with rs2070011 and strong LD with rs2066865 (FGG10034C/T)	149 Korean patients with CAD and controls	No association.	Not reported.	Lee *et al*. (1999)
				475 subjects with PE or DVT and controls	Not reported.	Increased susceptibility to fibrin clot embolisation and clot stability by altering Factor XIII-dependent cross-linking.	Carter *et al*. (2000)
				403 healthy Chinese subjects	In men homozyogtes for rare allele (A312) had lowest plasma fibrinogen levels *vs* other genotypes.	Not reported.	Liu *et al*. (2001)
				200 subjects, healthy male relatives of patients with premature CAD and controls	Not reported.	Showed no association with clot structure.	Mills *et al*. (2002)
				125 white patients with clinical diagnosis of acute stroke	No association.	Influenced relationship between fibrinogen and permeability. Ala312 produced less permeable clots in presence of higher fibrinogen levels *vs* Thr.	Lim *et al*. (2003)
				In vitro, purified system	Not reported.	Ala312 produced stiffer clots with thicker fibres *vs* Thr312.	Standeven *et al*. (2003)

				3788 healthy European and African American subjects	Only associated with decreased functional fibrinogen levels, no association with fibrinogen levels. In EAs only associated with decreased fibrinogen levels when measured by functional assay and not by immunoassay.	Not reported.	Reiner *et al*. (2006)
				4688 subjects (European and African American) with confirmed MI and stroke	Homozygotes for minor allele associated with lower fibrinogen levels in older EAs (data not published).	Not reported.	Carty *et al*. (2008)
				895 subjects, MI survivors (5 European countries)	Haplotype model defined by rs6050 showed a significant positive association with fibrinogen levels, but rs6050 did not show a significant association in a single SNP analysis	Not reported.	Jacquemin *et al*. (2008)
FGG 10034C/T	2066865	In the 3' UTR in intron 9, close to the pA2 signal used for the formation of γA	In strong but not complete LD with FGA Thr312Ala	3788 healthy European and African American subjects	Associated only with decreased functional fibrinogen levels, no association with fibrinogen levels	Not reported.	Reiner *et al*. (2006)
				1206 Dutch males with first MI and controls (SMILE study)	No effect on fibrinogen levels in controls	Not reported.	Uitte de Willige *et al*. (2006)
				249 first-ever IS or TIA patients and controls	No association with total fibrinogen levels. Associated only with a significantly decreased γ'/total fibrinogen ratio in	Not reported.	Cheung *et al*. (2008b)

					patients and controls.		
				895 subjects, MI survivors (5 European countries)	No significant association with fibrinogen levels.	Not reported.	Jacquemin *et al*. (2008)
				4688 subjects (European and African American) with confirmed MI and stroke	Addition of each minor allele associated with 10 mg/dL lower fibrinogen levels.	Not reported.	Carty *et al*. (2008)
FGG 9340T/C	1049636	Located within intron 9, close to boundary between exon 9 and 10, the splicing region of fibrinogen γ'	In tight LD with FGG 10034C/T (rs2066865)	3788 healthy European and African American subjects	↑fibrinogen levels in EAs.	Not reported.	Reiner *et al*. (2006)
				774 unselected survivors of a first MI and controls	Not reported.	A haplotype model containing the minor FGG 9340 C and FGA 2224 A alleles explained 8.8% of variation in permeability.	Mannila *et al*. (2006b)
				1206 Dutch males with first MI and controls (SMILE study)	No association with fibrinogen levels in controls.	Not reported.	Uitte de Willige *et al*. (2006)
				2774 survivors of first MI and controls	No influence on fibrinogen levels.	Not reported.	Mannila *et al*. (2007a)
				774 survivors of first MI and controls	Major allele T together with minor G allele of FGA 2224G/A associated with highest total fibrinogen levels (above 75th percentile) in patients.	Not reported.	Mannila *et al*. (2007b)
				249 frst-ever IS or TIA patients and controls	No association.	Not reported.	Cheung *et al*. (2008b)
				895 subjects, MI survivors (5 European countries)	No significant association with fibrinogen levels.	Not reported.	Jacquemin *et al*. (2008)
				4416 subjects (MONICA study)	Associated with lower fibrinogen levels in women but not in men.	Not reported.	Kolz *et al*., (2009)

Factor XIII A Val34Leu	5985	Codon 34 of exon 2. Close to thrombin activation site		*In vitro*	Not reported.	Leu homozygotes had shorter lag phase, faster polymerisation and slightly lower max absorbance *vs* Val homozygotes. Leu variants produced clots with slower permeability with thinner fibres and smaller pores *vs* Val variants with thicker fibres and larger pores.	Ariëns *et al*. (2000)
				200 subjects, healthy male relatives of patients with premature CAD and controls	Not reported.	No association with clot structure.	Mills *et al*. (2002)
				125 white patients with clinical diagnosis of acute stroke	No association with fibrinogen levels.	Modulated relationship between fibrinogen levels and clot permeability. For Val homozyogtes clot permeability decreased with increasing fibrinogen levels. For homozyotes of the Leu allele, clots with lower permeability were formed at low fibrinogen levels and had a tight FNS with thinner fibres. At higher fibrinogen levels thicker loosely packed fibres were formed in Leu *vs* Val variants.	Lim *et al*. (2003)
				774 unselected survivors of a first MI and controls	Homozygotes for minor T allele associated with highest fibrinogen levels in controls.	Not independently related to fibrin gel porosity or permeability.	Mannila *et al*. (2006b)
				2774 survivors of first MI and controls	Does not appear to influence fibrinogen levels	Not reported.	Mannila *et al*. (2007a)

Factor XIII B His95Arg	6003	Exon 3, codon 95 in Factor XIII B-subunit's second sushi domain		In the literature search no studies were found that investigated this polymorphism in relation to fibrinogen and/or clot properties.

Ala: alanine; Arg: arginine; CABG: coronary artery bypass grafting; CAD: coronary artery disease; CHD: coronary heart disease; DVT: deep vein thrombosis; EA: European American; ECTIM: Etude Cas-Temoins sur l'Infarctus du Myocarde; FGA: fibrinogen alpha gene; FGB: fibrinogen beta gene; FGG: fibrinogen gamma gene; FNS: fibrin network structure; G: guanine; His: histidine; IS: ischaemic stroke; LD: linkage disequilibrium; Leu: leucine; Lys: lysine; MI: myocardial infarction; MONICA: Monitoring Trends and Determinants in Cardiovascular Disease; PE: pulmonary embolism; rsID: reference SNP identifier; SMILE: Study of Myocardial Infarctions Leiden; SNP: single nucleotide polymorphism; T: thymine; TIA: transient ischaemic attack; UTR: untranslated region; Val: valine; Thr: threonine; pA2: polyadenylation site 2; Vs: versus.

2.2.3.2 Demographic and environmental factors

Environmental factors play an important role in the development of CVD, as is shown by migrant studies (Ajjan & Grant, 2005), and studies on urbanisation (Pieters *et al*., 2011). It has been shown that migrants are likely to acquire the risk factors that are prevalent in a specific demographic area (Ajjan & Grant, 2005; Pieters *et al*., 2011). For example, in South Africa, several environmental or CVD risk factors have been shown to increase with urbanisation (Pieters *et al*., 2011). Various environmental factors have been demonstrated to modify CVD risk by influencing fibrinogen concentration and fibrin network structure (Dunn *et al*., 2005; Undas, 2011a). The effect of environmental factors on fibrinogen concentration and/or fibrin network structure is, however, beyond the scope of this study. Demographic and environmental (including behavioural) factors that have been shown to influence fibrinogen concentration and or fibrin network structure are summarised in Table 2.2.

Table 2.2: Demograpic and environmental factors that have been shown to influence fibrinogen concentration and/or fibrin network structure

Environmental factors	Effect on fibrinogen concentration	Reference	Effect on fibrin network structure	Reference
Diabetes				
Type 1	↑	Ford *et al*. (1991); El Khwand *et al*. (1993); Ceriello *et al*. (1994); Schalkwijk *et al*. (1999)	↓ clot permeability (indicative of less porous structure)	Nair *et al*. (1991b); Jörneskog *et al*. (1996)
Type 2	↑	Ford *et al*. (1991); Collier *et al*. (1992); Donders *et al*. (1993); Avellone e*t al*. (1994); Crook *et al*. (1996); Morishita *et al*. (1996); Festa *et al*. (1999); Barazzoni *et al*. (2000); Barazzoni *et al*. (2003); Tessari *et al*. (2006); Marchi-Cappelletti and Suárez-Nieto (2010)	Shorter lag phase, ↑ maximum absorbance	Dunn *et al*. (2005)
			↑ rate of lateral fibrin aggregation (slope), lower lysis rate, ↓ inelasticity	Pieters *et al*. (2008b)
			↓ compaction	Marchi-Cappelletti and Suárez-Nieto, (2010)
			↓ clot permeability	Dunn *et al*. (2005); Marchi-Cappelletti and Suárez-Nieto (2010)
Liver disease				
Acute			↓ fibrin polymerisation rate	Lane *et al*. (1977)
Chronic	↓	Siddiqui *et al*. (2011)	↓ fibrin polymerisation rate	Lane *et al*. (1977)
Cirrhosis				
Mild and moderate	↑	De Maat *et al*. (1995)		
Advancing	↓	Arif *et al*. (2002)		
Severe/terminal	↓	De Maat *et al*. (1995); Arif *et al*. (2002)		

Vascular disease states*	↑	Collet *et al*. (2006); Undas *et al*. (2007); Undas *et al*. (2008); Rooth *et al*. (2011); Stępień *et al*. (2012)	↑ fibrin polymerisation	Undas *et al*. (2008); Undas *et al*. (2009a)
			↓ fibre mass-length ratio	Fatah *et al*. (1992); Fatah *et al*. (1996)
			Numerous, stiffer and shorter fibres	Collet *et al*. (2006)
			↑ fibre diameter and density	Undas *et al*. (2009a)
			↓ clot permeability	Fatah *et al*. (1992); Fatah *et al*. (1996); Undas *et al*. (2007); Undas *et al*. (2008); Undas *et al*. (2009a); Rooth *et al*. (2011)
			Slower/longer clot lysis rate	Collet *et al*. (2006); Undas *et al*. (2007); Undas *et al*. (2008); Rooth *et al*. (2011)
Ageing	↑	Meade *et al*. (1979); Krobot *et al*. (1992); Laharrague *et al*. (1993); Hager *et al*. (1994); Ishikawa *et al*. (1997); Fu and Nair (1998); Wirtz *et al*. (2008)	More, thinner fibres and form minor fibre lattices	Pretorius *et al*. (2010a)
	↓	James *et al*. (2000)		
Gender (female)	↑	Krobot *et al*. (1992); Laharrague *et al*. (1993); James *et al*. (2000); Tuut and Hense (2001)	↑ clot maximum absorbance, ↓ clot lysis rate	Alzahrani *et al*. (2012)
Hormones				
Growth hormone	↑	Jeejeebhoy *et al*. (1970)		
Thyroid hormones	↑	Chadarevian *et al*. (1999); Hooper *et al*. (2012)	↑ clot maximum absorbance, slower clot lysis rate	Hooper *et al*. (2012)
Oestrogen	↓	Meilahn *et al*. (1996)		
Insulin	↑	Ditschuneit *et al*. (1995)		

Body mass index (Obesity)	↑	Meade *et al*. (1979); Ditschuneit *et al*. (1995); James *et al*. (2000); Maple-Brown *et al*. (2010); Tofighi (2012)	Tighter, more rigid fibrin gel structure, resistant to lysis	Malan (1999)
Race/ethnicity				
Black *vs* Caucasian population	↑ ↓	Tracy *et al*. (1992); Carroll *et al*. (2009); Okwuosa *et al*. (2013) Cook *et al*. (2001)		
Seasonal variation				
Winter *vs* summer	↑	Stout and Crawford (1991); Fröhlich *et al*. (1997); Crawford *et al*. (2003)		
Exercise/physical activity	↓	Stratton *et al*. (1991); Zanettini *et al*. (1997); Prerost *et al*. (1999); Isasi *et al*. (2000); Shahram *et al*. (2011); Okwuosa *et al*. (2013)	↑ clot permeability, ↑fibre mass-length ratio (indicative of shorther, thicker fibres)	Moss *et al*. (2001)
Smoking	↑	Meade *et al*. (1979); Krobot *et al*. (1992); Eliasson *et al*. (1995); Cullen *et al*. (1998); Hunter *et al*. (2001); Tuut and Hense (2001)	↓ clot permeability Thinner fibres, ↑ fibre density ↑ clot strength ↑ clot lysis time	Undas *et al*. (2009b) Barua *et al*. (2010a) Barua *et al*., (2010b) Undas *et al*. (2009b); Barua *et al*. (2010a); Stępień *et al*. (2011)
Alcohol	↓	Meade *et al*. (1979); Krobot *et al*. (1992); Dimmitt *et al*. (1998); Mennen *et al*. (1999); Marques-Vidal *et al*. (2001); Mukamal *et al*. (2001); Sierskma *et al*. (2002); Wannamethee *et al*. (2003); Mezzano (2004); Mukamal *et al*. (2004); Hansen *et al*. (2005); Schröder *et al*. (2005); Pomp *et al*. (2008)	↓ clot lysis time	Pieters *et al*. (2010); De Lange *et al*. (2012)

Diet				
Prudent diet	↓	James *et al*. (2000)		
Complete meal (15% protein, 55% carbohydrates, 30% fat)	↑	Caso *et al*. (2009)		
Low GI meal (23% protein, 50% carbohydrates, 27% fat)	↓	Marsh *et al*. (2010)	↑ clot permeability, ↑ compaction, ↑ fibre mass-length ratio	Moss *et al*. (2001)
Dietary vitamin C	↓	Khaw and Woodhouse (1995)		
Supplementation:				
Niacin	↓	Philipp *et al*. (1998)		
Fish oil, omega-3 PUFAs	↓	Radack *et al*. (1989); Vanschoonbeek *et al*. (2004)	↑ permeability, ↓ $t_{50\%}$	Gajos *et al*. (2011)
Dietary fibre and metabolites: Pectin and acetate, SCFAs (acetate, propionate and butyrate)			↑ permeability, ↑compaction, ↑ lag phase, ↑ fibre thickness, ↓ lysis rate	Veldman *et al*. (1997); Veldman *et al*. (1999); De Wet (1999)
Vitamin C			↑ compaction	Loots *et al*. (2004)
Psychological factors				
Mental stress, psychiatric disorders	↑	Jern *et al*. (1989); Fenga *et al*. (2004)		
Socioeconomic status (low)	↑	Brunner *et al*. (1996); Maes *et al*. (1997); Wamala *et al*. (1999); Von Känel *et al*. (2009)		
Inflammatory markers				
IL-6	↑	Castell *et al*. (1989)	↓ clot permeability, ↓ compaction, ↑ fibre diameter and fibre mass-length ratio, ↓ clot lysis rate	Sjøland *et al*. (2007b)

CRP	↑	Maple-Brown *et al*. (2010); Pieters *et al*. (2011)	↓ clot permeability, ↓clot lysis rate ↓ compaction, ↑ fibre diameter and fibre mass-length ratio	Undas *et al*. (2007); Sjøland *et al*. (2007b) Sjøland *et al*. (2007b)
Orosmucoid			↓ permeability, ↓ fibre mass-length ratio	Fatah *et al*. (1992)
IL-1β, TNF-α	↓	Castell *et al*. (1989)	Faster onset and ↑ rate of clot formation, denser fibrin networks	Campbell *et al*. (2009)
Lipids				
TC	↑	Okazaki *et al*. (1994); Cushman *et al*. (1996); Pieters *et al*. (2011)		
LDL-C	↑	Cushman *et al*. (1996); Halle *et al*. (1996); Pieters *et al*. (2011); Okwuosa *et al*. (2013)	↓ clot permeability, ↓ fibre mass-length ratio, ↑ fibre thickness	Fatah *et al*. (1992); Bhasin *et al*. (2008)
TG	↑	Fogari *et al*. (1994); Cushman *et al*. (1996); Pieters *et al*. (2011) Okwuosa *et al*. (2013)		
HDL-C	↑	Cushman *et al*. (1996); Halle *et al*. (1996); Folsom *et al*. (2000); Okwuosa *et al*. (2013)	↑ clot permeability (more porous), ↓ fibre thickness, ↓ $t_{50\%}$	Bhasin *et al*. (2008); Ząbczyk *et al*. (2013)
Plasma proteins, ions and molecules				
Plasma proteins: *Albumin*			↓ lag time ↓ clot permeability, ↓ fibre thickness ↓ fibre mass:length ratio, ↓fibril radius	Wilf *et al*. (1985) Nair and Dhall (1991a) Galanakis *et al*. (1987)
Gamma-globulin, fibronectin			↓ lag time, ↑ clot permeability, ↑ fibre thickness	Wilf *et al*. (1985); Nair and Dhall (1991a)

Haemoglobin, ovalbumin *Prothrombin* *Tissue factor*			↓ lag time ↓ fibre mass:length ratio ↑ fibrin network density	Wilf *et al*. (1985) Wolberg *et al*. (2003) Campbell *et al*. (2009)
Ions: *Sodium chloride, Calcium chloride* *Calcium, magnesium* *Calcium* *Chloride* Cations: *Zinc*			↓ clot formation rate, ↓turbidity ↑ turbidity ↓ fibre size ↓ lateral aggregation, thinner fibres ↓ clotting time, ↑ fibre thickness, ↑ pores	Edsall and Lever (1951) Nair *et al*. (1986) Ryan *et al*. (1999) Di Stasio *et al*. (1998) Fatah and Hessel (1998)
Molecules: *Polyphosphate*			↑ turbidity, ↑ mass:length ratio, ↑ fibre thickness, ↑ clot lysis time ↓ turbidity, ↓permeability	Smith and Morrissey (2008) Mutch *et al*. (2010)
pH: *pH (↑)*			↑ clot formation rate ↓ network development	Edsall and Lever (1951) Nair *et al*. (1986)
Temperature: *Temperature (↑)*			↑ fibrin formation	Edsall and Lever (1951)
Temperature (↓)			↓ fibrin formation/network growth	Edsall and Lever (1951); Nair *et al*. (1986)

Homocysteine	↑	Sauls *et al*. (2007)	↓ clot permeability, ↓ clot lysis susceptibility	Undas *et al*. (2007)
			↑ compact network; shorter, thicker fibres, more branched fibres	Lauricella *et al*. (2002)
			Prolonged clotting time, ↑ velocity of coagulation propagation, ↑ clot firmness	Ebbesen *et al*. (2003)
			Shorter clotting time, thinner fibres	Sauls *et al*. (2003)
			Thinner, more tightly packed fibres	Sauls *et al*. (2006); Sauls *et al*. (2007)
			Stimulate fibrin polymerisation	Malinowska *et al*. (2011)
			Slower clot lysis	Sauls *et al*. (2003); Sauls *et al*. (2007); Malinowska *et al*. (2011)
Hypertension	↑	Cushman *et al*., (1996); Lip *et al*. (1997); Coban *et al*. (2004)		
Oxidation, oxidative/nitrative stress			↑ fibrin formation rate	Upchurch *et al*. (1998)
			↓ fibrin formation rate	Piryazev *et al*. (2009)
			↑ clot formation, ↑ clot stiffness, ↓ clot lysis time	Parastatidis *et al*. (2008)
Medications				
Antidiabetic: *Gliclazide*			↑ fibre thickness, ↓ clot permeability, ↑ clot lysis	Nair *et al*. (1991b)

Metformin			↑ clot lysis ↑ clotting time/lag phase, ↓ fibre thickness, ↓ pore size	Nair *et al*. (1991b) Standeven *et al*. (2002)
Aspirin (acetylsalicylic acid)			↑ clot porosity ↑ fibre thickness, looser clot structure, bigger pores ↑ clot lysis time	Williams *et al*. (1998) Ajjan *et al*. (2009) Bjornsson *et al*. (1989); Ajjan *et al*. (2009)
Cholesterol lowering: *Statins* *Fibrates* *Quinapril*	↑ ↓	Maison *et al*. (2002) Maison *et al*. (2002)	↑ permeability, ↓ clot lysis time ↑ permeability ↑ permeability	Undas *et al*. (2007) Undas *et al*. (2006b) Undas *et al*. (2006b)
Thrombin-inhibitors: *Argatroban, Bivalirudin*			↑ permeability, ↓ clot lysis time	He *et al*. (2010)
Factor Xa-inhibitor: *Danaparoid*			↑ permeability, ↓ clot lysis time	He *et al*. (2010)
Oral contraceptives	↑	Meade *et al*. (1979)	Netted, fine fibrin network	Pretorius *et al*. (2010b)
Hormone replacement therapy	↓	Lee *et al*. (1993); Meilahn *et al*. (1996); Folsom *et al*. (2000)	Netted, fine fibrin network	Pretorius *et al*. (2010b)
Migration / urbanisation	↑ ↓	Malan *et al*. (2008) Pieters *et al*. (2011)		

CRP: C-reactive protein; GI: glycaemic index; HDL-C: high-density lipoprotein cholesterol; IL-1β: interleukin-1β; IL-6: interleukin-6; LDL-C: low-density lipoprotein cholesterol; PUFA: polyunsaturated fatty acid; TC: total cholesterol; TG: triglycerides; TNF-α: tumour necrosis factor-α; SCFA: short chain fatty acid. *It remains unclear whether fibrinogen levels play a causal or consequential role in vascular disease states, as in some cases increased fibrinogen levels are predictive of vascular disease while in others increased fibrinogen is a result of factors that accompany vascular disease states such as inflammation.

2.2.4 Methods of measuring clot structure

The fibrin network can be characterised by its structural and mechanical properties as well as the kinetics of clot formation. Methods used to characterise the fibrin network structure will be summarised below with special focus on measuring the kinetics of clot formation as these were the techniques used in this thesis.

Structural properties

Structural properties of a fibrin network structure include fibre diameter, length and branching, as well as pore size. These properties can be measured by direct and indirect measures. Direct measures include imaging techniques, such as scanning and transmission electron microscopy (SEM and TEM) and light microscopy techniques *i.e.* confocal (fluoresence) and deconvolution microscopy (Chernysh & Weisel, 2008), as well as the recently applied total internal reflection fluoresence microscopy (TIRFM), which allows visualisation of single fibrinogen molecules (Hategan *et al.,* 2013). Indirect measures of fibrin network structure include the clot permeability assay or Darcy constant K_s (an indicator of pore size), fibre mass–length ratio, as well as maximum absorbance (Undas & Zeglin, 2006a) (an indicator of average fibre size) (Pieters *et al*., 2008b), measured by means of turbidimetry (related to the number of fibrin fibres, fibre thickness and fibre branching) (Undas & Zeglin, 2006a).

Mechanical / visco-elastic properties

The mechanical properties of a clot play an important role in its functioning and are of clinical significance in relation to clotting disorders and disease (Liu *et al*., 2010). The fibrin clot possesses viscoelastic properties (Weisel, 2008) that determine how a clot will respond in flowing blood (Weisel, 2004). A blood clot is an obstacle to flowing blood and has to resist the mechanical load or strain of the flowing blood *i.e.* whether it will deform, break apart (lyse) or become occlusive (embolise) under a certain strain (Riha *et al*., 1999; Weisel, 2004). Mechanical properties of fibrin networks thus include viscous properties, where the loss modulus or creep compliance is used to characterise the inelastic or irreversible component of the clot, and elastic properties, where the elastic or storage modulus is used to characterise the stiffness of the clot (Weisel, 2004; Undas & Zeglin, 2006a).

The mechanical properties of fibrin networks are said to depend on three network properties: a) the overall network structure composition, b) mechanical properties of

individual fibres and c) properties of joints between fibres (Liu *et al*., 2010). It has been shown, moreover, that the mechanical properties of fibrin are determined by those of the individual fibrin monomers, which are possibly further determined by the properties of structures within a fibrin monomer, including coiled-coil connectors, the folded globular nodules and relatively unstructured αC regions (Falvo *et al*., 2010).

Methods used to measure viscoelastic properties of blood clots include rheometry (Liu *et al*., 2010), *i.e.* rheometers, *e.g*. cone-and-plate (Guthold *et al*., 2004), rotational, capillary-tube (Kim, 2002; Aho, 2011) and concentric-cylinder rheometers (Kim, 2002); torsion pendulums (Janmey *et al*., 1992); and compaction (Nair & Shats, 1997; Sjøland, 2007a) and force measurements that are used to measure mechanical properties of single or individual fibres, such as laser tweezers (Collet *et al*., 2005b; Iowa State University, 2007), atomic force microscopy *(*AFM) (Piechocka *et al*., 2010) and a combined AFM/optical microscope technique (Carlisle, 2010; Liu *et al*., 2010).

Kinetics of clot formation

The kinetics of clot formation involves numerous reactions (Margolis *et al*., 1964) and plays a major role in determining the fibrin network structure (Sjøland, 2007a). The coagulation of blood occurs in two steps, namely thrombin generation from prothrombin and the conversion of fibrinogen to fibrin under the influence of thrombin (Hearon, 1948), to establish a three-dimensional fibrin gel network (Evans *et al*., 2008). The process of blood coagulation changes viscoelastic properties, which are very sensitive measures of fibrin polymerisation and clot structure. Fibrin gels are considered the main structural scaffolds of a blood clot and the time required for the conversion of fibrinogen into the fibrin clot network can be called the clotting time or “gel point” (the transition between a pre-gel, viscoelastic fluid, and a post-gel, viscoelastic solid) (Evans *et al*., 2008).

Methods used to measure the kinetics of clot formation include direct and indirect measures. Direct measures of viscoelasticity during blood coagulation include rheometry and wave propagation techniques. Techniques used in rheometry include: controlled stress and controlled strain rheometrical techniques (forced oscillation techniques) (Evans *et al*., 2006; Evans *et al*., 2008), free oscillation techniques for coagulation monitoring (Evans *et al*., 2006; Evans *et al*., 2008; Lindahl & Romström, 2009), the Fourier transform mechanical spectroscope (FTMS) for gel point detection

during coagulation (Weisel, 2010), rheological and (di)-electric measurements (Bender *et al*., 2012), and dynamic elastography (DE) (Schmitt *et al*., 2011). Wave propagation techniques include: sonorheometry (Evans *et al*., 2008), thickness-shear mode (TSM) resonator technique (Bandey *et al*., 2004), thromboelastography (TEG) (Luddington, 2005; Evans *et al*., 2006; Lermusiaux *et al*., 2006; Evans *et al*., 2008; Lindahl & Romström, 2009; Mazibuko, 2009) and rotational thromboelastography (ROTEM) (Lindahl & Romström, 2009; Mazibuko, 2009), as well as the eight-chain model of Arruda and Boyce (Brown *et al*., 2009; Purohit *et al*., 2011). Indirect measures used to study blood coagulation include spectrophotometry and photometric techniques *e.g*. nephelometry (Morais *et al*., 2006; Undas *et al*., 2007) and spectrophotometry (turbidity) (Weisel & Nagaswami, 1992; Morais *et al*., 2006; Carter *et al*., 2007; Sjøland, 2007a; Pieters *et al*., 2008b; Chernysh *et al*., 2011), which will be discussed in more detail.

Spectrophotometry (Turbidimetry)

The principles of turbidity and nephelometry are almost the same. Radiation passes through a transparent solution containing dispersed solid particles; the particles partly scatter radiation in all directions and this eventually results in decreased incident radiation and the turbid appearance of a sample (Morais *et al*., 2006). The principle of turbidimetry is thus based on optical changes of light absorbance of a solution over time, monitored by a spectrophotometer (Pieters *et al*., 2008b). It measures the decrease in intensity of incident radiation caused by particles scattering radiation. Turbidimetry is particularly useful when light scattering is extensive (many particles in the solution) as it gives more reliable results (Morais *et al*., 2006). Turbidimetry can, for example, be used to follow fibrin polymerisation over time by measuring the amount of light that passes through the clotting solution after plasma or a purified fibrinogen solution has been supplemented with thrombin (Sjøland, 2007a). Fibrin formation is indicated by an increase in the light absorbance of the clotting solutions, owing to light scattering that is induced by the evolving fibrin fibres. By plotting the light absorbance *versus* time, a sigmoid curve is formed (Sjøland, 2007a). The turbidity curve usually reveals the recorded lag phase, slope and maximum absorbance. The lag phase represents the time required for the fibrin monomers to form protofibrils and for protofibrils to grow sufficiently to allow lateral aggregation. The lag phase occurs between the time of thrombin addition and removal of fibrinopeptides, and when the onset of absorbancy increases (Weisel & Nagaswami, 1992; Sjøland, 2007a; Pieters *et al*., 2008b). The lag phase is followed by the slope – a phase associated with a rapid

increase in light absorbance or turbidity, representing the rapid formation of fibrin fibres through lateral aggregation of protofibrils that have reached a specific length. The rapid rate of light absorbance increases until the maximum rate of light absorbance is reached, termed maximum absorbance (calculated as the absorbance after a plateau has been reached minus absorption at baseline), which is an indication of average size of fibre or fibre bundles (Weisel & Nagaswami, 1992; Sjøland, 2007a; Pieters *et al*., 2008b).

Turbidimetric techniques are commonly used to measure the global properties of fibrin networks, such as characterising overall clot structure and its kinetics (Chernysh *et al*., 2011). It is a simple, quick technique with a high sensitivity (Morais *et al*., 2006). Turbidity measurements have, furthermore, shown good reproducibility, which makes this technique suitable for large-scale epidemiological and clinical studies (Carter *et al*., 2007) in comparison with the direct techniques, which are much more labour intensive and costly, making them impractical in the epidemiological setting. Maximum absorbance additionally provides some indirect information on clot structure in supplying proxy data for fibre cross-sectional area.

The ability of the turbidity technique to measure the kinetics of fibrin clot formation makes it useful, particularly in epidemiological studies, to determine how CVD risk factors can possibly contribute to changes in the process of clot formation and how such alteration affects final fibrin network structure and its role in the pathogenesis of CVD. For example, turbidity measurements have been used in studies to determine the genetic and environmental contribution to fibrin network structure and clot formation (Dunn & Ariëns, 2004; Dunn *et al*., 2005; Carter *et al*., 2007).

The following section will discuss the biochemistry of fibrinogen γ', determinants of its plasma concentration and the effect of fibrinogen γ' on fibrin network structure.

2.3 FIBRINOGEN GAMMA PRIME (γ')

2.3.1 Biochemistry of fibrinogen γ'

Fibrinogen γ', also called γB (Francis *et al*., 1980) or $\gamma^{57.5}$ (Lovely *et al*., 2002), is the minor variant of the γ chain of fibrinogen (Lawrence *et al*., 1993). The γ'-chain variant is expressed in hepatocytes only (Uitte de Willige *et al*., 2009b) and usually accounts for

about five to eight percent of total plasma fibrinogen γ chains (Peerschke *et al*., 1986; Mosesson, 2003). Although the normal circulating plasma concentration of fibrinogen γ' has not been established, a "reference interval for healthy individuals" determined in a recent study was 0.088-0.551 g/L (Lovely *et al*., 2010). In circulation, fibrinogen γ' usually occurs in combination with the major γA chain variant of the γ chain (γA/γ') (Farrell, 2012) or "peak 2" fibrinogen (Lovely *et al*., 2002), and this heterodimeric form of fibrinogen constitutes about eight to fifteen percent of the total fibrinogen level in a healthy person, whereas the homodimeric form (γ'/γ') or "peak 1" fibrinogen, contributes only to about 0.5% of total fibrinogen (Uitte de Willige *et al*., 2009b). Furthermore, fibrinogen γ' has a molecular weight of 57.5 kDa and is larger than the γA-chain variant with a molecular weight of 50.0 kDa (Peerschke *et al*., 1986). The larger molecular weight is attributed to the fibrinogen γ' chain's extended C-terminal amino acid sequence (Peerschke *et al*., 1986), which is the result of alternative mRNA splicing and polyadenylation of the fibrinogen γ-chain gene (Cooper *et al*., 2003; Uitte de Willige *et al*., 2009b), which will be discussed below.

The γ-chain gene consists of ten exons and nine introns; in approximately 90% of mRNA transcripts intron nine is spliced out (all nine introns are thus removed) and polyadenylation occurs at pA2, downstream of exon ten (Lovely *et al*., 2002; Uitte de Willige *et al*., 2005; Koch *et al*., 2008). As a result exon nine and exon ten are joined to give rise to the major variant of the γ chain, γA (Lovely *et al*., 2002). However, the fibrinogen γ' chain arises if intron nine is not spliced out from the remaining transcripts and polyadenylation occurs within intron nine at an alternative site, polyadenylation site 1 (pA1), before removal of intron nine can take place (Lovely *et al*., 2002). Because intron nine is not spliced out it encodes a 20-amino-acid C-terminal sequence, γ408-VRPEHPAETEYDSLYPEDDL-427, which replaces the four-amino-acid C-terminal sequence, γ408-AGDV-411, of the γA chain encoded by exon ten (Lawrence *et al*., 1993; Lovely *et al*., 2002) of the γ chain (Uitte de Willige *et al*., 2009b) (Figure 2.8).

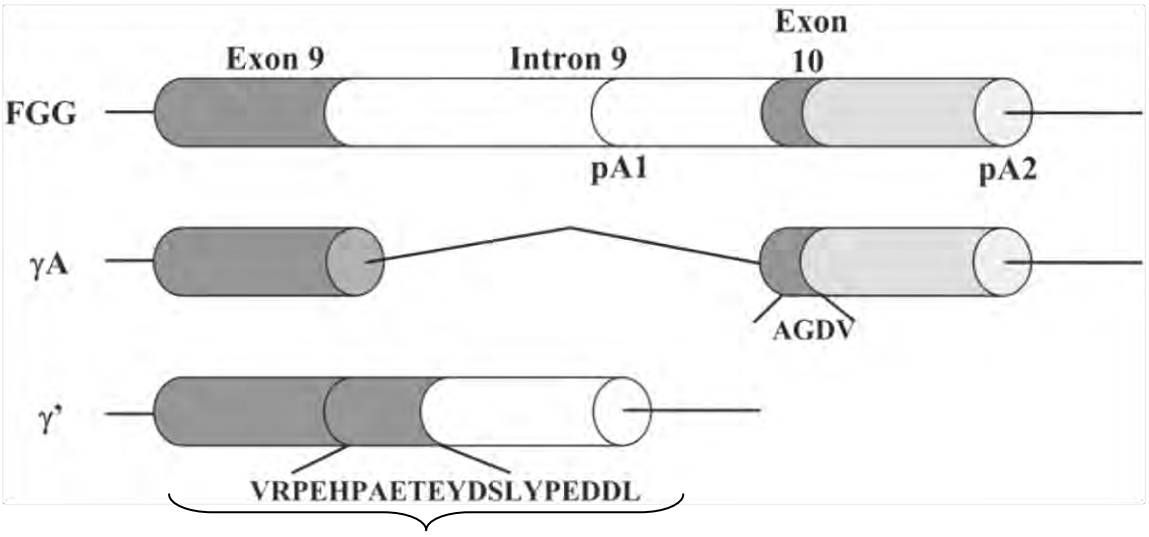

Figure 2.8: Formation of fibrinogen γ' by means of alternative mRNA processing [Adapted form Uitte de Willige *et al*. (2009b)]

FGG: fibrinogen gamma gene; pA1: polyadenylation site 1; pA2: polyadenylation site 2

The fibrinogen γ' chain thus consists of 427 amino acids, making it 16 amino acids longer than the γA chain, which consists of 411 amino acids (Mannila, 2006a) and consequently contributes to the γ' chain having a greater molecular weight than the γA chain. It is hypothesised that the γA to γ' mRNA level ratio is regulated by competition between spliceosome cleavage or removal of intron nine, which gives rise to γA, *versus* enzymatic cleavage and polyadenylation within intron nine at the 3' end of γ' mRNA, which gives rise to γ' (Lovely *et al*., 2002; Farrell, 2012). The elongated γ' chain, located at the C-terminus of the γ chain, protrudes from the D region of the fibrinogen molecule (Figure 2.9); it has been suggested that this extension could possibly reach a length of 30 to 40 angstrom (Å) or more, depending on folding (Uitte de Willige *et al*., 2009b).

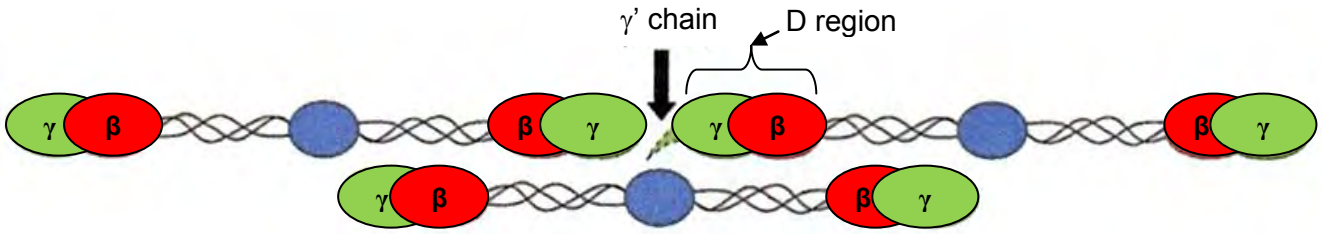

Figure 2.9: Location of the γ' chain on the fibrinogen molecule [Adapted form Uitte de Willige *et al*. (2009b)]

The extended fibrinogen γ' chain has been shown to be more acidic (Blombäck, 1996), and highly anionic and contains seven aspartic acid (Asp) and glutamic acid (Glu) residues (Lovely *et al*., 2002) located within the last 17 amino acids (Lovely *et al*., 2003). Additionally, it contains two sulphated tyrosines (Lovely *et al*., 2002; Mosesson, 2005). The γ' chain also contains a high-affinity binding site for thrombin (Mosesson, 2005) and a binding site for the B subunit of Factor XIII (Undas & Ariens, 2011b). The high-affinity thrombin-binding site is located between amino acid residues 414 and 427 in the C-terminus of the γ' chain and within this amino acid part lie the sulphated tyrosine residues, at Tyr418 and 422, further increasing thrombin's binding potential to the γ' chain (Lovely *et al*., 2003; Mosesson, 2005). Binding of thrombin and Factor XIII to fibrinogen γ' affects their activity and can result in altered final clot structure (Uitte de Willige *et al*., 2009b). The γ' chain additionally lacks the platelet-binding site (a unique αIIbβ3 targeting sequence, HHLGGAKQAGDV) present in γA residues 400 to 411 and has, therefore, a reduced platelet aggregation activity compared with γA (Kirschbaum *et al*., 1992; Uitte de Willige *et al*., 2009b; Hantgan *et al*., 2010).

2.3.2 Factors affecting fibrinogen γ' concentration

Fibrinogen is a known risk factor for CVD and determinants of its concentration have been extensively studied. Fibrinogen γ', however, has only relatively recently been found to be associated with CVD, independently of total fibrinogen concentration (Uitte de Willige *et al*., 2009b; Farrell, 2012) and, therefore, evidence regarding determinants of its concentration is still limited (Uitte de Willige *et al*., 2009b). Circulating plasma levels of fibrinogen γ' are highly variable among healthy populations and, although the contribution of environmental factors to its variance is still unknown, heritability has recently been shown to contribute up to 54% of its variance (Ozel *et al*., 2011).

2.3.2.1 Genetics

Introduction to fibrinogen γ' genetics

In a search for genetic determinants of fibrinogen γ' levels, a GWAS has revealed significant association and linkage signals in the fibrinogen gene locus on chromosomes 4 and 16 (Ozel *et al*., 2011). Furthermore, a recent GWAS by Lovely *et al*. (2011) identified 54 SNPs in or near the fibrinogen gene locus associated with fibrinogen γ' levels; these include known SNPs, such as FGA 6534A/G or Thr312Ala (rs6050), FGA

2224G/A (rs2070011), FGB -249C/T (rs1800788), FGG 10034C/T (rs2066865) and FGG 9340T/C (rs1049636).

Polymorphisms that have been shown to have a profound effect on fibrinogen γ' levels were selected from the literature for the purpose of this study, and will be discussed further below. Background information regarding the SNPs has already been discussed in section 2.2.3 and therefore the following section will focus specifically on the effect of the selected SNPs on levels of fibrinogen γ'.

Two polymorphisms in the FGG gene that have so far been associated with fibrinogen γ' levels are FGG 10034C/T (rs2066865) and FGG 9340T/C (rs1049636). Both SNPs are located in regions involved with γ' splicing (Reiner *et al*., 2006; Mannila *et al*., 2007b; Lange *et al*., 2008), indicating their possible influence on fibrinogen γ' levels. Furthermore, the location of the minor T allele of FGG 10034C/T in the CstF consensus 2a sequence is suggestive of its potential to increase the production of γA compared with γ' (Uitte de Willige *et al*., 2005). Indeed, the majority of evidence has shown that the T allele of FGG 10034C/T is associated with reduced fibrinogen γ' levels, as well as reduced γ'/total fibrinogen ratios (Uitte de Willige *et al*., 2005; Mosesson *et al*., 2007; Cheung *et al*., 2008b; Smalberg *et al*., 2013). It is suggested that FGG 10034C/T affects the development of thromboses such as VT (Uitte de Willige *et al*., 2005; Smalberg *et al*., 2013) and thrombotic microangiopathy (Mosesson *et al*., 2007) *via* reduced fibrinogen γ' levels and γ'/total fibrinogen ratio while FGG 10034CC might represent a protective effect (Cheung *et al*., 2008b; Nowak-Göttl *et al.*, 2009).

The role of FGG 10034C/T on fibrinogen γ' levels and thrombosis might, however, be influenced by its rare T allele, which is in LD with two other SNPs in the FGG gene that have both been associated with fibrinogen γ' levels, FGG 7874G/A (rs2066861), located at intron eight, and FGG 9615C/T (rs2066864), located at intron nine (Lovely *et al*., 2011). Furthermore, FGG 10034C/T is also in LD with SNP FGA Thr312Ala (rs6050) (Nowak-Göttl *et al.*, 2009; Farrell, 2012), which has itself been associated with fibrinogen γ' levels (Lovely *et al*., 2011). Ala312 (tagging SNP for FGA-H1 haplotype) has additionally been shown to produce clots with properties similar to biochemical properties predicted for clots produced in the presence of reduced fibrinogen γ' levels (Nowak-Göttl *et al.*, 2009). While the latter polymorphism seems to be co-inherited with

FGG 10034C/T (Nowak-Göttl *et al.*, 2009) and although it appears that an interaction between these two SNPs could play a role in the risk for thrombosis (Farrell, 2012), it should be taken into account that FGG 10034C/T can influence fibrinogen γ' levels independently of fibrinogen levels (Nowak-Göttl *et al.*, 2009), while fibrinogen levels (decreased or increased) do play a role in the way FGA Thr312Ala alters clot structure, *i.e*. protective or prothrombotic (Lim *et al*., 2003).

Polymorphism FGG 10034C/T is also in LD with FGG 9340T/C (rs1049636), but despite their LD they have rather contrasting effects on fibrinogen γ' levels. Although both are located in regions involved with γ' splicing, one seems to down-regulate fibrinogen γ' levels while the other up-regulates fibrinogen γ' levels (Mannila *et al*., 2007b; Lovely *et al*., 2011). Polymorphism FGG 9340T/C seems to have the stronger association with fibrinogen γ' levels, since after controlling for the top SNP - rs7681423 (located in the 5' of FGG), which showed the strongest association with fibrinogen γ' levels in a recent GWAS - the genome-wide (GW) significance of the association of FGG 10034C/T with fibrinogen γ' levels disappeared along with others, but the GW significance of FGG 9340T/C remained (Lovely *et al*., 2011).

Polymorhism FGG 9340T/C is considered to be an independent predictor of fibrinogen γ' levels, and its contribution to the variance of fibrinogen γ' levels has been shown to be about 10.4% (Mannila *et al*., 2007b). It has been associated with increased fibrinogen γ' levels, and plasma γ' levels have been shown to increase with increasing numbers of the minor C allele; however, there is evidence that demonstrates no association of FGG 9340T/C with γ'/total fibrinogen ratio (Cheung *et al*., 2008b; Smalberg *et al*., 2013).

The effect of FGG 9340T/C on fibrinogen γ' levels has been shown, furthermore, to be boosted by an interaction with SNP FGA 2224G/A, an interaction that seems to result in a phenomenon where fibrinogen γ' levels in homozygotes for the major T allele of FGG 9340T/C increase with increasing numbers of the major G allele of FGA 2224G/A (Mannila *et al*., 2007b). The boosting effect on fibrinogen γ' levels, however, did not seem to occur between the minor alleles of these two SNPs, as the minor C allele of FGG 9340T/C was associated with higher fibrinogen γ' levels and the minor A allele of FGA 2224G/A was associated with lower fibrinogen γ' levels (Mannila *et al*., 2007b). Both of these SNPs, furthermore, exhibit an effect on clot permeability (Mannila *et al*.,

2006b), and although one could argue that the effect of these two SNPs on clot permeability might be mediated by their effect on fibrinogen γ' levels, evidence regarding the effect of fibrinogen γ' on permeability is still scarce and conflicting. It has been demonstrated that the γ' variant is associated with increased permeability when compared with the γA variant (Collet *et al*., 2004), but this finding was not supported by others (Mannila *et al*., 2007b).

Polymorphism FGB 1038G/A (rs1800791), which is in LD with FGG 9340T/C and FGA 2224G/A (Mannila *et al*., 2006a; Mannila *et al*., 2007b), also showed an association with fibrinogen γ' levels. Homozygotes for the minor A allele in a MI patient group were associated with elevated fibrinogen γ' levels that were almost similar to those of homozygotes for the minor C allele of FGG 9340T/C. In healthy subjects, however, homozygotes for the minor C allele of FGG 9340T/C were associated with much higher fibrinogen γ' levels compared with homozygotes for the minor allele of FGB 1038G/A (Mannila *et al*., 2007b). In a recent GWAS, however, no association between FGB 1038G/A and fibrinogen γ' levels was revealed (Lovely *et al*., 2011). This could thus suggest that the observation by Mannila *et al*. (2007b) may be reflective of an association of FGG 9340T/C/ and FGB 1038G/A with fibrinogen γ' levels that is only in a disease state such as MI. Together with FGG 9340T/C, FGB 1038G/A does seem to affect the risk of MI, independently of fibrinogen levels (Mannila *et al*., 2006b), but the role of fibrinogen γ' levels in this matter has not been investigated. However, where fibrinogen γ' levels independently predicted the risk of MI, only FGG 9340T/C and FGA 2224G alleles further increased the risk of MI in patients with elevated fibrinogen γ' levels (Mannila *et al*., 2007b).

In addition to the above SNPs, GW significance for an association of FGB -249C/T (rs1800788) and FGA 6534A/G (rs6050) with fibrinogen γ' was also demonstrated, but after controlling for top SNPs (including FGB 9340T/C (rs1049636)) GW significance was lost (Lovely *et al*., 2011), which also reflects the possible interaction of FGB -249C/T and FGA 6534A/G with other polymorphisms.

Lastly, in a comparison of the genetic control of fibrinogen γ' and total fibrinogen, a GWAS revealed that fibrinogen and fibrinogen γ' might be under different genetic control as the top SNP (rs1800789 on the FGB gene) associated with fibrinogen levels was not

associated with fibrinogen γ' levels (Lovely *et al*., 2011). Furthermore, other SNPs, such as FGG 10034C/T and FGB -249C/T, seem to be associated with fibrinogen γ' levels rather than with fibrinogen levels. In contrast, SNPs such as FGB 1038G/A (rs1800791), FGA 2224C/T (rs2070011) and FGG 9340T/C (rs1049636) are associated with both total fibrinogen and fibrinogen γ' levels, suggesting that genetic control may be similar, at least in part.

2.3.2.1 Demographic and environmental factors

Evidence regarding the role of environmental determinants of fibrinogen γ' levels is fairly recent and, as yet, still poorly understood (Uitte de Willige *et al*., 2009b) and although much still needs to be uncovered (Lovely *et al*., 2002), it already seems to play an important role, particularly in the association between fibrinogen γ' and CVD. It has been suggested, moreover, that the contribution of environmental factors to the relationship between fibrinogen γ' levels and CVD is greater than that of genetic factors (Lovely *et al*., 2011).

Fibrinogen γ', although an isoform of the fibrinogen molecule, is not only a reflection of total fibrinogen level variation but also shows a degree of independent functionality, as shown in CVDs such as MI (Mannila *et al*., 2007b) and intracerebral haemorrhage (ICH) (Van den Herik *et al*., 2012), where fibrinogen γ' levels, but not the γ'/total fibrinogen ratio, were independently associated with the disease. However, there are also CVDs such as IS, PE, unstable angina pectoris (Cheung *et al*., 2008b; Cheung *et al*., 2009; Van den Herik *et al*., 2011) and DVT (Uitte de Willige *et al*., 2005), where both fibrinogen γ' levels and γ'/total fibrinogen ratio were associated with disease while in portal vein thrombosis (PVT) only γ'/total fibrinogen ratio and fibrinogen levels were associated with disease (Smalberg *et al*., 2013). Consequently, fibrinogen γ', expressed not only as an absolute concentration but also as a fraction of the total fibrinogen content (γ'/total fibrinogen ratio), should be investigated in order to determine whether it is the absolute fibrinogen γ' levels or the γ'/total fibrinogen ratio that exerts the physiological effects of fibrinogen γ'.

Since γA/γ' levels can vary independently of total fibrinogen levels (Lovely *et al*., 2002), environmental determinants affecting fibrinogen levels might not necessarily affect fibrinogen γ' levels. Although there are only a very limited number of studies in the

literature that investigate the association of environmental factors with fibrinogen γ' levels, the findings will be discussed below.

Diabetes

Diabetes, as well as pre-diabetic conditions such as the metabolic syndrome (MetS) has been associated with fibrinogen γ' levels and is associated with increased fibrinogen γ' levels (Lovely *et al*., 2010). Additionally, as in the case of fibrinogen, blood glucose levels increase with increasing fibrinogen γ' levels (Lovely *et al*., 2010).

Age

Although fibrinogen has been associated with ageing, conflicting evidence exists regarding the association between fibrinogen γ' levels and age, with one study finding a positive association (Lovely *et al*., 2010) while another did not (Lovely *et al*., 2002).

Gender

Evidence regarding the relationship between fibrinogen γ' levels and gender is also conflicting. In CAD and non-CAD patient groups, fibrinogen γ' levels were increased in both the men and women, thus demonstrating no effect of gender on fibringoen γ' levels (Lovely *et al*., 2010), while another study showed that women had significantly higher fibrinogen γ' levels in comparison with men (Mannila *et al*., 2007b). However, there is also evidence of no relationship between gender and fibrinogen γ' levels (Lovely *et al*., 2002).

Body mass index

Only one study thus far has investigated the association of fibrinogen γ' levels with body mass index (BMI), finding a positive association in Caucasians participating in the Framingham offspring study (Lovely *et al*., 2010).

Smoking

Smoking, as in the case of fibrinogen, has been significantly associated with increased fibrinogen γ' levels in Caucasians participating in the Framingham offspring study (Lovely *et al*., 2010).

Inflammatory markers

A relationship between fibrinogen γ' levels and inflammation has been demonstrated during the acute phase of IS when fibrinogen γ' levels as well as the γ'/total fibrinogen ratio were elevated along with C-reactive protein (CRP) levels (Cheung *et al*., 2008b; Van den Herik *et al*., 2011). These authors hypothesised that mRNA processing of fibrinogen γ' may be altered during the acute-phase reaction.

Lipids

Despite increasing total cholesterol (TC) levels resulting in increased fibrinogen levels, they did not affect fibrinogen γ' levels in the study by Lovely *et al*. (2010). Triglycerides (TG), however, have been shown to increase significantly with increasing fibrinogen γ' levels (Lovely *et al*., 2010). Furthermore, increased fibrinogen γ' levels were associated with decreased high-density lipoprotein (HDL) cholesterol levels (Lovely *et al*., 2010).

Hypertension

Available evidence so far has found no association between hypertension or systolic blood pressure and fibrinogen γ' levels in Caucasians participating in the Framingham offspring study (Lovely *et al*., 2010).

2.3.3 The effect of fibrinogen γ' on fibrin network structure

The association of fibrinogen γ' levels with thrombosis risk has in part been ascribed to its effect on fibrin network structure (Falls & Farrell, 1997; Cooper *et al*., 2003). The effect of fibrinogen γ' on the final fibrin network structure seems to be linked to its influence on the process of clot formation or kinetics of clot formation. The following section will, therefore, discuss the possible effects of fibrinogen γ' on the kinetics of clot formation and how it can alter the final fibrin network structure.

Clot formation is initiated by thrombin which first cleaves FPA and secondly FPB resulting in polymerisation and lateral aggregation of fibrin monomers, as mentioned in section 2.2.2. The effect of fibrinogen γ' on thrombin cleavage of FPA and FPB, however, is conflicting. Some studies found the release of FPA to be similar for γA/γA, γA/γ' and γ'/γ' (Cooper *et al*., 2003; Gersh *et al*., 2009), while other evidence showed a slower release of FPA in the presence of γA/γ' compared with γA/γA (Siebenlist *et al*., 2005). It has been suggested that a slower release of FPA can result in thinner, more

branched fibres (Siebenlist *et al*., 2005). The release of FPB has, furthermore, also been demonstrated in some studies to be slower for γA/γ' than γA/γA (Cooper *et al*., 2003; Siebenlist *et al*., 2005), while others showed an increased release of FPB from γ'/γ' in comparison with γA/γA and γA/γ' (Gersh *et al*., 2009). A slower release of FPB can possibly result in reduced lateral aggregation, which in turn also leads to thinner fibres (Cooper *et al*., 2003).

Evidence regarding the effect of fibrinogen γ' on the kinetics of fibrin clot formation has so far demonstrated a decreased polymerisation rate in γA/γ' than in γA/γA clots (Cooper *et al*., 2003; Allan *et al*., 2012), and this was even lower for γ'/γ' clots in a recombinant purified system (Gersh *et al*., 2009). The lag phase of polymerisation, however, does not seem to be affected by fibrinogen γ' (Cooper *et al*., 2003; Gersh *et al*., 2009). Furthermore, most studies demonstrate a prolonged clotting time for γA/γ'compared with γA/γA clots (Falls & Farrell, 1997; Siebenlist *et al*., 2005). These findings support the above-mentioned hypothesis that slower FPB release can result in reduced lateral aggregation. Moreover, γA/γ' is associated with a decreased maximum absorbance (Cooper *et al*., 2003; Gersh *et al*., 2009; Allan *et al*., 2012). Reduced maximum absorbance from turbidity analysis is an indicator of thinner fibrin fibres at constant fibrinogen concentration. The effect of fibrinogen γ' on fibre diameter, as measured by SEM *in vitro*, is generally in agreement with this. Several studies showed that clots containing fibrinogen γ' have thinner fibres (Cooper *et al*., 2003; Siebenlist *et al*., 2005; Allan *et al*., 2012). However, evidence from studies using the recombinant form of fibrinogen γ' is conflicting. On the one hand, no difference in fibre diameter between γA/γ' and γA/γA was found, and only γ'/γ' was associated with thinner fibres (Gersh *et al*., 2009), whereas, on the other hand, some researchers found an increased fibre diameter for γ'/γ' in comparison with γA/γA (Collet *et al*., 2004).

Several studies showed that fibrinogen γ' produced clots with increased branching (Cooper *et al*., 2003; Siebenlist *et al*., 2005). Furthermore, fibres of clots containing fibrinogen γ' also produced tight interconnecting bundles with large pores (Gersh *et al*., 2009; Allan *et al*., 2012).

Formation of a fibrin network structure is followed by cross-linking by Factor XIIIa to ensure clot stability. Factor XIII cross-linking additionally alters fibrin clot structure and

mechanical properties. While subunit A of Factor XIII is known to bind to fibrin, the B subunits of Factor XIII have been shown additionally to bind to fibrinogen γ' (Siebenlist *et al*., 1996; Mosesson, 2003). However, evidence regarding the effect of fibrinogen γ' on the process of Factor XIII activation and cross-linking is contradictory. While some evidence showed a slower Factor XIII activation for γA/γ' than for γA/γA (Siebenlist *et al*., 2005), others demonstrated faster Factor XIII activation in the presence of γA/γ' (Moaddel *et al*., 2000). Regarding the effect of fibrinogen γ' on γ-chain cross-linking by Factor XIIIa, some investigations demonstrated that γ-chain cross-linking of γA/γ' and γA/γA was similar (Siebenlist *et al*., 2005; Allan *et al*., 2012), while others demonstrated either slower cross-linking of γA/γ' (Mosesson, 2003) or more rapid cross-linking of γA/γ' (Moaddel *et al*., 2000). Factor XIII γ-chain cross-linking seems, furthermore, to alter fibrin network structure by increasing fibre diameter, in comparison with normal fibrin that usually displays reduced fibre diameter upon cross-linking (Standeven *et al*., 2007). Evidence is still lacking as to the influence of fibrinogen γ' in this regard.

Furthermore, cross-linking of the α chain, which follows cross-linking of the γ chain, also seems to be affected by fibrinogen γ'. Cross-linking of the α chain has been shown to be reduced in γA/γ' clots (Allan *et al*., 2012) and, consequently, resulted in reduced formation of α-polymers in γA/γ' clots (Siebenlist *et al*., 2005; Allan *et al*., 2012). In contrast to these findings, Moaddel *et al*. (2000) found the number of α-polymers formed to be much greater in γA/γ' than in γA/γA fibrin, ascribing it to more rapid cross-linking of γA/γ'. Altered α-chain cross-linking in the presence of fibrinogen γ' has also been shown to affect clot structure. Decreased α-chain cross-linking in clots containing fibrinogen γ' can result in γA/γ' clots that have thinner fibres and, moreover, the reduced α-chain cross-linking together with the presence of thinner fibres leads to less stiff γA/γ' clots compared with γA/γA (Allan *et al*., 2012). In addition to the effect of α-chain cross-linking on clot structure, α-polymer chain cross-linking also seems to affect resistance to clot lysis (Francis & Marder, 1988; Muszbek *et al*., 2011). However, there is evidence demonstrating no relationship of resistance to fibrinolysis with the degree of α-chain cross-linking (Siebenlist *&* Mosesson, 1994; Standeven *et al*., 2007).

Evidence regarding the effect of fibrinogen γ' on permeability is scarce and also conflicting. Homodimeric γ'/γ' fibrinogen has been associated in some studies with

increased permeability (Collet *et al*., 2004) while others did not show any association between fibrinogen γ' and permeability (Mannila *et al*., 2007b). Despite these discrepancies, evidence from purified systems supports the finding that clots containing fibrinogen γ' are more resistant to lysis, as demonstrated by a slower clot lysis rate (Falls & Farrell, 1997; Collet *et al*., 2004; Siebenlist *et al*., 2005).

From the above evidence it is clear that fibrinogen γ' has the potential to influence the process of clot formation and structure, a process considered by some to be independent of thrombin binding and Factor XIIIa cross-linking (Allan *et al*., 2012). The conflicting results found regarding the effect of fibrinogen γ' on clot structure may be due to differences in study design, fibrinogen source and/or analytical techniques used.

Most studies investigating the relationship between fibrinogen γ' and clot structure/function used *in vitro* experimental models with either plasma purified (Cooper *et al*., 2003; Siebenlist *et al*., 2005; Allan *et al*., 2012) or recombinant purified fibrinogen (Collet *et al*., 2004; Gersh *et al*., 2009). Although the use of purified fibrinogen containing fixed fibrinogen and fibrinogen γ' concentrations allows the detailed study of possible mechanisms underlying the effects of fibrinogen γ' on fibrin network structure, evidence for the *in vivo* role of fibrinogen γ' in altering fibrin network structure, *i.e*. in the presence of varying plasma concentrations of fibrinogen, is needed to clarify the *in vivo* relationships between these variables.

The last section of this literature chapter provides an overview of CVD prevalence in black South Africans and the relationship of both fibrinogen and fibrinogen γ' with CVD.

2.4 CARDIOVASCULAR DISEASE (CVD)

2.4.1 CVD in black South Africans

The prevalence of CVD is still lower among the black South African population than in South Africans of European descent, but the prevalence as well as the risk of CVD is increasing in this group (Mbewu, 2009). Indeed, the former belief that Africans might be protected against CVD seems to be changing in South Africa, too (Mbewu, 2009). Cardiovascular disease is predicted to become the main contributor to the overall morbidity and mortality of those above 50 years of age in South Africa. Younger age groups, however, are also affected to such an extent that it is estimated that death from

CVD will increase by more than 40% in the 35- to 64-year age group (Maredza *et al*., 2011). Furthermore, the growing burden of CVD in black South Africans seems to affect more women than men (Doyal & Hoffman, 2009).

The bigger picture of CVD in Africans reveals not only an increased prevalence, but also its changing nature. For example, 50 years ago, poverty-related rheumatic heart disease (a result of overcrowded houses, poor housing conditions and undernutrition), and cardiomyopathies dominated, but currently, hypertensive heart disease and haemorrhagic stroke are taking the lead (Mbewu, 2009). As is the case in other African countries, stroke is the major CVD event in black South Africans, emerging as a higher risk than ischaemic heart disease (IHD) (Pieters & Vorster, 2008a; Mbewu, 2009) and, compared with what is seen in developed countries, haemorrhagic stroke is more common in Africans than in white populations (Connor *et al*., 2009; Mbewu, 2009).

Changes observed in terms of CVD in Africans have been ascribed to rapid epidemiological transition characterised and fuelled by 1) decreased infant and child mortality, resulting in an increase in the population over 60 years, also called "demographic transition"; 2) a decrease in deaths from communicable diseases, leading to socioeconomic development; 3) improved socioeconomic development, resulting in environmental and behavioural changes towards an unhealthy lifestyle, including an unhealthy diet, smoking and physical inactivity; 4) prolonged exposure to an unhealthy lifestyle as a result of increased life expectancy eventually increasing the prevalence of CVD (Mbewu 2009; Maredza *et al*., 2011).

In the black South African population, however, this transition is different because of other disease burdens such as the human immunodeficiency virus (HIV) / acquired immunodeficiency syndrome (AIDS), tuberculosis (TB) and maternal and perinatal conditions that further influence CVD risk (Maredza *et al*., 2011). In addition to these burdens, black South Africans are also facing urbanisation, which is accompanied by CVD risk factors, such as smoking, obesity, hypertension, hypercholesterolaemia and hyperfibrinogenaemia (Pieters & Vorster, 2008a; Pieters *et al*., 2011). Urban areas, however, are not alone in facing the increased prevalence of CVD; rural areas, although to a lesser extent, are also affected by CVD and its risk factors (Maredza *et al*., 2011). Cardiovascular disease is thus no longer increasing only among the wealthier, but also

among the poorer black South Africans, as poverty is strongly linked to its development (Vorster & Kruger, 2007).

Despite a lower prevalence of CVD in black South Africans, available evidence has shown that lifestyle CVD risk factors, such as hypertension, dyslipedaemia, obesity, westernised diet, smoking, physical inactivity and psychosocial stress, are present and increasing (Vorster & Kruger, 2007; Pieters *et al*., 2011). From the haemostatic profile it is known that fibrinogen levels are raised in black South Africans (Pieters & Vorster, 2008a; Pieters *et al*., 2011) and, although scarce, evidence regarding fibrin network structure in black South Africans shows that obese black urban women present with a fibrin gel structure that is tighter, more rigid and more resistant to lysis than it is in Caucasians (Malan, 1999). Additionally, there are also protective factors present in black South Africans, including higher HDL-cholesterol levels, lower TC levels, (TC, however, increases with urbanisation) and genetically determined low homocysteine levels (Pieters & Vorster, 2008a; Nienaber-Rousseau *et al*., 2013). The association of the above-mentioned risk factors with CVD has been established mainly in Caucasians, while associations in other ethnicities, such as black South Africans, are, not yet clear. Of particular interest is the fact that fibrinogen levels are increased in black South Africans, and while increased fibrinogen is an established CVD risk factor in Caucasians, little investigation has been done in Africans to clarify these relationships.

2.4.2 Fibrinogen and CVD

Fibrinogen is a known independent predictor of CVDs (Stec *et al*., 2000) such as MI, stroke (especially non-haemorrhagic stroke), VTE and peripheral arterial disease (Danesh *et al*., 2005; De Moerloose *et al*., 2010). A moderately strong association exists between fibrinogen levels and CVD risk and the impact of rising fibrinogen levels on CVD risk is such that for each 1 g/L increase in fibrinogen levels the risk of CVD is almost doubled (Danesh *et al*., 2005). Moreover, the association between increased fibrinogen levels and CVD risk affects both Caucasian and African populations (De Moerloose *et al*., 2010; Pieters *et al*., 2011).

Despite the association between fibrinogen levels and CVD, the nature of this association is still not fully understood (Ernst & Resch, 1993). Several possible mechanisms have been suggested for the role of fibrinogen in the pathogenesis of CVD, but whether this role is causal or consequential has yet to be proved (Ernst &

Resch, 1993; Lowe, 2011). The main possible mechanisms suggested so far for the association between increased fibrinogen levels and CVD risk include 1) increasing platelet aggregation, 2) increasing plasma viscosity and RBC aggregation, 3) impairment of vascular/endothelial function, 4) participation in the inflammatory process and 5) altering fibrin network structure (Ernst & Resch, 1993; Stec *et al*., 2000; Pulanić & Rudan, 2005; De Moerloose *et al*., 2010; Papageorgiou *et al*., 2010; Lominadze *et al*., 2010). These possible mechanisms will be discussed briefly below.

1) Increasing platelet aggregation

The role of fibrinogen in platelet aggregation is important for haemostasis (Pulanić & Rudan, 2005). Upon vascular wall injury, fibrinogen binds to glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$) receptor binding sites of activated platelets, which results in platelet adhesion to the endothelium of the vascular wall, platelet cross-linking and aggregation (Gawaz, 2004; Pulanić & Rudan, 2005), followed by thrombus formation, which is needed for vascular wall repair (Ruggeri & Mendolicchio, 2007). However, in the presence of pathological conditions such as inflammation (Rumbaut & Thiagarajan, 2010) or fatty streak formation associated with CVD, platelet activity (*i.e*. increased platelet adhesion and aggregation) is increased and can result in thrombus formation and ischaemic events (Willoughby *et al*., 2002). Increased fibrinogen levels are associated with increased platelet aggregation (Mikhailidis *et al*., 1985; Endenburg *et al*., 1996) and increased platelet activity has been shown to be present in pathological conditions where fibrinogen levels are increased (Mikhailidis *et al*., 1985), thus pointing towards a possible mechanism by which increased fibrinogen levels can contribute to CVD risk.

In addition to the above proposed mechanism, fibrinogen has also been shown to increase the degranulation of activated platelets in the presence of platelet agonist, adenosine diphosphate (ADP), thus increasing platelet reactivity (Schneider *et al*., 1999), which in turn increases the likelihood of thrombus formation, providing another possible mechanism by which elevated fibrinogen levels may predispose to CVD events (Schneider *et al*., 1999).

2) Increasing plasma/blood viscosity and red blood cell aggregation

As in the case of platelet activity or aggregation, plasma and blood viscosity, as well as RBC aggregation, are associated with CVD risk and are also increased by elevated fibrinogen levels (De Moerloose *et al*., 2010; Lominadze *et al*., 2010, Davalos &

Akassoglou, 2012). Furthermore, increased blood viscosity (or reduced blood flow) results in increased blood flow shear stress, which in turn activates both endothelial cells (ECs) and platelets (Lominadze *et al*., 2010). Disturbing blood flow and shear stress may also contribute to the upregulation of EC genes and proteins associated with atherogenesis and may also contribute to the "hyperviscosity syndrome" which includes, among others, heart disease and stroke (Davalos & Akassoglou, 2012). Additionally, increased RBC aggregation is evident in conditions such as hypertension and hypercholesterolaemia and it has been suggested that elevated fibrinogen levels are the cause (Carvalho *et al*., 2011).

Following activation of ECs as a result of disturbed blood flow, a series of reactions takes place, such as release or activation of adhesion molecules, including fibrinogen receptors $\alpha_v\beta_3$ and intercellular adhesion molecule-1 (ICAM-1) (Lominadze *et al*., 2010), and when fibrinogen binds to ICAM-1, the stage is set for inflammatory and atherosclerotic plaque formation reactions (Papageorgiou *et al*., 2010). Therefore, increased fibrinogen levels can change blood rheology and so influence the risk of CVD by reducing blood flow, increasing the risk of thrombosis and contributing to the process of atherogenesis (Ernst & Resch, 1993).

3) Impairment of vascular/endothelial function

Upregulation of adhesion molecules and integrin receptors as a result of activated ECs controls the physiological changes in the vascular wall, such as vasodilation and vasoconstriction (Davalos & Akassoglou, 2012). Fibrinogen influences vascular reactivity by binding to ICAM-1 and inducing vasoconstriction, but binding to integrins $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ on the contrary, can cause vasodilation (Lominadze *et al*., 2010).

Upon binding of fibrinogen to the ICAM-1 receptor on the EC surface, fibrinogen-ICAM-1 interactions and fibrinogen degradation products mediate the adhesion of molecules such as leukocytes, macrophages and platelets to the EC surface, which results in the release of vasoactive mediators (Gawaz, 2004; Pulanić & Rudan, 2005; De Moerloose *et al*., 2010). Degradation products of fibrinogen further enhance EC permeability by generating processes such as mitogenesis and synthesis of collagen, as well as by attracting leukocytes (Papageorgiou *et al*., 2010). Modulation of EC permeability leads to the deposition of fibrinogen and fibrin into the subendothelial space, which then provides a docking place for the accumulation of low-density lipoprotein (LDL) and

apo(a) (Pulanić & Rudan, 2005) and so contributes to the lipid nucleus of atherosclerotic lesions (Papageorgiou *et al*., 2010). Therefore, if increased, fibrinogen levels contribute to impairment of the vascular wall and EC functioning, possibly resulting in a proinflammatory state characterised by conditions such as hypertension and atherosclerosis, which can lead to events that include stroke and MI (Davalos & Akassoglou, 2012).

4) Participation in the inflammatory process

Fibrinogen is seen as a high-risk marker for vascular inflammatory diseases, as well as a proinflammatory state indicator (Davalos & Akassoglou, 2012). Fibrinogen's proinflammatory function occurs through interactions with certain leukocyte receptors (Mac-1 and $\alpha_x\beta_2$) that determine how it participates in the inflammatory process (Kamath & Lip, 2003; Kaneider *et al*., 2010). Leukocyte adhesion to the EC surface is mediated by ICAM-1 and fibrinogen (Kamath & Lip, 2003). Mac-1 receptors on leukocytes facilitate the binding of leukocytes to fibrinogen and result in the accumulation of fibrinogen at sites of inflammation and injury when vascular permeability and damage have already occurred (Chavakis *et al*., 2001). Furthermore, the binding of fibrinogen to Mac-1 receptors on leukocytes, including monocytes, is followed by the activation of nuclear factor-kappa B (NF-$_k$B) (Pulanić & Rudan, 2005; Davalos & Akassoglou, 2012) (an important transcriptor factor in the inflammatory process) (Jennewein *et al*., 2011), which leads to expression of proinflammatory cytokines IL-6 and tumour necrosis factor (TNF)-α (Davalos & Akassoglou, 2012). These proinflammatory cytokines in turn are also stimuli for the expression of fibrinogen (Papageorgiou *et al*., 2010). Mac-1 receptors that are found on monocytes and neutrophils also facilitate their binding to fibrinogen (Chavakis *et al*., 2001) and result in chemotactic responses (Kamath & Lip, 2003; Pulanić & Rudan, 2005).

In addition to leucocyte receptors, fibrinogen also affects the process of inflammation *via* receptor $\alpha_{IIb}\beta_{3,}$ which is also found on mast cells (Oki *et al*., 2006). Mast cells are important inflammatory effector cells that affect atherosclerotic plaque progression and stability and contribute to the progression of CVDs (Bot *et al*., 2008). Binding of the four-amino-acid C-terminal sequences of the fibrinogen γA chain, γ408-AGDV-411, to this receptor can activate mast cells (Davalos & Akassoglou, 2012) and enhance the effect of mast cells on CVD development. The fibrinogen–$\alpha_{IIb}\beta_3$ interaction also

increases fibrinogen uptake into mast cells (Davalos & Akassoglou, 2012) and has been shown to increase IL-6 levels (Oki *et al*., 2006).

Fibrinogen could thus contribute to CVD by affecting the inflammatory process through binding to various receptors with resulting fibrinogen-receptor interactions that can eventually lead to or enhance a process such as atherosclerosis.

5) Altering fibrin network structure

Fibrinogen is known to predict fibrin clot properties (Undas *et al*., 2010) and increased fibrinogen may contribute to thrombosis by altering the fibrin network structure. Fibrin network alterations in the presence of increased fibrinogen levels that have been associated with thrombosis risk and CVD events include thinner fibres (Ryan *et al*., 1999; Collet *et al*., 2006), increased fibre density or tight fibrin network, reduced clot pore size (indicated by reduced permeability) (Ajjan & Grant, 2005; Ariëns, 2011), increased clot stiffness and increased clot resistance to lysis (Ariëns, 2011). For example, in CAD patients with higher fibrinogen levels, the fibrin network consisted of thinner, shorter and more fibrin fibres (Collet *et al*., 2006), and in stroke patients, increased fibrinogen levels have been shown to result in an increased clot lysis time (Pilgeram *et al*., 1973). Additionally, in patients with MI, increased fibrinogen levels were associated with reduced clot permeability (fibrin gel porosity) (Fatah *et al*., 1992). Even in relatives of patients with CAD, fibrinogen levels were strongly correlated with clot formation parameters such as a shorter lag phase (associated with a tight fibrin network) and greater maximum absorbance (indicative of increased clot density), as well as reduced clot permeability (Mills *et al*., 2002). Therefore increased fibrinogen can contribute to CVD by altering the kinetics of clot formation as well as final clot properties.

2.4.3 Fibrinogen γ' and CVD

Unlike fibrinogen, an established predictor of CVD, fibrinogen γ' is an emerging risk marker for thrombotic diseases. The association between fibrinogen γ' and CVD is still to be elucidated and is, therefore, only a suggested potential risk factor for CVD (Farrell, 2012). In contrast with increased fibrinogen levels, which pose the greater risk for CVD, both increased and decreased levels of fibrinogen γ' are associated with thrombotic diseases, arterial and venous thrombosis, respectively (Uitte de Willige *et al*., 2005;

Mosesson *et al*., 2007; Mannila *et al*., 2007b; Cheung *et al*., 2008b). However, it is important to note that, as previously mentioned, it is not the fibrinogen γ' concentration alone that may play a role in CVD development and events, but also the γ'/total fibrinogen ratio (Drouet *et al*., 1999; Uitte de Willige *et al*., 2005; Cheung *et al*., 2008b; Cheung *et al*., 2009; Van den Herik *et al*., 2011). In arterial disease states such as IS, PE and unstable angina pectoris, for example, both the fibrinogen γ' concentration and γ'/total fibrinogen ratio were elevated (Cheung *et al*., 2008b; Cheung *et al*., 2009; Van den Herik *et al*., 2011). However, in the case of MI, only elevated fibrinogen γ' concentration and not γ'/total fibrinogen ratio independently predicted MI risk (Mannila *et al*., 2007b); also, in patients with ICH, only fibrinogen γ' concentration and not γ'/total fibrinogen ratio was increased (Van den Herik *et al*., 2012). In contrast to arterial disease states, in venous disease states such as DVT both the fibrinogen γ' concentration and γ'/total fibrinogen ratio were decreased (Uitte de Willige *et al*., 2005). Additionally, in PVT only the γ'/total fibrinogen ratio was decreased while the fibrinogen γ' concentration was similar in patients and controls (Smalberg *et al*., 2013). Although more data are emerging, the possible associations between fibrinogen γ' and CVD have been investigated primarily in Caucasians and data in African populations are still lacking.

The biochemical properties of the fibrinogen γ' chain seem to favour a mechanistic role in the development of CVD that can be either prothrombotic or antithrombotic (Farrell, 2012). Mechanisms suggested so far for the association between fibrinogen γ' levels and CVD risk include 1) modulation of thrombin acitivity, 2) modification of platelet activity, 3) mediating Factor XIII activity (Uitte de Willige *et al*., 2009b) 4) participation in the inflammatory process (Farrell, 2012) and, 5) alteration of fibrin network structure (Uitte de Willige *et al*., 2009b; Farrell, 2012). As with fibrinogen, it is still unclear whether fibrinogen γ' levels play a causal or consequential role in CVD.

1) Modulation of thrombin acitivity

Fibrinogen γ' plays an important role in thrombin binding. The fibrinogen γ' chain contains a high-affinity binding site for thrombin, as mentioned previously, and its biochemical properties, such as a negative charge and sulphation, enhance thrombin binding (Uitte de Willige *et al*., 2009b). Binding of fibrinogen γ' to thrombin can have a two-way effect on thrombin activity that results in both prothrombotic and antithrombotic

functions (Uitte de Willige *et al*., 2009b; Farrell, 2012). Fibrinogen γ' can reduce thrombin generation and also inhibit thrombin activity with consequent inhibition of the intrinsic pathway of coagulation and thrombus formation. Furthermore, the inhibitory effect was shown to be greater for γA/γ' than for γA/γA (Uitte de Willige *et al*., 2009b). Additionally, fibrinogen γ' can also inhibit Factor VIII cleavage, which also results in a reduced rate of coagulation (Farrell, 2012). The ability of fibrinogen γ' to inhibit coagulation thus suggests an antithrombotic role in CVD.

In its prothrombotic role, fibrinogen γ' bound to thrombin serves as a reservoir for active thrombin in the blood clot and also allows thrombin to remain functionally active (Uitte de Willige *et al*., 2009b). Fibrinogen γ' further offers protection of thrombin against inactivation by antithrombin, also allowing it to remain functionally active and so enhance thrombus formation (Uitte de Willige *et al*., 2009b; Farrell, 2012).

2) Modification of platelet activity

Compared with the γA chain, the γ' chain does not contain the platelet-binding site, $\alpha_{IIb}\beta_3$, which is needed for platelet adhesion and aggregation and, therefore, fibrinogen γ' does not increase platelet aggregation, but rather decreases or inhibits thrombin-induced platelet activation and aggregation. This effect of fibrinogen γ' on platelet activity reduces the risk of thrombus formation and suggests another possible antithrombotic role of fibrinogen γ' against CVD, such as protecting against VT (Uitte de Willige *et al*., 2009b; Farrell, 2012).

3) Mediating Factor XIII activity

Fibrinogen γ' also posesses a binding site for Factor XIII, as mentioned earlier, and the interaction of fibrinogen γ' with Factor XIII through its B subunits enables it to transport Factor XIII into the fibrin clot. Factor XIII is known to increase resistance to clot lysis, and increasing the amount of Factor XIII deposited in a blood clot by the mechanism above can promote clot resistance to lysis. As with the fibrinogen γ'-thrombin binding, γA/γ' showed a higher affinity for Factor XIII binding compared to γA/γA, but also demonstrated a greater enhancing effect on Factor XIII activation. Furthermore, in the presence of Factor XIII, γA/γ' produced clots with decreased clotting and lysis rates (Uitte de Willige *et al*., 2009b). Thus fibrinogen γ' can predispose to CVD by mediating and exerting the effect of Factor XIII on clot structure and properties.

It seems, therefore, that the relationship between fibrinogen γ' and thrombosis depends on the type of vascular disease. It is suggested that prothrombotic mechanisms of fibrinogen γ', such as altered fibrin structure and increased Factor XIII activity, may prevail in arterial disease while the antithrombotic mechanisms, such as reduced thrombin generation and platelet activation, play a role in the relationship of fibrinogen γ' with venous disease (Uitte de Willige *et al*., 2009b).

4) Participation in the inflammatory process

Fibrinogen γ' is strongly associated with inflammation. During the inflammatory state it seems that fibrinogen γ' levels are regulated by the inflammatory process as it increases and decreases when CRP levels increase and decrease in CVD events such as stroke (Farrell, 2012). The rise in fibrinogen γ' levels during inflammation is thus suggested to be an acute-phase response and a possible risk marker for inflammation and CVD (Alexander *et al*., 2011; Farrell, 2012).

5) Alteration of fibrin network structure

The presence of the fibrinogen γ' chain has been shown to alter fibrin network structure and the majority of observed alterations seem to favour a prothrombotic state. Fibrinogen γ', particulary the heterodimer γA/γ', has been shown to result in a fibrin network with thinner fibres, more branching and smaller pores than γA/γA (Uitte de Willige *et al*., 2009b). Additionally, clots containing both γA/γ' and γ'/γ' have been shown to produce fibres that are non-uniformly arranged with tight interconnecting bundles that have large open pores (Gersh *et al*., 2009; Allan *et al*., 2012). Although it has been suggested that these alterations in fibrin network structure result either in a prothrombotic or antithrombotic state, respectively (Gersh *et al*., 2009), this might partly explain the different roles of fibrinogen γ' observed in arterial and venous thrombosis (Allan *et al*., 2012).

Moreover, clots formed in the presence of γA/γ' have been shown to have a higher viscosity, despite reduced stiffness, when compared with γA/γA, and this is suggestive of clots that are less resistant to deformation (Allan *et al*., 2012). In addition to the above, it was shown that γ'/γ' clots are much stiffer (Collet *et al*., 2004) and also more resistant to lysis than γA/γA clots (Falls & Farrell, 1997; Collet *et al*., 2004). Overall, it is

thus clear that fibrinogen γ' may predispose to CVD risk by altering the fibrin network structure.

2.5 CONCLUSION

From epidemiological studies, conducted mainly in Caucasians, it is evident that increased fibrinogen is a risk factor for CVD and increased fibrinogen has also been associated with several CVD risk factors. Furthermore, various determinants of fibrinogen concentration, including genetics and environmental factors, have also been identified, with the main body of evidence coming from Caucasian participants. From the available evidence of the few studies conducted in Africans across the world, however, it is clear that differences exist in CVD risk between Africans and Caucasians. To date not much is known about the relationship between fibrinogen and CVD and CVD risk factors, especially fibrin network structure, and determinants of fibrinogen levels in other ethnicities such as black South Africans.

Compared with Caucasians, Africans present with increased fibrinogen levels despite historically lower, but lately also increasing, prevalence of CVD. This is partly due to their exposure to lifestyle changes associated with urbanisation, which contribute, to the increased prevalence of several CVD risk factors (*e.g*. obesity, dyslipidaemia, hypertension, smoking and diabetes) that may also influence fibrinogen levels. Furthermore, evidence from the prevalence of fibrinogen polymorphisms as possible determinants of fibrinogen levels investigated in Africans, mainly Africans residing in Europe or America, although demonstrating conflicting results, does seem to indicate that genetic regulation of fibrinogen is different from Caucasians. No data, however, exist on the prevalence of or influence of fibrinogen polymorphisms on fibrinogen levels in black South Africans. In addition to fibrinogen levels, the influence of its γ'-chain variant on CVD risk has been investigated also mainly in Caucasians. Data regarding environmental determinants of fibrinogen γ' are scarce and, furthermore, the effect of fibrinogen γ' on fibrin network structure has been investigated mostly by using purified and recombinant fibrinogen *in vitro*, while very little is known about its role in plasma *in vivo.* Moreover, the role of fibrinogen γ' in CVD development, causal or consequential, is also poorly understood and it seems that both the fibrinogen γ' concentration and γ'/total fibrinogen ratio play a role in CVD. To date, however, no data exist regarding the role of fibrinogen γ' and γ'/total fibrinogen ratio in black South Africans.

It is, therefore, the aim of this study to determine fibrinogen functionality, including analysis of total fibrinogen, fibrinogen γ' and γ'/total fibrinogen ratio, selected fibrinogen polymorphisms and the kinetics of clot formation (fibre diameter, slope and lag time) and lysis from plasma. From the above-mentioned data it will be possible to gain a better understanding of fibrinogen functionality and its role in CVD in black South Africans and, in addition, to provide new data on its role in a plasma system.

CHAPTER 3:

Evidence that fibrinogen γ' regulates plasma clot structure and lysis and relationship to cardiovascular risk factors in black Africans

Authors: Marlien Pieters, Retha C. Kotze, Johann C. Jerling, Annamarie Kruger and Robert A. S. Ariëns

This chapter includes:

- author instructions of the journal, *Blood* (Impact factor: 9.8)
- the article titled "Evidence that fibrinogen γ' regulates plasma clot structure and lysis and relationship to cardiovascular risk factors in black Africans", published 2013 121:3254-3260 (the published article is provided as Addendum E, p 269)
- online supplemental material published in *Blood*

AUTHOR INSTRUCTIONS: BLOOD

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In this section:

Jump to:	Manuscript preparation	Manuscript length restrictions	Editing services for non-English speakers
	Manuscript organization	Important guidelines for image preparation	Supplemental data

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Acknowledgments

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ARTICLE

Evidence that fibrinogen γ' regulates plasma clot structure and lysis, and relationship to cardiovascular risk factors in black Africans

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Key points:

This paper describes for the first time:

- the effect of fibrinogen γ' on clot structure in plasma (previously shown in purified systems);
- the respective roles of total fibrinogen, fibrinogen γ' concentration and ratio on clot structure and lysis rates.

Abstract

Fibrinogen γ' is known to influence fibrin clot structure in purified experimental models but little is known regarding its influence on clot structure in plasma. Furthermore, the environmental and biological factors that affect its concentration are poorly described. We analysed fibrinogen γ', total fibrinogen concentration and fibrin clot structure in 2010 apparently healthy black South Africans and related them to traditional CVD risk factors. Fibrinogen γ' generally increased with increasing fibrinogen concentration, however a decreased γ'/total fibrinogen ratio was found at the highest total fibrinogen concentrations. Clot maximum absorbance increased with total fibrinogen and fibrinogen γ', but decreased with γ'/total fibrinogen ratio. Clot lysis time showed a stronger relationship with fibrinogen γ' than with total fibrinogen, whereby increased fibrinogen γ' delayed clot lysis times. CVD risk factors (excluding fibrinogen) explained 20% and 3% respectively of the variance in fibrinogen γ' and the γ'/total fibrinogen ratio, with CRP making the biggest contribution. More than 50% of the variance in fibrinogen γ' and γ'/total fibrinogen ratio is explained by factors other than total fibrinogen concentration or other traditional CVD risk factors. Our data show that fibrinogen γ' modulates plasma clot structure and fibrinolysis, and is also influenced by other factors than fibrinogen.

Introduction

Fibrinogen gamma prime (γ'; previously also called γB or γ57.5) arises from a splice variant of the γ chain mRNA resulting from an alternative polyadenylation signal in intron 9.[1,2] The alternative polyadenylation leads to the translation of a unique 20-amino-acid C-terminal extension encoded by intron 9, which substitutes the 4 γA amino acids of exon 10.[2-4] Approximately 8-15% of total fibrinogen is comprised of γ' fibrinogen, of which the majority is in the heterodimeric γA/γ' form.[3] Fibrinogen γ' is associated with both venous[5] and arterial thrombosis.[6-10] This association with different thrombotic disorders has in part been ascribed to the effects of γ' on clot structure, cross-linking by Factor XIIIa, thrombin activity or fibrinolysis.[11]

The relationship between γ' fibrinogen and clot structure/function has been investigated mainly using *in vitro* experimental models with purified fibrinogen.[12-14] While all these studies agree that γ' fibrinogen influences clot structure, some differences were observed in the type of changes, possibly due to differences in experimental designs, source material *i.e.* fibrinogen purified from plasma or recombinant fibrinogen, and selective co-purification of other plasma proteins such as FXIII with plasma derived fibrinogen. Some of these discrepancies may, however, also be the result of a heterogeneous, non-uniform clot structure that was observed in clots containing γ' fibrinogen in subsequent *in vitro* studies.[15;16] While the use of purified fibrinogen and fixed protein concentrations allow for the detailed study of mechanisms underpinning the effect of γ' fibrinogen on clot structure, data on the effect of γ' fibrinogen in varying plasma concentrations in the presence of varying total fibrinogen concentrations on clot structure are needed to provide insight into the *in vivo* relationships between these variables.

Furthermore, the factors that contribute to γ' fibrinogen formation and to γ'/total fibrinogen ratio *in vivo* are poorly understood. Although a constituent of total fibrinogen, variation in γ' fibrinogen concentration is not merely a reflection of changes in total fibrinogen levels but is also a result of independent control mechanisms[17;18]. In a study comparing ischaemic stroke patients in the acute phase with healthy controls for example, both γ' fibrinogen and γ'/total fibrinogen ratio were increased[9], while in intracerebral haemorrhage patients absolute γ' fibrinogen levels but not the γ'/total fibrinogen ratio were increased.[19] Fibrinogen γ' is thought to be related to

cardiovascular disease (CVD) risk independent of total fibrinogen concentration.[11;20] Therefore not only factors that affect γ' fibrinogen levels but also factors that could potentially influence the γ'/total fibrinogen ratio should be determined.

Our aim was to analyse whether γ' fibrinogen, total fibrinogen, and γ'/total fibrinogen ratio relate to plasma clot structure in a large population-based study of apparently healthy black South Africans. For this, we used the South African arm of the international Prospective Urban and Rural Epidemiology (PURE) study.[21;22] We also aimed to analyse the relationship between γ' fibrinogen and cardiovascular risk factors in this well-characterised epidemiological study.

Materials and methods

Study cohort

PURE is a large-scale cohort study that tracks changing lifestyles, risk factors and chronic disease in rural and urban areas of 17 countries in transition over 12 years. The data reported here are from the baseline data of 2010 randomly selected participants (1260 women and 750 men) from well-established rural (n = 1006, living under tribal law) and urban (n = 1004, living in informal and formal settlements surrounding cities) communities in the North West Province of South Africa collected over a twelve week period in 2005. These participants were recruited from 6000 randomly selected households from the two communities based on representativeness and feasibility for long-term follow-up, according to the guidelines stipulated in the overarching PURE study.[21;22] Apparently healthy black South Africans between the ages of 35 and 65 years were eligible to participate. Exclusion criteria were use of chronic medication for non-communicable diseases and/or any self-reported acute illness. The study was approved by the Ethics Committee of the North-West University, SA and subjects signed informed consent before taking part in the study. All data were treated confidentially and all analyses were performed with coded data.

Blood processing

Fasting blood samples were collected with minimum stasis from the antecubital veins of participants between 07:00 and 11:00. For the analysis of lipids and C-reactive protein (CRP), blood was collected in tubes without anticoagulant. Blood was collected in EDTA tubes for the determination of homocysteine and glycosylated haemoglobin (HbA1c) and in fluoride tubes for glucose measurements. For the analysis of γ'

fibrinogen, total fibrinogen and turbidimetric measurement of clot formation and lysis, blood was collected into citrate tubes and kept on ice until centrifugation (< 30 minutes). This procedure did not significantly influence plasma fibrinogen level (data not shown). Samples were centrifuged at 2000 x g for 15 minutes at 10°C within 30 minutes of collection. Aliquots were frozen on dry ice, stored in the field at -18°C and then after 2-4 days at -82°C until analysis.

Laboratory and clot structure/fibrinolysis analysis

Details regarding methods used to analyse CRP, serum lipids, total homocysteine, glucose and HbA1c were published previously.[23] Fibrinogen was measured using a modified Clauss method (Multifibrin U-test, BCS coagulation analyzer, Dade Behring, Deerfield, USA). Turbidimetric analysis (A405nm) was used to determine plasma fibrinolytic potential of tissue factor induced clots, lysed by exogenous tissue plasminogen activator (tPA) with the method of Lisman et al[24] with slightly modified tissue factor and tPA concentrations in order to obtain comparable clot lysis times (CLTs) of about 60 min (intra-assay CV = 3.6%, between plate CV= 4.5%). Final concentrations were tissue factor (125 x diluted – an estimated final concentration of 59 pM according to Duckers et al[25]; Dade Innovin, Siemens, Marburg, Germany), $CaCl_2$ (17 mmol/l), tPA (100 ng/ml; Actilyse, Boehringer Ingelheim, Ingelheim, Germany) and phospholipid vesicles (10 µmol/l; Rossix, Mölndal, Sweden). CLT was defined as the time from the midpoint in the transition from the initial baseline to maximum turbidity, which is representative of clot formation, to the midpoint in the transition from maximum turbidity to the final baseline turbidity, which represents the lysis of the clot.[24] Kinetics of clot formation, lag time, slope and maximum absorbance, were additionally calculated from the turbidity curves (Online Supplement 1). When clotting plasma with thrombin, lag time represents the time required for fibrin fibres to grow sufficiently to allow lateral aggregation. Since we clotted our plasma with tissue factor, lag time additionally included the time it took for activation of the coagulation cascade and was taken at the point where absorbance increased 0.015 from baseline. The slope, calculated at half maximum absorbance, represents the rate of lateral aggregation and the maximum absorbance, calculated as the maximum absorbance at plateau minus the baseline, is an indication of average fibre size. Fibrinogen γ' was measured according to the method of Uitte de Willige[5], - an ELISA using a 2.G2.H9 mouse monoclonal coating antibody against the human γ' sequence from Santa Cruz Biotechnology (Santa Cruz, USA) for antigen capture and a goat polyclonal HRP-conjugated antibody against

human fibrinogen from Abcam for development (Cambridge, USA). The coefficient of variance for all assays was < 10%.

Statistical analysis

The computer software package Statistica® (Statsoft Inc., Tulsa, Oklahoma, USA) was used for statistical analyses. A p-value ≤ 0.05 was regarded as statistically significant. Normally distributed variables are reported as mean (95% confidence interval). Non-parametric data was log transformed to improve normality and reported as median [25th-75th percentile]. T-tests for independent samples for parametric data and the Mann-Whitney U test for non-parametric data were used for comparison between two groups. Analysis of variance (ANOVA) with Tukey's Honest Significant Difference post hoc test was used for comparison between three or more groups. Analysis of co-variance (ANCOVA) was used when comparison between groups required adjustment for confounders. Pearson correlations and, univariate regression were used to determine associations between γ' fibrinogen, γ'/total fibrinogen ratio and total fibrinogen and markers of clot structure using normally distributed data or log-transformed data for non-parametric variables. Forward Stepwise Multiple Regression analysis was used to determine the main contributors to the variance in γ' fibrinogen and γ'/total fibrinogen ratio using parametric and log transformed data.

Results

The mean total fibrinogen concentration of the population was 3.7 g/L (±2.18). The mean γ' fibrinogen and γ'/total fibrinogen ratio of the population was 0.38 g/L (±0.27) and 12.1% (±8.11) respectively. Table 1 presents the correlation between γ' fibrinogen, γ'/total fibrinogen ratio and total fibrinogen and markers of clot formation and structure in plasma. Lag time did not show noteworthy correlations with any of the fibrinogen variables. Slope showed the strongest correlation with total fibrinogen (r = 0.37). Maximum absorbance showed a positive correlation with total fibrinogen (r = 0.58) and γ' fibrinogen (r = 0.36), and a negative correlation with γ'/total fibrinogen ratio (r = -0.21). The correlation between γ' fibrinogen and maximum absorbance, although less strong remained significant after adjustment for total fibrinogen (r = 0.17, p<0.0001 – data not shown). Clot lysis time showed the strongest correlation with γ' fibrinogen (r = 0.36). Fibrinogen γ' correlated positively with both γ'/total fibrinogen ratio (r = 0.53) and total

fibrinogen (r = 0.40) while γ'/total fibrinogen ratio correlated negatively with total fibrinogen (r = -0.56).

The contribution of fibrinogen variables to the variance in the markers of clot formation and structure was analysed using univariate regression models (Table 2). The results of the regression models are in agreement with the correlations observed. None of the fibrinogen variables contributed significantly to the variance in lag time (all 1% or less). Total fibrinogen was the largest contributor to variance in slope explaining 14% of the variance. Total fibrinogen was also the largest contributor to the variance in maximum absorbance, explaining 33% of the variance, with γ' fibrinogen explaining 13.5%. The γ'/total fibrinogen ratio additionally explained 4.5% of the variance in maximum absorbance, with an increase in the ratio being associated with a decreased maximum absorbance. Fibrinogen γ' was the largest contributor to CLT, explaining 12% of its variance.

The association of γ' fibrinogen, γ'/total fibrinogen ratio and total fibrinogen with CVD risk markers was analysed by stratification of the CVD risk markers and comparison of the distribution of the fibrinogen variables between the strata (Table 3 and Online Supplement 2). If total fibrinogen differed between the strata an ANCOVA was performed for γ' fibrinogen and γ'/total fibrinogen ratio adjusting for total fibrinogen. All three fibrinogen variables increased significantly with abdominal obesity and increasing body mass index (BMI) and CRP categories and the significance for γ' fibrinogen and γ'/total fibrinogen ratio remained after adjustment for total fibrinogen (Table 3). Individuals with low high-density lipoprotein (HDL) cholesterol had higher levels of all fibrinogen variables and the significance for γ' fibrinogen and γ'/total fibrinogen ratio also remained after adjustment for total fibrinogen. Participants with the metabolic syndrome (using the criteria recommended by Alberti et al[26]) also had higher levels of all fibrinogen variables than those without metabolic syndrome and the significance for γ' fibrinogen and γ'/total fibrinogen ratio remained after adjustment for total fibrinogen. The same pattern was observed for increasing HbA1c categories (Online Supplement 2). Women had higher γ' fibrinogen, γ'/total fibrinogen ratio and total fibrinogen than men. Total fibrinogen increased with 10-year age categories, while γ' fibrinogen did not. Individuals with increased total cholesterol had increased total fibrinogen but not γ' fibrinogen. Individuals with hyperhomocysteinaemia had lower γ' fibrinogen and γ'/total

fibrinogen ratio but similar total fibrinogen compared to individuals with normal homocysteine levels. Total fibrinogen was moderately increased in individuals with hypertension while no difference was observed for γ' fibrinogen.

To determine the respective contribution of the CVD risk factors to the variance in γ' fibrinogen and the γ'/total fibrinogen ratio, the CVD risk factors presented in Table 3/Online Supplement 2 with and without total fibrinogen and or γ' fibrinogen were entered into forward stepwise regression models (Table 4). The CVD risk factors together with fibrinogen explained 26.3 % of the variance in γ' fibrinogen, with total fibrinogen explaining 16%, followed by CRP (5%), HDL-cholesterol (3%), gender (2%), HbA1c (1%) and homocysteine (1%). When fibrinogen was excluded from the model, the CVD risk factors alone explained 19.9% of the variance in γ' fibrinogen, with CRP now explaining 13.5 % and little change in the contribution of the other CVD risk factors. When total fibrinogen or γ' fibrinogen was entered together with the CVD risk factors, the models explained 39.3%, and 37%, respectively of the variance in γ'/total fibrinogen ratio. The CVD risk factors alone explained only 3% of the variance of which CRP and HDL-cholesterol made the largest contribution.

Discussion

Our data provide evidence that γ' fibrinogen influences fibrin clot structure in plasma. It has been shown that γ' fibrinogen changes fibrin structure *in vitro* in purified systems at levels of 100%,[12-16] however, whether such effects would also occur at physiological levels of 10-15% γ' fibrinogen and in the presence of other plasma proteins was hitherto unknown. We used a large, well-characterised population-based study to show that this is indeed the case. We found associations of γ'/total fibrinogen ratio with lower maximum absorbency and prolongation of lysis time, in agreement with previously published *in vitro* effects. We also found that γ' fibrinogen levels associate with other cardiovascular risk factors and not only total fibrinogen levels.

Fibrinogen γ' levels correlated positively with total fibrinogen, although the γ'/total fibrinogen ratio decreased with increasing fibrinogen concentration, suggesting that while γ' fibrinogen increases as total fibrinogen increases, it does so to a relatively lesser extent (*i.e.* γ' levels were not a constant fraction of total fibrinogen levels throughout the entire range of fibrinogen concentrations), resulting in the decreased

ratio at higher total fibrinogen levels. The median γ' fibrinogen concentration in this black African population is higher to what was previously reported for Caucasians from the Framingham Offspring study (0.38 vs 0.23 g/L). This is in agreement with the relatively high total fibrinogen concentration (3.7 g/L) found in this and other studies investigating fibrinogen concentration in black Africans.[23;27]

Turbidity curves are often used as a measure of clot formation and structure in large datasets due to its high throughput methodology.[28] Its relevance is further supported by the fact that final clot structure is to a large extent kinetically controlled and therefore information on clot formation is critical in determining clot structure.[29;30] None of the fibrinogen variables, showed noteworthy associations with lag time. This is likely due to the fact that the samples were clotted with tissue factor which resulted in lag time not only representing the time required for the formation and growth of protofibrils from monomers, but also the time required for activation of the coagulation cascade. Tissue factor rather than thrombin was used to clot the samples as it allows for comprehensive analysis of plasma fibrinolytic potential (reported as CLT) including all coagulation and lysis components in plasma. Additionally, since clot formation variables and lysis times were determined in the same experiment it allows for better comparison of the different variables. *In vitro* experiments using purified fibrinogen also found no difference in lag time between clots made from γ' or γA fibrinogen.[12;15]

Of the fibrinogen variables, total fibrinogen correlated best with slope (rate of lateral aggregation), although the fibrinogen variables only explained a small percentage of the variance observed in the slope. These results indicate an increased rate of lateral aggregation with increasing fibrinogen concentration. This is in agreement with a kinetic model developed by Weisel et al[29] which indicates an increase in the maximum rate of protofibril addition per fibre with increasing fibrinogen concentration. An increase in γ' fibrinogen was associated with a small increase in rate of lateral aggregation, while an increase in the γ'/total fibrinogen ratio was associated with a small decrease. When adjusting for difference in total fibrinogen, the association between γ' fibrinogen and slope disappeared, suggesting that *in vivo* (at least in the plasma setting) γ' fibrinogen does not significantly affect rate of lateral aggregation despite decreased rates observed in purified models for clots made of γ' compared to γA fibrinogen.[12;15;16] It may be that the effects of γ' fibrinogen in plasma is relatively small compared to the effect of increasing fibrinogen concentration in this setting.

The variance in maximum absorbance was explained to a much larger extent (>30%) by the fibrinogen variables than either lag time or slope. An increase in both γ' fibrinogen and total fibrinogen was associated with an increased maximum absorbance with total fibrinogen contributing to a larger extent than γ' fibrinogen. An increase in the γ'/total fibrinogen ratio was, however, associated with a decrease in maximum absorbance. In agreement with this, turbidity curves obtained from purified fibrinogen models showed decreased maximum absorbance in clots containing γ' compared with γA fibrinogen, indicating that the γ'/total fibrinogen ratio is indeed an accurate indicator for the relative amount of γ' in the plasma clot.[12;15;16] Reduced maximum absorbance from turbidity analysis is an indicator of thinner fibrin fibres at constant fibrinogen concentration. The effect of γ' fibrinogen on fibre diameter as measured by scanning electron microscopy *in vitro* is generally in agreement with this. Several studies showed that clots containing γ' fibrinogen have thinner fibres.[12;14-16] Others, however, using a recombinant homodimer form of fibrinogen γ', found no difference in fibre diameter.[13] Several studies showed that fibrinogen γ' fibrinogen produced clots with increased branching.[14;16] Additionally it was found that clots containing γ' fibrinogen were non-homogenously arranged into tight interconnecting bundles with tighter pores with bundled fibres and large open pores in other areas of the clot.[15;16] While the use of plasma samples is of immense value in determining the *in vivo* relationship between γ' fibrinogen, total fibrinogen and clot structure, the interpretation of experimental results, especially from an indirect technique such as turbidity, is complicated due to the complex nature of plasma, and the interplay of factors that determine maximum absorbency including fibrinogen concentration and average fibrin fibre diameter. Because of the high throughput design of turbidity analysis, and the time-consuming nature of more direct methods (such as microscopy), it can, however, play a significant role in providing information regarding clot structure in an epidemiological study setting with large subject numbers.

There has been only one previous study that investigated the effect of γ' fibrinogen concentration on clot structure in plasma. Mannila et al[8] investigated the effect of γ' fibrinogen concentration on plasma permeability (Ks) and fibre mass-length ratio (μ calculated from Ks) in 60 control patients from the Stockholm Coronary Artery Risk Factor (SCARF) study. Fibrinogen γ' concentration was not found to be associated with Ks or μ. However, the authors did not analyse γ'/total fibrinogen ratio and the overall study size was probably too small to detect the relatively moderate effects of

physiological levels of γ' fibrinogen on clot structure. Our current study is much larger in size and showed significant effects of γ' fibrinogen on plasma clot structure. In particular, the γ'/total fibrinogen ratio showed similar effects on clot structure as previous *in vitro* studies, with an association of increased γ' fibrinogen content with thinner fibres (lower maximum absorbance) and prolonged fibrinolysis.

Although CLT correlated positively with both γ' fibrinogen and total fibrinogen, fibrinogen γ' had the strongest association with CLT, explaining 12% of its variance. This suggests γ' fibrinogen to have a larger effect on CLT than total fibrinogen. An increased CLT indicates decreased lysis rate, which is in agreement with results from studies using purified fibrinogen which showed decreased lysis rates for clots containing γ' compared to γA fibrinogen.[13;14;31] Falls and Farrell[31] additionally found decreased lysis rates in afibrinogenaemic plasma to which γA/γ' fibrinogen was added compared with plasma containing an equal concentration of γA/γA fibrinogen. They ascribed the decreased fibrinolysis rate to increased FXIII binding to γ' fibrinogen and subsequent increased cross-linking. Allan et al[16], however, found no difference in γ-chain cross-linking between γA/γA and γA/γ' containing clots and decreased α-chain cross-linking in γA/γ' clots, indicating that other factors likely play a role in the decrease of fibrinolysis rates by γ' fibrinogen. Our current data provide clear evidence for a role of γ' fibrinogen in the regulation of fibrinolysis rates also in plasma obtained from a large number of subjects with varying γ' fibrinogen levels. As γ' fibrinogen level increases in individuals, CLT also increases and γ' fibrinogen has a larger effect on CLT than total fibrinogen. The results furthermore suggest that this association between γ' fibrinogen and CLT is not only the result of a denser clot network formed in the presence of higher γ' fibrinogen concentration as total fibrinogen had a larger impact on maximum absorbance than γ' fibrinogen and maximum absorbance did not correlate with CLT (data not shown). The mechanism by which γ' fibrinogen influences fibrinolysis rates should be further investigated using experimental models of clot formation and fibrinolysis.

In general, the association of γ' fibrinogen with CVD risk factors followed the same trend as that of fibrinogen. Increased levels were observed in women compared to men, subjects with increased BMI, increased waist circumference, elevated CRP and HbA1c categories and in individuals with metabolic syndrome as well as in individuals with low

HDL-cholesterol. The association between γ' fibrinogen and these CVD risk factors remained significant after adjustment for total fibrinogen, and is accompanied also by an increase in the γ'/total fibrinogen ratio, suggesting that these associations are not merely reflecting the association of the CVD risk factors with total fibrinogen, but that they are likely independent relationships. These results are in agreement with the study of Lovely et al[32] who also found significant associations between γ' fibrinogen and BMI, (decreased) HDL-cholesterol, diabetes, blood glucose and gender in Caucasians. In two other studies, γ' fibrinogen did, however, not differ between genders[7;33] and while we and others[7;33] found no association between γ' fibrinogen and age, a positive association was found by another group.[32] In this study population, total fibrinogen increased over 10-year age categories and in individuals with increased total cholesterol (>5.2 mmol/L), while no increase was observed for the γ' fibrinogen, resulting in a decreased γ'/total fibrinogen ratio. In hyperhomocysteinaemic individuals on the other hand, decreased γ' fibrinogen was observed with no change in total fibrinogen consequently also decreasing the γ'/total fibrinogen ratio.

Multiple regression analysis indicated that fibrinogen concentration explained the largest percentage of the variation in γ' fibrinogen and that the CVD risk factors alone, excluding total fibrinogen, explained 20% and 3% of the variances of γ' fibrinogen and γ'/total fibrinogen ratio, respectively, suggesting that the γ'/total fibrinogen ratio in apparently healthy black Africans is not strongly affected by CVD risk factors other than γ' fibrinogen and total fibrinogen. The γ'/total fibrinogen ratio was, however, found to be different between several CVD patient groups and controls of Caucasian descent.[5;9;34] Of the other CVD risk factors, CRP was found to be the biggest determinant of both γ' fibrinogen and γ'/total fibrinogen ratio. These results are in agreement with a case-control study by Cheung et al[34] who found a significant association between CRP and γ'/total fibrinogen ratio in the acute phase of ischaemic stroke. These authors hypothesised that mRNA processing of γ' fibrinogen may be altered during the acute phase reaction.

In conclusion, γ' fibrinogen levels increase as total fibrinogen increases, although to a lesser extent, resulting in a decrease in γ'/total fibrinogen ratio at high fibrinogen concentrations in this apparently healthy black African population. Increases in both γ'

fibrinogen and total fibrinogen were associated with increased maximum absorbance, in agreement with the formation of clots composed of increased fibrin material. However, the γ'/total fibrinogen ratio was associated with decreased maximum absorbance, in agreement with clots made of thinner fibrin fibres. Increased γ' fibrinogen levels were associated with prolonged clot lysis. Traditional CVD risk factors (excluding fibrinogen) explained 20% and 3% respectively of the variance in γ' fibrinogen and γ'/total fibrinogen ratio, with CRP making the largest contribution. These data show that physiological levels of γ' fibrinogen influence fibrin clot structure in plasma and that factors other than fibrinogen, likely involved in the inflammatory response, regulate plasma γ' fibrinogen concentration. Our findings support future studies of the role of γ' fibrinogen in thrombosis.

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Authorship

Contribution:

MP; performed research, collected data, analysed and interpreted data, performed statistical analysis and wrote manuscript

RCK; analysed and interpreted data, performed statistical analysis

JCJ; designed research, collected data

AK; designed research, collected data

RASA; analysed and interpreted data and critically reviewed the manuscript

Conflict-of-interest disclosure:

None of the authors have any conflict of interest to declare

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Table 1 Correlation between γ' fibrinogen, γ'/total fibrinogen ratio and total fibrinogen and measures of clot structure

Variable	γ' fibrinogen		γ'/total fibrinogen ratio		Total fibrinogen	
	r	p	r	p	r	p
Lag time (min)	0.0517	0.04	-0.0245	0.32	0.08	0.002
Slope	0.1712	<0.0001	-0.1829	<0.0001	0.3651	<0.0001
Max abs	0.3623	<0.0001	-0.2091	<0.0001	0.5802	<0.0001
Clot lysis time	0.3568	<0.0001	0.1720	<0.0001	0.1631	<0.0001
γ' fibrinogen	-	-	0.5319	<0.0001	0.403	<0.0001
γ'/total fibrinogen ratio	0.5319	<0.0001	-	-	-0.561	<0.0001
Total fibrinogen	0.403	<0.0001	-0.561	<0.0001	-	-

Table 2 Univariate regression results of γ' fibrinogen, γ'/total fibrinogen ratio and total fibrinogen with measures of clot structure

Univariate Regression	Lag time (min)			Slope			Maximum absorbance			Clot lysis time (min)		
	β	95% CI	%*	β	95% CI	%*	β	95% CI	%*	β	95% CI	%*
γ' fibrinogen	0.25	0.08; 0.42	1	0.06	0.04; 0.08	2.5	0.11	0.09; 0.12	13.5	7.30	6.37; 8.24	12
γ'/total fibrinogen ratio	-0.11	-0.26; 0.05	<1	-0.06	-0.08; -0.05	3.5	-0.06	-0.07; -0.04	4.5	3.35	2.45; 4.25	3
total fibrinogen	0.32	0.16; 0.49	1	0.13	0.12; 0.15	14	0.17	0.15; 0.18	33	3.28	2.31; 4.25	2.5

* **%** variance explained (R^2 x 100)

Table 3 The association between γ' fibrinogen, γ'/total fibrinogen ratio and total fibrinogen and traditional CVD risk factors

CVD Risk Factor	γ' fibrinogen (mg/ml)	γ'/total fibrinogen ratio (%)	Total fibrinogen (mg/ml)
BMI, kg/m^2:			
<18.5	0.28 (0.20-0.42) (n=303)	9.24 (9.06-13.4) (n=288)	2.95 (2.20-4.80) (n=306)
18.5-24.9	0.28 (0.21-0.41) (n=732)	10.3 (7.19-14.7) (n=712)	2.60 (2.10-4.10) (n=741)
25-29.9	0.35 (0.24-0.46) (n=298)	10.6 (7.39-15.3) (n=285)	2.90 (2.30-5.00) (n=302)
≥30	0.38 (.029-0.56) (n=345)	10.4 (7.33-15.1) (n=327)	3.70 (2.60-6.20) (n=338)
unadjusted p	<0.0001	0.001	<0.0001
p value adjusted for Fbg	<0.0001	<0.0001	-
Waist circumference, cm:			
Normal	0.28 (0.21-0.40) (n=1150)	9.98 (7.12-14.3) (n=1112)	2.60 (2.10-4.10) (n=1167)
Abdominal obesity	0.38 (0.28-0.53) (n=605)	10.4 (7.28-15.2) (n=574)	3.30 (2.50-5.90) (n=559)
unadjusted p	<0.0001	0.067	<0.0001
p value adjusted for Fbg	<0.0001	<0.0001	-
HDL-cholesterol, mmol/L:			
Normal (≥1.0)	0.29 (0.22-0.43) (n=1224)	9.76 (6.84-14.2) (n=1175)	2.80 (2.20-4.80) (n=1228)
Low (<1.0)	0.35 (0.26-0.51) (n=514)	11.1 (8.03-15.3) (n=496)	3.00 (2.30-5.50) (n=521)
unadjusted p	<0.0001	<0.0001	0.009
p value adjusted for Fbg	<0.0001	<0.0001	-
Metabolic syndrome:			
Yes	0.38 (0.27-0.52) (n=425)	10.4 (7.54-15.1) (n=397)	3.30 (2.50-5.80) (n=415)
No	0.29 (0.22-0.42) (n=1290)	10.0 (7.08-14.5) (n=1251)	2.80 (2.20-4.50) (n=1309)
unadjusted p	<0.0001	0.06	<0.0001

p value adjusted for Fbg	<0.0001	<0.0001	-
C-reactive protein, mg/L:			
≤3	0.27 (0.21-0.37) (n=822)	10.5 (7.77-14.8) (n=797)	2.50 (2.00-3.20) (n=832)
>3	0.36 (0.26-0.52) (n=907)	9.73 (6.77-14.3) (n=866)	3.60 (2.50-6.10) (n=909)
unadjusted p	<0.0001	0.007	<0.0001
p value adjusted for Fbg	<0.0001	<0.0001	-

BMI – body mass index; HDL – high density lipoprotein; Fbg - fibrinogen

Table 4 Multiple regression results for γ' fibrinogen, γ'/total fibrinogen ratio and total fibrinogen and traditional CVD risk factors

Variable	% Variance explained		
	Model 1 CVD risk factors plus total fibrinogen	Model 2 CVD risk factors plus γ' fibrinogen	Model 3 CVD risk factors
γ' fibrinogen			
Total variance explained	26.3		19.9
Risk factors:			
- total fibrinogen	16		Not included
- C-reactive protein	5		13.5
- HDL-cholesterol	3		3
- gender	2		2
- HbA1c	1		1
- homocysteine	1		0.5
γ'/total fibrinogen ratio			
Total variance explained	39.3	37	3
Risk factors:			
- γ' fibrinogen	Not included	28.8	Not included
- total fibrinogen	31	Not included	Not included
- C-reactive protein	4	7	0.5
- HDL-cholesterol	2	-	2
- gender	1	0.5	-
- homocysteine	0.5	-	1
- HbA1c	0.5	-	-
- age	-	1	0.5

- variable did not enter forward stepwise model

Online Supplemental material

Online Supplement 1

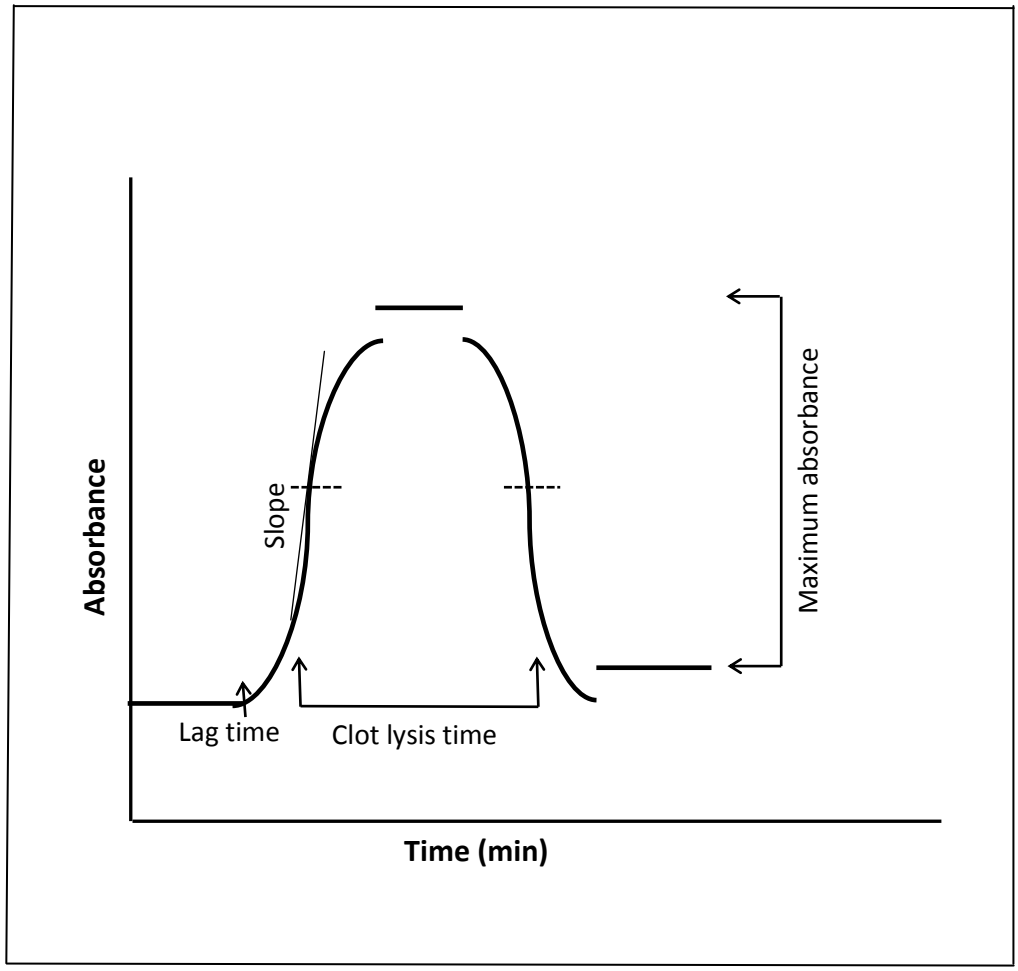

Figure: Variables calculated from Turbidity Curves. The following measurements were obtained from turbidity curves of fibrin polymerisation triggered by the addition of tissue factor (59 pM) to plasma and lysis induced by tPA. Lag time was taken as the time point when absorbance increased 0.015 from baseline. Slope was calculated from the slope of the line at the midpoint (----) between initial baseline and maximum absorbance at plateau. Maximum absorbance was calculated as the increase in absorbance from initial baseline absorbance to maximum at plateau. Clot lysis time was calculated as the time from the midpoint (----) in the transition from the initial baseline to maximum absorbance to the midpoint (----) in the transition from maximum to the final baseline absorbance.

Online Supplement 2

The association between γ' fibrinogen, γ'/total fibrinogen ratio and total fibrinogen and traditional CVD risk factors

CVD Risk Factor	γ' fibrinogen (mg/ml)	γ'/total fibrinogen ratio (%)	Total fibrinogen (mg/ml)
Gender:			
Men	0.28 (0.20-0.39) (n=660)	9.77 (7.09–13.9) (n=631)	2.60 (2.10-3.70) (n=673)
Women	0.349 90.25-0.48) (n=1111)	10.3 (7.17-15.1) (n=1070)	3.10 (2.30-5.50) (n=1109)
unadjusted p	<0.0001	0.048	<0.0001
p value adjusted for Fbg	<0.0001	<0.0001	-
Age, years:			
30-39	0.30 (0.22-0.44) (n=329)	10.7 (7.95-15.4) (n=322)	2.70 (2.20-3.90) (n=334)
40-49	0.30 (0.22-0.42) (n=691)	105. (7.59-14.7) (n=671)	2.70 (2.10-4.30) (n=700)
50-59	0.32 (0.24-0.48) (n=468)	9.65 (6.50-14.2) (n=442)	3.10 (2.40-5.40) (n=461)
60+	0.33 (0.24-0.46) (n=283)	9.08 (6.16-13.9) (n=266)	3.30 (2.50-5.80) (n=287)
unadjusted p	0.28	0.01	<0.0001
p value adjusted for Fbg	0.78	0.78	-
Total cholesterol, mmol/L:			
<5.2	0.30 (0.22-0.44) (n=1.03)	10.5 (7.39-15.1) (n=995)	2.20 (2.80-4.80) (n=1046)
≥5.2	0.32 (0.23-0.46) (n=708)	9.62 (6.94-14.0) (n=676)	3.00 (2.40-5.35) (n=703)
unadjusted p	0.94	0.01	0.004
p value adjusted for Fbg	0.20	0.20	-
HbA1c, mmol/L:			
Quartile 1 (<5.3)	0.28 (0.20-0.39) (n=441)	10.4 (7.27-14.3) (n=425)	2.50 (2.10-3.70) (n=443)
Quartile 2 (5.3 – 5.49)	0.28 (0.21-0.41) (n=291)	9.73 (7.09-13.3) (n=278)	2.80 (10.4-4.30) (n=292)
Quartile 3 (5.5 – 5.79)	0.31 (0.23-0.45)	10.1 (7.21-15.1)	2.90 (2.30-4.50)

	(n=471)	(n=455)	(n=474)
Quartile 4 (≥5.8)	0.36 (0.26-0.55)	10.2 (7.05-15.0)	3.30 (2.50-5.90)
	(n=552)	(n=528)	(n=556)
unadjusted p	<0.0001	0.75	<0.0001
p value adjusted for Fbg	<0.0001	<0.0001	-
Homocysteine, µmol/L:			
Normal (≤15)	0.32 (0.23-0.46)	10.3 (7.31-14.7)	2.90 (2.30-5.25)
	(n=1527)	(n=1438)	(n=1538)
High (>15)	0.27 (0.20-0.41)	8.73 (5.63-13.3)	2.90 (2.20-4.80)
	(n=190)		(n=191)
unadjusted p	0.0002	0.0009	0.67
Blood pressure, mmHg:			
Normal	0.31 (0.23-0.46)	10.5 (7.59-15.1)	2.80 (2.20-4.80)
	(n=927)	(n=904)	(n=955)
Hypertension	0.31 (0.23-0.44)	9.8 (6.64-14.0)	3.00 (2.30-5.30)
	(n=830)	(n=785)	(n=815)
unadjusted p	0.19	0.01	0.057

Fbg - fibrinogen

CHAPTER 4:

Genetic polymorphisms influencing total and γ' fibrinogen levels and fibrin clot properties in Africans

Authors: Retha C. Kotzé, Cornelie Nienaber-Rousseau, Zelda De Lange, Moniek P. De Maat, Tiny Hoekstra and Marlien Pieters

This chapter includes:

- author instructions of the journal, *British Journal of Haematology* (Impact factor 4.9)
- letter of acceptance of manuscript from *British Journal of Haematology* (July, 2014) the article titled "Genetic polymorphisms influencing total and γ' fibrinogen levels and fibrin clot properties in Africans", accepted for publication (Br J Haematol. 2014 Aug 25. doi: 10.1111/bjh.13104. [Epub ahead of print])
- online supplemental material as submitted online to *British Journal of Haematology*
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ARTICLE

Genetic polymorphisms influencing total and γ' fibrinogen levels and fibrin clot properties in Africans

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Abstract

Inter-ethnic variation in fibrinogen levels is hypothesised to be the result of differences in genetic background. No information is available regarding the contribution of genetics to fibrinogen γ' in Africans. Only limited information is available on the interaction between genotypes and total and γ' fibrinogen concentration in determining fibrin clot properties. Our aim was to investigate the effect of polymorphisms in the fibrinogen and Factor XIII genes on total and γ' fibrinogen and clot properties (turbidimetry) in 2010 black Africans, as well as to determine their interactions. Significant associations were observed between rs1049636 (*FGG* gene) and total fibrinogen levels and between rs2070011 (*FGA* promoter area) and fibrinogen γ' levels. Significant associations were observed between SNPs in the *FGA* (rs2070011), *FGB* (rs1800787) and *FGG* (rs1049636) genes and fibre size. Significant interactions were found between total and/or γ' fibrinogen levels and SNPs in the *FGA* (rs2070011), *FGB* (rs2227385, rs1800787, rs1800788, rs4220) and Factor XIII genes (rs5985) in determining clot properties. The different SNPs influenced the relationships between total and γ' fibrinogen levels with clot properties in opposing directions. Genetic influences may be ethnic-specific and should not only focus on fibrinogen concentration, but also on functionality in determining its role in CVD.

Keywords: fibrinogen, genetics of thrombosis and haemostasis, epidemiology, fibrinolysis

Introduction

Fibrinogen levels are known to differ between whites and African Americans as well as Africans, with higher levels observed in the black ethnicities (Folsom *et al*, 1992; Vorster *et al*, 1998; Kaptoge *et al*, 2007; Pieters & Vorster, 2008). The γ chain gene of fibrinogen (*FGG*) has two alternative splice variants, γA and γ', resulting from an alternative polyadenylation signal in intron 9 (Francis *et al*, 1980). Approximately 8–15% of total fibrinogen is comprised of γ' fibrinogen, of which the majority is in the heterodimeric γA/γ' form (Chung & Davie, 1984). The variability in total fibrinogen and fibrinogen gamma prime (γ') in Africans is only partly explained by known cardiovascular disease (CVD) risk factors (Pieters *et al*, 2011). Of these, C-reactive protein (CRP) has been shown to make the largest contribution (Pieters *et al*, 2011; Pieters *et al*, 2013).

Both total and γ' fibrinogen are also genetically regulated and genetic factors are considered to explain 30 to 40% of the variation in fibrinogen concentration in white populations (Reed *et al*, 1994; Friedlander *et al*, 1995; Freeman *et al*, 2002). However, in Africans, very little is known about the contribution of genetic factors to the variation in total and γ' fibrinogen concentration. Fibrinogen single nucleotide polymorphisms (SNPs) have been shown to account for approximately 1.4 to 3.8% and 2% of variance in total fibrinogen levels in African Americans (Reiner *et al*, 2006; Wassel *et al*, 2011) and non-Hispanic blacks (Jeff *et al*, 2012), respectively. This tends to be mostly lower (Reiner *et al*, 2006; Wassel *et al*, 2011) than the contribution in white populations, which varies from 1.6 to 15% (Reiner *et al*, 2006; Wassel *et al*, 2011). To our knowledge, no studies have reported evidence on the contribution of genetic factors to fibrinogen γ' variability in black ethnicities. Since African populations are reported to contain greater genetic variability than non-African populations (Chen *et al*, 1995), we hypothesise that the association between fibrinogen and Factor XIII SNPs and total and γ' fibrinogen levels may be different in our African population from what has been found in individuals of European descent.

Genetic polymorphisms affect not only the concentration of total and γ' fibrinogen, but also their functionality, resulting in altered clot structure. For example, minor alleles of the fibrinogen α-chain (*FGA*) gene polymorphism 2224G/A (rs2070011) and fibrinogen γ-chain (*FGG*) gene polymorphism 9340T/C (rs1049636) have been associated with

reduced clot permeability (Mannila *et al*, 2006), while the Ala312 allele of *FGA* 6534/Thr312Ala (rs6050) has been associated with increased clot stiffness (Standeven *et al*, 2003), and the Lys448 allele of the fibrinogen β-chain (*FGB*) gene polymorphism βArg448Lys (rs4220) resulted in a compact fibrin network structure resistant to lysis (Ajjan *et al*, 2008). A polymorphism of the Factor XIII subunit A gene (*F13A1*), Val34Leu (rs5985), has also been shown to result in altered clot structure (Ariëns *et al*, 2000).

Furthermore, despite the limited data available, fibrinogen concentration has the potential to influence the effect polymorphisms may exert on clot structure. For example, for *F13A1* Val34Leu (rs5985), homozygotes of the Val allele produced clots with decreased clot permeability in the presence of a wide range of fibrinogen levels, while homozygotes of the Leu allele formed clots with lower permeability and a tight fibrin network structure with thinner fibres only at low fibrinogen levels (Lim *et al*, 2003). To the best of our knowledge, such interactions between fibrinogen γ' concentration and polymorphisms on clot structure has not yet been investigated.

The aim of this study was, therefore, firstly to investigate, in a black South African population, the effect of fibrinogen and Factor XIII genetic polymorphisms on total and γ' fibrinogen concentrations, as well as on clot properties (measured by turbidimetry). SNPs were selected based on known associations published in the literature for individuals of European descent (Van't Hooft *et al*, 1999; Cook *et al*, 2001; Lim *et al*, 2003; Uitte de Willige *et al*, 2005; Mannila *et al*, 2006; Reiner *et al*, 2006; Mannila *et al*, 2007). Our second aim was to determine whether interactions exist between total and γ' fibrinogen levels and genetic polymorphisms in determining clot structure and properties.

Materials and methods

Study population

The Prospective Urban and Rural Epidemiological (PURE) study is a large prospective cohort tracking changing lifestyles, risk factors and chronic disease in urban and rural areas of 21 countries in transition over 12 years. For the South African arm of this study, a census of 6000 African households was undertaken, starting from a randomly

selected address in each of four (two rural and two urban) communities. Apparently healthy black South African men and women aged 35–65 years were included. Exclusion criteria were use of chronic medication for non-communicable diseases and/or any self-reported acute illness. Blood was collected from 2010 randomly selected participants during a 12-week blood collection period in 2005. The study was approved by the Ethics Committee of the North-West University of South Africa and, prior to taking part in the study, study procedures were explained to all the participants in their home language, after which they signed informed consent forms in accordance with the Declaration of Helsinki. Data were treated confidentially and data analyses were performed with coded data.

Blood collection

Fasting blood samples were collected from the antecubital vein by qualified nurses between 07:00 and 11:00. Blood was collected in citrate tubes and centrifuged at 2000 x g for 15 minutes at 10°C within 30 minutes of collection. Aliquots were frozen on dry ice, stored in the field at -18°C and then, after 2–4 days, at -82°C until analysis. Following centrifugation, the leucocyte layer was transferred into a separate aliquot from which DNA was isolated using the FlexiGene™ DNA extraction kit (QIAGEN® Valencia, CA).

Laboratory and clot structure/fibrinolysis analysis

Fibrinogen was measured using a modified Clauss method (Multifibrin U-test, BCS coagulation analyser, Dade Behring, Deerfield, IL, USA). Fibrinogen γ' was measured with an ELISA, using a 2.G2.H9 mouse monoclonal coating antibody against the human γ' sequence from Santa Cruz Biotechnology (Santa Cruz, USA) for antigen capture and a goat polyclonal antibody against human fibrinogen from Abcam for development (Antibody 7539, Cambridge, USA) (Uitte de Willige *et al*, 2005; Pieters *et al*, 2013). Pooled normal plasma with a known γ' fibrinogen concentration was used for the standard curve. To determine plasma fibrinolytic potential, turbidimetric analysis (A405 nm) was conducted according to the method of Lisman *et al* (2005), with slightly modified tissue factor and tissue plasminogen activator (tPA) concentrations in order to obtain clot lysis times (CLTs) of 60 minutes, as published previously (De Lange *et al*, 2012). Final concentrations were tissue factor (125x diluted – an estimated final

concentration of 59 pM; Dade Innovin, Siemens Healthcare Diagnostics Inc., Marburg, Germany), $CaCl_2$ (17 mmol/l), tPA (100 ng/ml; Actilyse, Boehringer Ingelheim, Ingelheim, Germany) and phospholipid vesicles (10 µmol/l; Rossix, Mölndal, Sweden). Kinetics of clot formation, lag time, slope and maximum absorbance were additionally calculated from the turbidity curves, as previously described (Pieters *et al*, 2013). Lag time represents the time required for fibrin fibres to grow sufficiently to allow lateral aggregation as well as activation of the coagulation cascade (since tissue factor was used to activate clotting). The slope represents the rate of lateral aggregation and the maximum absorbance is an indication of average fibre size. The CV for all assays was <10%.

Single nucleotide polymorphism (SNP) selection

Based on literature describing the influence of SNPs on total and γ' fibrinogen levels and/or clot structure (Van't Hooft *et al*, 1999; Cook *et al*, 2001; Lim *et al*, 2003; Uitte de Willige *et al*, 2005; Mannila *et al*, 2006; Reiner *et al*, 2006; Mannila *et al*, 2007), the following SNPs were selected for genotyping: in the fibrinogen gene: *FGA* 2224G/A (rs2070011), *FGA* 6534A/G (rs6050), *FGB* 1038G/A (rs1800791), *FGB* Arg448Lys, G/A (rs4220), *FGB* -148C/T (rs1800787), *FGG* 10034C/T (rs2066865) and *FGG* 9340T/C (rs1049636). Additionally, in the Factor XIII gene: His95Arg, A/G (rs6003) and Val34Leu, C/A (rs5985) were selected for genotyping. The promoter area of the *FGB* gene was also sequenced to identify possible novel polymorphisms in Africans.

Gene sequencing

Sequencing of 2 kB of the promoter region of the *FGB* gene was done in a subgroup of 28 randomly selected participants (used as a representative group of the study population) on a 3130*xl* GeneticAnalyser (AppliedBiosystems, CA, USA) (Sanger *et al*, 1977). Primers used for sequencing are presented in Table SI. Fourteen polymorphisms were detected (Table SII). The haplotype block structure, as determined by Haploview, was used to identify haplotype-tagging SNPs (Barrett *et al*, 2005). The following four haploptype-tagging SNPs were consequently selected for further analysis in all participants: *FGB* 40A/G (rs2227385), *FGB* 749A/G (rs2227388), *FGB* 1038G/A (rs1800791) and 1643C/T (rs1800788) (Table SII).

Genotyping

Genotyping was performed by allelic discrimination with TaqMan-based assays (Thermo Fischer Scientific, Walthem MA, USA) using the MyIQBioRad real-time PCR machine (Bio-Rad Laboratories Inc., Hercules, CA, USA) and a BeadXpress™ platform by means of the IlluminaVeraCodeGoldenGate Genotyping Assay using the VeraCode technology (Illumina®, San Diego, CA, USA). SNPs *FGB* 1038G/A (rs1800791), *FGB* 1643C/T (rs1800788), *FGB* 40A/G (rs2227385) and *FGB* 749A/G (rs2227388) were determined through the first method with appropriate quality control measures taken *i.e*. inclusion of non-template and positive control samples. The well-known SNP, *FGB* -455G/A (rs1800790), which is known to affect fibrinogen concentrations, was initially selected for analysis, but showed no variation in this population. The remaining SNPs were sent through to Illumina Technical Support for evaluation using the Assay Design Tool (Illumina®, San Diego, CA, USA) and each SNP was scored (design scores varying from 0–1) by the Assay Design Tool, based on compatibility with successful GoldenGate genotyping. SNPs that met the quality criteria, *i.e*. a gene call score of >0.5 and samples with a call rate of $\geq$0.9, were included in the analysis. Owing to insufficient sample volume and analytical fall-out of random samples, genotyping could not be performed on all samples. Exact sample numbers are given in each table.

Statistical analysis

The computer software package, Statistica (Statsoft, Tulsa, OK, USA), was used. Normally distributed data are reported as mean (95% confidence interval or standard deviation). Nonparametric data were log transformed to improve normality and reported as median (25^{th}–75^{th}percentile). T-tests were employed when comparing differences between two groups. One-way analysis of variance (ANOVA) and Tukey's Honest Significant Difference *post-hoc* tests were used when comparing more than two groups. When comparison between groups required adjustment for confounders, analysis of co-variance (ANCOVA) was used. Significance was set at p≤0.01 following Bonferroni adjustment to account for multiple testing. To investigate possible gene-environment interactions between fibrinogen and Factor XIII SNP genotypes and total and γ' fibrinogen in determining clot properties, interaction terms were entered into an ANCOVA with full factorial analysis. In addition, regression slopes were compared. For these interaction analyses, if sample sizes were too small, the homozygous mutant

genotype groups were combined with the heterozygous group. Chi-square tests were used to determine if the different polymorphisms were in Hardy-Weinberg equilibrium. Haploview software was used to calculate pairwise linkage disequilibrium (LD), which was presented as standardised disequilibrium (D') and correlation coefficient squared (r^2) values. The SAS System software (SAS Institute Inc., Cary, NC, USA) was used to calculate 95% confidence intervals of the SNP frequencies.

Results

Baseline characteristics

Baseline descriptive characteristics are presented in Table I. Data of the total study population did not differ significantly from those of individuals for whom genotyping data were available (data not shown). Total and γ' fibrinogen and γ' ratio were significantly higher in women than in men. Significantly higher maximum absorbance levels (p=0.0005) and longer CLTs (p<0.0001) were observed in women, but no differences between the genders were observed for lag time (p=0.79) or slope (p=0.60).

Genetic variation in the PURE-SA study population

Table II presents the genotype distribution and minor allele frequencies (MAF) of the measured SNPs. We identified five novel SNPs in the *FGB* promoter area that have not been described in the Seattle database of the National Heart, Lung and Blood Institute's Program for Genomic Applications (http://pga.gs.washington.edu/) before. All polymorphisms analysed were in Hardy-Weinberg equilibrium. LD between the measured SNPs is indicated by the Haploview LD structure in Fig S1. Owing to the relatively low r^2 values, however, which are most likely due to the differences in MAF, it can be assumed that these SNPs may not have been in complete LD and may still have differing effects on total and γ' fibrinogen concentration and clot properties. The results of the individual SNPs will therefore be presented instead of making use only of representative tagging SNPs.

Effect of SNPs on plasma total and γ' fibrinogen levels and γ' ratio

Table III presents the SNPs that affected plasma total and γ' fibrinogen levels, or γ' ratio. A significant association was observed between SNP rs2070011 and fibrinogen γ' levels

(p=0.008). Subjects homozygous for the minor A allele had significantly higher fibrinogen γ' levels. SNP rs1049636 had a significant effect on total fibrinogen levels (p=0.0009), with homozygotes for the minor C allele presenting with significantly higher fibrinogen levels. SNP rs1800791 also showed an association with total fibrinogen levels (p=0.04), but after Bonferroni adjustment this was no longer considered significant. Data of the remaining SNPs investigated in this study are presented in Table SIII.

Effect of SNPs on clot properties

SNPs for which significant effects on clot properties were detected are presented in Table IV. SNP rs1800787 demonstrated a significant effect on maximum absorbance (p=0.001), with carriers of the minor allele having a significantly higher maximum absorbance. Similar results were seen for SNP rs2070011, which also demonstrated a significant effect on maximum absorbance (p=0.01), but this disappeared after adjustment for fibrinogen γ' (p=0.06). SNP rs1049636 also had a significant effect on maximum absorbance (p=0.01), with significantly higher maximum absorbance observed in the homozygous minor allele carriers. After adjustment for total fibrinogen, the difference was no longer significant. Of the Factor XIII gene polymorphisms, SNP rs5985 demonstrated an effect on lag time (p=0.04), although after the Bonferroni adjustment the effect was no longer significant. Data of the remaining SNPs investigated in this study are presented in Table SIV.

Gene-environment interactions for SNPs and fibrinogen variables on clot properties

Potential gene-environment interactions between SNPs and fibrinogen variables in determining clot properties were investigated next. Significant interactions are presented in Table V. Significant interactions that affected slope (rate of lateral aggregation) included the following: an interaction between SNP rs2070011 in the *FGA* promoter area and total fibrinogen levels (p=0.01). In general, there was an increase in slope as fibrinogen concentration increased. This association differed across genotypes with a bigger increase in slope observed in the homozygous wild-type group, as fibrinogen concentration increased. Two SNPs in the *FGB* gene, one in exon 8 (rs4220) (p=0.01) and one in the promoter area (rs1800787) (p=0.003), showed interactions with the relative amount of fibrinogen γ' (γ' ratio). In general the γ' ratio had

a negative association with slope. Slope, however, decreased more with an increase in γ' ratio in the homozygous wild-type carriers. The *F13A1* SNP, rs5985, showed an interaction with fibrinogen γ' in determining maximum absorbance (p=0.004). Homozygotes for the minor allele, in comparison with major allele carriers, had a larger increase in maximum absorbance as fibrinogen γ' levels increased. Three SNPs in the promoter area of the *FGB* gene had interactions with total fibrinogen and/or fibrinogen γ' in determining CLT. For rs1800788 (p=0.01) and rs1800787 (p=0.01), CLT increased more with increasing total fibrinogen γ' and fibrinogen concentration respectively in the minor allele carriers than in the major allele carriers. While for rs2227385, CLT was positively associated with both total fibrinogen (p=0.0003) and fibrinogen γ' (p=0.005) in those homozygous for the major allele. These associations were weakened in the presence of the minor alleles. For the sake of completeness of this thesis, additional regression slopes of the gene-environment interactions discussed above are provided on pages 169-173.

Discussion

This study describes the effect of known fibrinogen and Factor XIII SNPs on total and γ' fibrinogen and clot properties in continental Africans in whom we observed genetic variation that is different from what was found in previous research in white populations. We provide evidence that these SNPs influence the relationship of both plasma total and γ' fibrinogen with clot properties, providing a possible explanation for the fact that Mendelian randomisation studies could not conclusively link fibrinogen concentration to CVD (Van der Bom *et al*, 1998).

Most of the known fibrinogen SNPs reported in white populations have also been identified in this study population; however, the MAF for most SNPs was much lower than previously reported data (Iso *et al*, 1995; Van't Hooft *et al*, 1999; Blake *et al*, 2001; Green, 2001; Ariëns *et al*, 2002; Uitte de Willige *et al*, 2009; http://www.ncbi.nlm.nih.gov/projects/SNP). In contrast, the MAF of rs6050 and rs2066865 in the *FGA* and *FGG* genes, respectively, were higher in our population than in white populations (Green, 2001; Reiner *et al*, 2006; Lange *et al*, 2008; Lovely *et al*, 2011), while no variation has been reported for SNPs rs2227385 and rs2227388, situated in the promoter area of the *FGB* gene, in white populations (http://www.ncbi.nlm.nih.gov/projects/SNP). Furthermore, no variation was observed for

the well-known *FGB* gene SNP, -455G/A (rs1800790) (Undas & Zeglin, 2006). The SNP rs1800789, in the *FGB* gene, the top SNP associated with total fibrinogen levels, as identified by genome-wide association studies (GWAS), was not selected to be analysed in the total study population because of its low frequency. According to Dehghan *et al* (2009), this SNP is in high LD with the functional SNP rs1800787, also in the promoter area of the *FGB* gene, which we did analyse. This SNP has been shown to influence fibrinogen concentration by directly affecting gene transcription in basal and IL-6-stimulated conditions in luciferase expression studies (Verschuur *et al*, 2005). Its association with total fibrinogen concentration in our population is, however, inconclusive, with substantially increased total fibrinogen levels observed in the homozygous minor allele carriers, but due to the small sample number (n=9) remaining statistically inconclusive.

For the *F13B* SNP, rs6003, the minor (95Arg) allele had a higher frequency than the wild-type (95His) allele in white populations, which is in agreement with previous research in Nigerians (Ryan *et al*, 2009).

There were differences in the LD observed in this population and that reported in studies investigating participants of European descent. In comparison with other studies, no LD was observed between rs1800791, rs1800787, rs4220, rs1049636 and rs2070011 (Behague *et al*, 1996; Mannila *et al*, 2005; Mannila *et al*, 2006); however, LD not previously reported, was observed between rs1800791, rs2227388 and rs2227385. These findings are not entirely unexpected since LD patterns of Africans are considered to be more complex and thus different from those of other ethnicities (Teo *et al*, 2010). This may also explain the fact that the associations of the measured SNPs with total and γ' fibrinogen observed in this study differ somewhat from what has been reported in white populations (as discussed below), since common variant association testing is strongly dependent upon LD (Teo *et al,* 2010).

Regarding the association of fibrinogen SNPs with fibrinogen levels, we support previous findings of an association between rs1049636 in intron 9 of the *FGG* gene (minor C-allele) and rs1800791 (minor A-allele) (although not significantly so) in the *FGB* gene promoter area with increased total fibrinogen levels (Reiner *et al*, 2006; Van't Hooft *et al*, 1999). However, since it is a haplotype-tagging SNP, rs1049636 may not be the functional SNP but may act only as a proxy for other functional SNPs at different

locations in the fibrinogen genes influencing the expression of fibrinogen (Reiner *et al*, 2006). SNP rs1800791, furthermore, may increase fibrinogen levels through its involvement in the binding of nuclear proteins to the promoter of the *FGB* gene, which results in increased *FGB* transcription (Van't Hooft *et al*, 1999). Contrary to other studies that found an association between *FGB* gene SNPs (rs1800787, rs4220), *FGA* gene SNPs (rs6050, rs2070011), rs2066865 in the *FGG* gene and rs5985 in *F13A1* and total fibrinogen levels (Iso *et al*, 1995; Carter *et al*, 1997; Cook *et al*, 2001; Liu *et al*, 2001; Kain *et al*, 2002; Keavney *et al*, 2006; Mannila *et al*, 2006; Mannila *et al*, 2007; Carty *et al*, 2008; Jacquemin *et al*, 2008; Wypasek *et al*, 2012), we observed no such associations.

From a recent GWAS by Lovely *et al* (2011), after controlling for the top SNP rs7681423, rs1049636, situated in the splicing region for fibrinogen γ' in intron 9 of the *FGG* gene, became the second most significant SNP associated with fibrinogen γ' levels, although we did not find such an association; instead, we demonstrated an association between increased fibrinogen γ' levels and rs2070011 in the promoter area of the *FGA* gene, which was not in LD with rs1049636. These results are in agreement with the study of Mannila *et al* (2007), although they found decreased fibrinogen γ' levels with increasing numbers of the minor A allele while we observed significantly higher fibrinogen γ' levels. Furthermore, in contrast to previous studies showing an association between fibrinogen γ' levels and another SNP in intron 9 of the *FGG* gene, rs2066865 and rs1800791 in the *FGB* gene promoter area (Uitte de Willige *et al*, 2005; Mannila *et al*, 2007), we did not observe such associations.

In agreement with Mannila *et al* (2006), we demonstrated associations between rs1049636 in the *FGG* gene and rs2070011 in the promoter area of the *FGA* gene in determining clot properties as well as a newly identified association between rs1800787 and clot properties. However, after adjusting for fibrinogen and fibrinogen γ' levels, respectively, these SNPs were no longer significantly associated with the clot properties, suggesting that the observed differences are probably the result of the difference in fibrinogen and fibrinogen γ' levels between genotypes. In support of this suggestion, the other SNPs analysed in this study, that were in LD with rs1049636 and rs2070011, also had no association with clot properties. Also for rs1800787 the observed increased maximum absorbance may be a result of increased fibrinogen

concentration, although this could not be conclusively proven, because of the small sample number of homozygous minor allele carriers.

Furthermore, a study by Lim *et al* (2003) showed, in stroke patients, that the effects of rs5985 and rs6050 on clot structure seem to be the result of a possible interaction between the SNPs and fibrinogen levels. However, in our study, no significant interactions were detected between rs5985 or rs6050 with total fibrinogen levels in determining clot properties. Instead, a significant interaction was demonstrated between rs5985 and fibrinogen γ' levels in determining clot density. Lim *et al* (2003) did not investigate fibrinogen γ' in their study. The presence of the minor allele significantly increased the positive association between clot density and absolute fibrinogen γ' concentration. These results suggest that the role of fibrinogen and fibrinogen γ' in vascular disease may be mediated by genetic components influencing protein functionality.

Furthermore, the different SNPs seem to influence the relationships between total and γ' fibrinogen levels with clot properties in opposing directions. For example, the presence of the minor allele of SNP rs2070011 resulted in a deterioration in the association between slope and the fibrinogen variables, while the association between slope and fibrinogen γ' was enhanced in the presence of the minor allele of others (rs1800787 and rs4220). The same is seen for CLT. The presence of the minor allele of SNP rs1800787 enhanced the effect of fibrinogen on CLT, while for SNP rs2227385, the effect was decreased. Also for fibrinogen γ', the presence of the minor allele of SNP rs1800788 enhanced the effect of fibrinogen γ' on CLT, but for SNP rs2227385, the association was decreased. These data suggest that increased fibrinogen concentration would not by default increase thrombosis risk, but, depending on the effect of SNPs on its functionality, may have either no effect or could even potentially decrease the risk attributed to/dependent on clot structure and lysis.

A limitation of the study is that we cannot exclude the possibility that the effects observed may reflect those of other functional SNPs not analysed, since some of the SNPs we investigated were haplotype-tagging SNPs. Only selected SNPs in the fibrinogen and Factor XIII genes were analysed and therefore the effects of SNPs outside these genes were not investigated.

In conclusion, the effect of individual SNPs in determining total and γ' fibrinogen levels as well as clot properties varied somewhat in our African population compared with what has been reported for white populations. This can possibly be attributed to the greater genetic variability in Africans, with other additional genes contributing to total and γ' fibrinogen variance. Since the genetic background of Africans is so diverse, it cannot be evaluated by simply using variants from other ethnicities. Although only a few individual SNPs contributed significantly to the variation in total and γ' fibrinogen levels and clot properties independently, interactions between the analysed SNPs and total or γ' fibrinogen levels revealed varying (and opposing) effects on clot properties. These data suggest that the influence of genetic factors on fibrinogen should not only focus on effects on concentration, but also on functionality in determining the role of total and γ' fibrinogen in CVD.

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Author contributions

RCK and CN interpreted the data and wrote the manuscript; ZDL interpreted the data and approved the final version of the manuscript; MPDM wrote the manuscript; TH analysed the data and approved the final version of the manuscript; MP analysed the data, interpreted the data and wrote the manuscript.

Disclosure

The authors have no conflict of interest to declare.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Pair-wise linkage disequilibrium structure presenting the D' (95% confidence bounds) and the r^2.

Table SI. Sequencing primers for the fibrinogen beta chain gene.

Table SII. Results of sequencing in 28 random subjects.

Table SIII. Effect of individual SNPs on fibrinogen γ', γ' ratio and total fibrinogen.

Table SIV. Effect of individual SNPs on clot properties.

References

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Table I. Basic descriptive characteristics of the study population

Variable	Total population (n=2010)	Men (n=749)	Women (n=1261)	p-value
Age (years)	48.3 (41.9-56.4)	48.8 (42.1-57.4)	47.9 (41.7-56.0)	0.13
HIV positive (%)	16.2	15.5	16.7	0.48
Blood pressure (mmHg)				
Systolic	134 ± 24.5	136 ± 24.0	132 ± 24.7	0.004
Diastolic	87.7 ± 14.5	86.9 ± 14.9	88.2 ± 14.3	0.043
Body mass index (kg/m^2)	23.0 (19.3-28.9)	19.8 (18.1-22.4)	26.0 (21.5-31.8)	<0.0001
Waist circumference (cm)	77.5 (70.2-87.7)	74.4 (69.9-81.3)	81.0 (70.6-91.3)	<0.0001
HbA1c (%)	5.50 (5.30-5.80)	5.50 (5.20-5.70)	5.60 (5.30-5.90)	<0.0001
Serum total cholesterol (mM)	5.01 ± 1.38	4.81 ± 1.33	5.13 ± 1.39	<0.0001
Serum HDL-cholesterol (mM)	1.52 ± 0.63	1.58 ± 0.66	1.48 ± 0.62	0.001
Serum LDL-cholesterol (mM)	2.92 ± 1.17	2.68 ± 1.16	3.04 ± 1.18	<0.0001
Serum triglycerides (mM)	1.08 (0.82-1.55)	0.98 (0.77-1.41)	1.14 (0.85-1.65)	0.0008
Serum IL-6 (pg/ml)	2.84 (0.75-5.76)	2.83 (0.75-6.30)	2.86 (0.75-5.54)	0.77
Serum CRP (mg/l)	3.29 (0.96-9.34)	2.50 (0.74-8.03)	3.69 (1.24-10.3)	0.0003
Plasma homocysteine (μM)	9.18 (7.45-12.1)	10.2 (8.30-13.2)	8.76 (7.09-11.2)	<0.0001
Plasma fibrinogen (g/l)	2.90 (2.30-5.00)	2.60 (2.10-3.70)	3.10 (2.30-5.50)	<0.0001
Plasma fibrinogen γ' (g/l)	0.31 (0.23-0.45)	0.28 (0.20-0.39)	0.34 (0.25-0.48)	<0.0001
γ' ratio (%)	10.2 (7.14-14.6)	9.77 (7.09-13.9)	10.3 (7.17-15.1)	0.049
Lag time (min)	6.46 ± 1.97	6.48 ± 2.04	6.45 ± 1.93	0.79
Slope	8.91 (6.48-12.0)	8.66 (6.38-11.9)	9.10 (6.50-12.0)	0.60
Maximum absorbance	0.43 ± 0.16	0.41 ± 0.16	0.44 ± 0.15	0.0005
Clot lysis time (min)	57.3 ± 11.2	52.9 ± 11.6	59.9 ± 10.2	<0.0001

Normally distributed data reported as: mean ± SD and non-parametric data as: median (25^{th}-75^{th} percentile). CRP, C-reactive protein; HbA1c, glycosylated haemoglobin; HIV, human immunodeficiency virus; HDL-cholesterol, high-density lipoprotein cholesterol; IL-6, Interleukin-6; LDL-cholesterol, low-density lipoprotein cholesterol; γ', gamma prime.

Table II. Genotype distributions and minor allele frequency (MAF) of investigated SNPs

SNP	Genotype	Genotype count	Genotype (%)	95% CI of SNP frequency (%)	MAF	HW (p-value)	LD
FGB -148 C>T (rs1800787)	CC	1397	88.3	86.7-89.9	0.06	0.41	
	CT	176	11.1	9.57-12.7			
	TT	9	0.60	0.20-0.94			
FGA 2224 G>A (rs2070011)	GG	1105	68.6	66.4-70.9	0.17	0.82	^
	GA	462	28.7	26.5-30.9			
	AA	43	2.70	1.88-3.46			
FGB Arg448Lys (rs4220)	GG	1367	84.6	82.9-86.4	0.08	0.87	*
	GA	236	14.6	12.9-16.3			
	AA	12	0.80	0.32-1.16			
FGG 10034 C>T (rs2066865)	CC	876	54.6	52.1-57.0	0.27	0.09	*
	CT	596	37.1	34.8-39.5			
	TT	133	8.30	6.94-9.64			
FGA 6534 A>G (rs6050)	AA	801	49.9	47.5-52.4	0.30	0.21	*
	AG	644	40.2	37.7-42.5			
	GG	159	9.90	8.45-11.4			
FGG 9340 T>C (rs1049636)	TT	1154	71.4	69.2-73.6	0.16	0.35	*
	TC	414	25.6	23.5-27.8			
	CC	48	3.00	2.14-3.80			
FGB 40 A>G (rs2227385)	AA	1773	94.7	93.6-95.7	0.03	0.49	#
	AG	100	5.3	4.32-6.36			
	GG	0	0	0			
FGB 749 A>G (rs2227388)	AA	1316	70.2	68.1-72.2	0.16	0.94	#@^
	AG	513	27.3	25.3-29.4			
	GG	47	2.50	1.80-3.21			
FGB 1038 G>A (rs1800791)	GG	1580	83.6	81.9-85.3	0.09	0.74	#
	GA	299	15.8	14.2-17.5			
	AA	11	0.60	0.24-0.93			
FGB 1643 C>T (rs1800788)	CC	1706	90.26	88.9-91.6	0.05	0.73	@
	CT	181	9.58	8.25-10.9			
	TT	3	0.16	0.00-0.43			
F13B His95Arg (rs6003)	AA	204	12.7	11.1-14.3	0.66	0.10	
	AG	682	42.4	40.0-44.9			
	GG	721	44.9	42.4-47.3			
F13A1 Val34Leu (rs5985)	CC	1165	73.5	71.3-75.7	0.14	0.98	
	CA	389	24.5	22.4-26.7			
	AA	31	1.96	1.27-2.64			

*^#@ Symbols indicate LD between SNPs. A, adenine; Arg, arginine; C, cytosine; CI, confidence interval; *F13A1*, Factor XIII subunit A gene; *F13B*, Factor XIII subunit B gene; *FGA*, fibrinogen alpha chain; *FGB*, fibrinogen beta chain; *FGG*, fibrinogen gamma chain; G, guanine; His, histidine; HW, Hardy-Weinberg equilibrium; LD, linkage disequilibrium; Leu, leucine; Lys, lysine; MAF, minor allele frequency; SNP, single nucleotide polymorphism; T, thymine; Val, valine.

Table III. Significant effects of individual SNPs on fibrinogen γ′, γ′ ratio and total fibrinogen

Genotype	Fibrinogen γ' (g/l)	γ' ratio	Fibrinogen (g/l)
rs2070011			
GG	0.30 (0.23-0.44) *	10.1 (7.14-14.7)	2.80 (2.30-5.00)
	(n=1021)	(n=987)	(n=1112)
GA	0.32 (0.23-0.46) ^{0}	10.3 (7.42-15.0)	2.90 (2.20-5.00)
	(n=428)	(n=405)	(n=422)
AA	0.39 (0.33-0.55) * ^{0}	11.1 (6.55-17.6)	3.70 (2.80-6.20)
	(n=40)	(n=36)	(n=37)
p-value	0.008	0.50	0.11
p-value after adjustment for fibrinogen γ'	-	0.52	0.52
rs1049636			
TT	0.31 (0.23-0.45)	10.3 (7.22-14.8)	2.80 (2.20-4.50) *
	(n=1058)	(n=1020)	(n=1069)
TC	0.31 (0.23-0.47)	10.3 (7.05-15.1)	3.00 (2.20-5.30) ^{0}
	(n=392)	(n=371)	(n=383)
CC	0.32 (0.22-0.57)	8.30 (5.67-13.6)	5.00 (2.80-6.25) * ^{0}
	(n=45)	(n=43)	(n=44)
p-value	0.61	0.08	0.0009
p-value after adjustment for fibrinogen	0.93	0.93	-
rs1800791			
GG	0.31 (0.23-0.45)	10.2 (7.21-14.5)	2.90 (2.20-5.00) *
	(n=1462)	(n=1402)	(n=1460)
GA	0.31 (0.23-0.46)	10.3 (7.11-15.1)	3.00 (2.30-5.00)
	(n=274)	(n=265)	(n=286)
AA	0.35 (0.28-0.50)	6.97 (5.39-10.2)	5.30 (2.90-6.40) *
	(n=11)	(n=11)	(n=11)
p-value	0.21	0.40	0.04
p-value after adjustment for fibrinogen	0.48	0.48	-

Data reported as median (25^{th}-75^{th} percentile). * ^{0} Means with the same symbol differ significantly. A, adenine; C, cytosine; G, guanine; SNP, single nucleotide polymorphism; T, thymine; γ', gamma prime.

Table IV. Significant effects of individual SNPs on clot properties

Genotype	Clot lysis time (minutes)	Lag time (minutes)	Slope	Maximum absorbance
rs1800787				
CC	57.3 [56.7-57.9] (n=1317)	6.36 [6.25-6.47] (n=1345)	9.83 [9.58-10.1] (n=1316)	0.43 [0.42-0.44] * (n=1318)
CT	56.2 [54.3-58.0] (n=165)	6.53 [6.23-6.83] (n=170)	9.93 [9.30-10.6] (n=168)	0.47 [0.44-0.50] * (n=165)
TT	57.3 [49.8-64.7] (n=9)	7.06 [5.51-8.60] (n=9)	9.76 [8.00-11.5] (n=9)	0.48 [0.42-0.54] (n=9)
p-value	0.27	0.16	0.82	0.001
rs2070011				
GG	57.4 [56.7-58.1] (n=1034)	6.43 [6.30-6.55] (n=1061)	9.69 [9.41-9.96] (n=1038)	0.43 [0.42-0.43] * (n=1035)
GA	56.9 [55.9-57.9] (n=440)	6.29 [6.11-6.47] (n=446)	10.2 [9.79-10.7] (n=438)	0.45 [0.43-0.46] * (n=440)
AA	59.1 [55.2-63.0] (n=43)	6.91 [6.34-7.48] (n=42)	9.11 [7.88-10.3] (n=42)	0.45 [0.41-0.50] (n=43)
p-value	0.93	0.90	0.22	0.01
p-value adjusted for fibrinogen γ'	0.97	0.81	0.21	0.06
rs1049636				
TT	57.3 [56.7-58.0] (n=1082)	6.37 [6.25-6.48] (n=1106)	9.76 [9.49-10.0] (n=1081)	0.43 [0.42-0.44] * (n=1083)
TC	57.2 [56.0-58.3] (n=394)	6.47 [6.28-6.67] (n=401)	9.93 [9.48-10.4] (n=395)	0.44 [0.43-0.46] (n=394)
CC	57.7 [54.3-61.0] (n=46)	6.58 [6.09-7.08] (n=48)	10.1 [8.74-11.5] (n=48)	0.48 [0.43-0.53] * (n=46)
p-value	0.94	0.27	0.42	0.01
p-value adjusted for fibrinogen	0.88	0.84	0.72	0.33
rs5985				
CC	57.0 [56.3-57.7] (n=1105)	6.31 [6.20-6.42] * (n=1130)	9.91 [9.65-10.2] (n=1110)	0.43 [0.42-0.44] (n=1105)
CA	57.8 [56.6-58.9] (n=360)	6.61 [6.39-6.84] * (n=366)	9.74 [9.23-10.2] (n=357)	0.44 [0.42-0.45] (n=361)
AA	58.8 [54.9-62.7] (n=28)	6.22 [5.61-6.83] (n=30)	8.67 [7.45-9.90] (n=28)	0.40 [0.32-0.48] (n=28)
p-value	0.39	0.04	0.30	0.55

Data reported as mean [95% confidence interval]. * Means with the same symbol differ significantly. A, adenine; C, cytosine; G, guanine; SNP, single nucleotide polymorphism; T, thymine; γ', gamma prime.

Table V. Gene-environment interactions for SNPs and fibrinogen variables on clot properties

Clot property	Interactions	Interaction p-value	N	Homozygous wild type Slope (95%CI)	N	Heterozygote Slope (95%CI)	N	Homozygous mutant Slope (95%CI)
Slope	rs2070011:							
	fibrinogen	0.01	974 (GG)	0.15 (0.13; 0.17)	399 (GA)	0.11 (0.08; 0.14)	36 (AA)	0.01 (-0.09; 0.12)
	fibrinogen γ'	0.08	994 (GG)	0.07 (0.05; 0.09)	416 (GA)	0.03 (-0.00; 0.07)	40 (AA)	-0.06 (-0.20; 0.08)
	γ' ratio	0.94	961 (GG)	-0.07 (-0.09; -0.05)	394 (GA)	-0.07 (-0.10; -0.04)	36 (AA)	-0.05 (-0.15; 0.05)
	rs1800787*:							
	fibrinogen	0.40	1222 (CC)	0.14 (0.12; 0.15)	163	0.11 (0.07; 0.16)		
	fibrinogen γ'	0.22	1260 (CC)	0.05 (0.03; 0.07)	165	0.09 (0.04; 0.13)		
	γ' ratio	0.003	1209 (CC)	-0.08 (-0.09; -0.06)	158	0.00 (-0.04; 0.05)		
	rs4220*:							
	fibrinogen	0.29	1189 (GG)	0.14 (0.12; 0.16)	225	0.11 (0.07; 0.16)		
	fibrinogen γ'	0.22	1229 (GG)	0.05 (0.03; 0.07)	226	0.08 (0.04; 0.13)		
	γ' ratio	0.01	1174 (GG)	-0.08 (-0.09; -0.06)	222	-0.02 (-0.06; 0.02)		
Max abs	rs5985:							
	fibrinogen	0.07	1029 (CC)	0.16 (0.14; 0.17)	335 (CA)	0.16 (0.13; 0.18)	25 (AA)	0.26 (0.16; 0.36)
	fibrinogen γ'	0.004	1051 (CC)	0.11 (0.09; 0.13)	344 (CA)	0.10 (0.06; 0.13)	27 (AA)	0.29 (0.16; 0.41)
	γ' ratio	0.37	1013 (CC)	-0.05 (-0.06; -0.03)	327 (CA)	-0.07 (-0.10; -0.04)	25 (AA)	-0.05 (-0.18; 0.08)

CLT	rs2227385:							
	fibrinogen	0.0003	1557 (AA)	3.85 (2.85; 4.86)	84 (AG)	-4.51 (-8.97;-0.05)	0 (GG)	-
	fibrinogen γ'	0.005	1594 (AA)	7.69 (6.72; 8.65)	89 (AG)	1.80 (-2.77; 6.37)	0 (GG)	-
	γ' ratio	0.37	1533 (AA)	3.20 (2.26; 4.14)	84 (AG)	5.03 (1.12; 8.94)	0 (GG)	-
	rs1800787*:							
	fibrinogen	0.01	1223 (CC)	2.68 (1.55; 3.80)	160	6.47 (3.34; 9.60)		
	fibrinogen γ'	0.14	1256 (CC)	7.10 (5.97; 8.24)	162	9.31 (6.55; 12.1)		
	γ' ratio	0.61	1205 (CC)	3.51 (2.45; 4.57)	155	2.74 (-0.30; 5.78)		
	rs1800788*:							
	fibrinogen	0.26	1500 (CC)	3.21 (2.20; 4.23)	156	5.18 (1.77; 8.59)		
	fibrinogen γ'	0.01	1534 (CC)	6.99 (6.01; 7.98)	165	11.3 (8.04; 14.5)		
	γ' ratio	0.19	1479 (CC)	3.14 (2.20; 4.08)	153	5.44 (1.94; 8.94)		

*Due to small sample numbers in the homozygous-mutant group, heterozygous and homozygous-mutant groups were combined. CLT, clot lysis time; Max abs, maximum absorbance; SNP, single nucleotide polymorphism; γ',gamma prime.

Online Supplemental material

Filename	Format	Size	Description
Bjh_TabSI-SIV_FigS1.SuppInfo.doc	Word document	214K	**Table SI.** Sequencing primers for the fibrinogen beta chain gene.
			Table SII. Results of sequencing in 28 random subjects.
			Table SIII. Effect of individual SNPs on fibrinogen γ', γ' ratio and total fibrinogen.
			Table SIV. Effect of individual SNPs on clot properties.
			Fig S1. Pair-wise linkage disequilibrium structure presenting the D' (95% confidence bounds) and the r^2.

Table SI. Sequencing primers for the fibrinogen beta chain gene

Primer name	Sequence 5'-3'	Annealing temperature
*FGB*_01_F	5' - GGT CAG AAG GAG GAT GGA TT - 3'	59°C
*FGB*_01_R	5' - CGC CTA CTA TGT CTG TCT TGC - 3'	59°C
*FGB*_08_F	5' - GAA TTT GGG AGG ACA ACC TA - 3'	62°C
*FGB*_08_R	5' - AAA GAT GGC AGG TTA TCA GG - 3'	62°C
*FGB*_11_F	5' - TGG ATT ACT GAC TGG TGT TCC - 3'	59°C
*FGB*_11_R	5' - TGT TAT TTT AAC ACT GGT GAA GTC T - 3'	59°C
*FGB*_13_F	5' - TCG GAG CTT GTG TAG TTT CC - 3'	59°C
*FGB*_13_R	5' - TCA TAC AAC TAT TAT TCT TTG TTG GTC - 3'	59°C

A, adenine; C, cytosine; F, forward; *FGB*, fibrinogen beta chain; G, guanine; R, reverse; T, thymine.

Table SII. Results of sequencing in 28 random subjects

Position Seattle	Common names	Rs number	SNP	Genotype (n)		TagSNP
40		rs2227385	A>G	A/G	(2)	Yes
333-334*			A Insertion	-/I	(1)	
354*			C>T	C/T	(1)	
432/433*			A Insertion	-/I	(1)	
432/433*			A Deletion	-/D	(1)	
472	-1420G>A, -1428G>A	rs1800789	G>A	A/G	(1)	
675		rs2227387	C>T	C/T	(1)	
749		rs2227388	A>G	A/G G/G	(11) (2)	Yes
938*			C>G	C/G	(1)	
899	-993C>T -1001C>T	rs2227389	T>C	C/T	(1)	
1038	-854G/A; -862G>A	rs1800791	G>A	G/A	(1)	Yes
1233*			C>T	C/T	(1)	
1643	-249C>T; -257C>T	rs1800788	C>T	C/T	(2)	Yes
1744	-148C>T, HindIII RFLP; Alu RFLP; -156C>T	rs1800787	C>T	C/T	(1)	

* Not reported in Seattle database. A, adenine; C, cytosine; I, insertion; D, deletion; G, guanine; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; T, thymine.

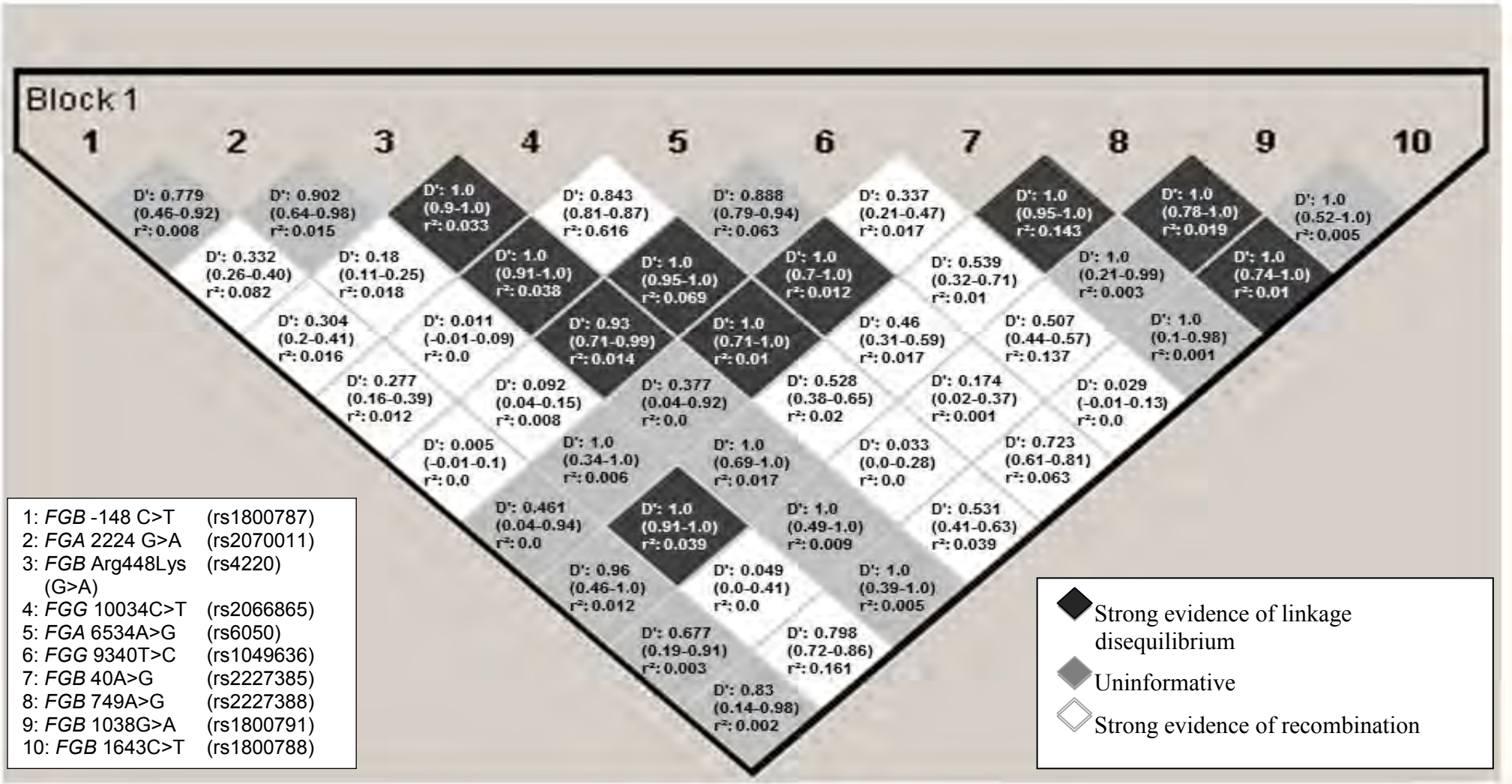


Fig S1. Pair-wise linkage disequilibrium structure presenting the D' (95% confidence bounds) and the r^2

Table SIII. Effect of individual SNPs on fibrinogen γ', γ' ratio and total fibrinogen

Genotype	Fibrinogen γ' (g/l)	γ' ratio	Fibrinogen (g/l)
rs1800787			
CC	0.31 (0.23-0.44)	10.3 (7.26-14.7)	2.90 (2.20-4.80)
	(n=1297)	(n=1244)	(n=1294)
CT	0.33 (0.22-0.48)	9.50 (6.27-14.6)	2.90 (2.20-5.90)
	(n=159)	(n=152)	(n=161)
TT	0.40 (0.31-0.46)	10.3 (5.76-16.6)	5.15 (2.75-6.15)
	(n=8)	(n=8)	(n=8)
p-value	0.43	0.52	0.13
rs4220			
GG	0.31 (0.23-0.45)	10.3 (7.21-14.8)	2.90 (2.20-5.00)
	(n=1263)	(n=1207)	(n=1256)
GA	0.30 (0.22-0.45)	9.95 (7.03-14.7)	2.90 (2.20-5.00)
	(n=219)	(n=214)	(n=227)
AA	0.33 (0.22-0.46)	10.0 (8.64-14.3)	3.10 (2.35-4.05)
	(n=12)	(n=12)	(n=12)
p-value	0.85	0.86	0.97
rs2066865			
CC	0.31 (0.22-0.45)	10.3 (7.04 - 14.8)	2.90 (2.30 – 5.00)
	(n=813)	(N=783)	(N=816)
CT	0.32 (0.24-0.46)	10.3 (7.39-14.3)	2.80 (2.20-5.30)
	(n=551)	(n=522)	(n=619)
TT	0.32 (0.23-0.43)	9.34 (7.58-15.2)	2.90 (2.20-4.50)
	(n=120)	(n=118)	(n=122)
p-value	0.18	0.62	0.92
rs6050			
AA	0.31 (0.22-0.46)	10.3 (7.04-15.03)	2.90 (2.20-5.30)
	(n=742)	(n=712)	(n=742)
AG	0.30 (0.24-0.45)	10.3 (7.46-14.5)	2.80 (2.20 4.50)
	(n=594)	(n=566)	(n=594)
GG	0.32 (0.23-0.44)	9.41 (7.32-15.1)	3.00 (2.40-5.00)
	(n=147)	(n=144)	(n=148)
p-value	0.77	0.80	0.66
rs2227385			
AA	0.31 (0.23-0.45)	10.2 (7.21-14.6)	2.90 (2.20-5.00)
	(n=1638)	(n=1575)	(n=1651)
AG	0.32 (0.22-0.48)	10.3 (6.38-15.4)	2.90 (2.40-5.00)
	(n=92)	(n=87)	(n=90)
GG	(n=0)	(n=0)	(n=0)

p-value	0.87	0.91	0.89
rs2227388			
AA	0.31 (0.23-0.46) (n=1220)	10.1 (7.16-14.6) (n=1167)	2.90 (2.20-5.30) (n=1221)
AG	0.31 (0.22-0.44) (n=470)	10.4 (7.22-14.9) (n=455)	2.90 (2.30-4.80) (n=479)
GG	0.27 (0.19-0.42) (n=44)	9.22 (6.30-12.5) (n=43)	2.75 (2.30-5.65) (n=44)
p-value	0.32	0.30	0.58
rs1800788			
CC	0.31 (0.23-0.46) (n=1577)	10.2 (7.17-14.7) (n=1520)	2.90 (2.30-5.00) (n=1590)
CT	0.32 (0.22-0.41) (n=167)	10.1 (6.59-13.5) (n=155)	2.95 (2.10-4.65) (n=164)
TT	0.39 (0.31-0.78) (n=3)	10.9 (9.69-11.1) (n=3)	3.60 (2.80-8.00) (n=3)
p-value	0.21	0.93	0.46
rs6003			
AA	0.31 (0.24-0.46) (n=188)	10.4 (7.17-15.5) (n=184)	3.00 (2.20-5.40) (n=195)
AG	0.31 (0.22-0.45) (n=628)	10.1 (7.24-14.8) (n=604)	2.80 (2.20-4.80) (n=631)
GG	0.32 (0.23-0.46) (n=640)	10.2 (7.13-14.7) (n=640)	2.90 (2.25-5.00) (n=664)
p- value	0.57	0.81	0.94
rs5985			
CC	0.31 (0.23-0.45) (n=1082)	10.3 (7.26-14.9) (n=1042)	2.90 (2.30-5.00) (n=1081)
CA	0.30 (0.23-0.44) (n=357)	9.86 (7.09-14.6) (n=340)	2.80 (2.10-5.00) (n=360)
AA	0.28 (0.24-0.39) (n=29)	9.29 (5.46-14.8) (n=27)	3.00 (2.15-4.65) (n=28)
p-value	0.71	0.89	0.84

Data reported as median (25^{th}-75^{th} percentile). A, adenine; C, cytosine; G, guanine; SNP, single nucleotide polymorphism; T, thymine; γ', gamma prime.

Table SIV. Effect of individual SNPs on clot properties

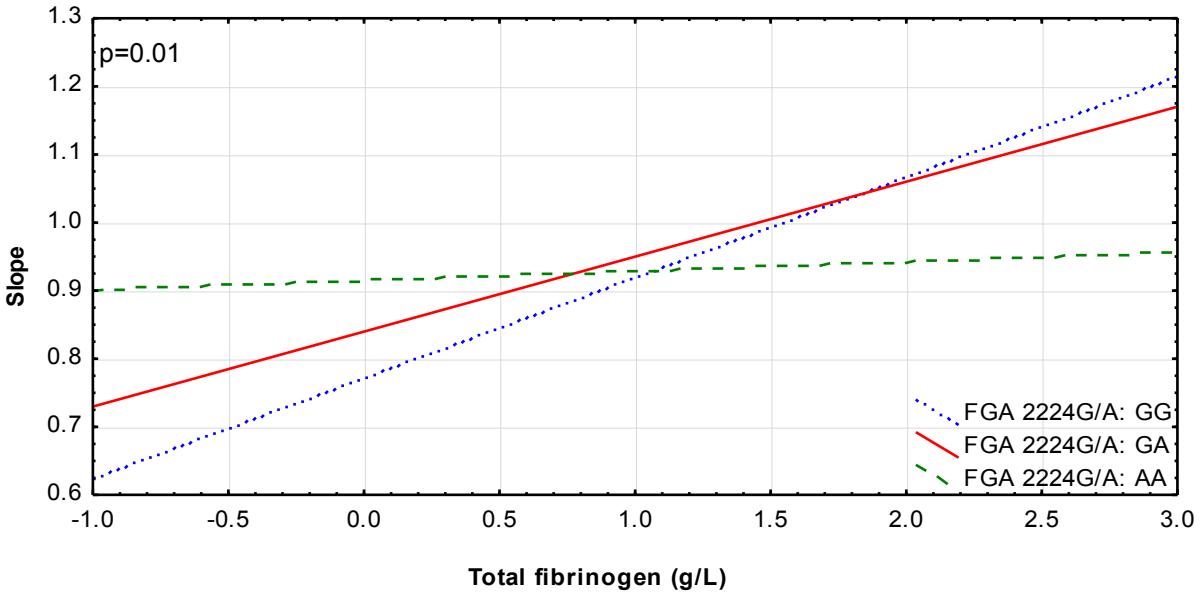
Genotype	Clot lysis time	Lag time	Slope	Maximum absorbance
rs4220				
GG	57.1 [56.5-57.8] (n=1289)	6.42 [6.31-6.52] (n=1317)	9.86 [9.61-10.1] (n=1290)	0.43 [0.43-0.44] (n=1290)
GA	57.9 [56.4-59.3] (n=220)	6.28 [6.01-6.55] (n=225)	9.66 [9.08-10.2] (n=221)	0.43 [0.40-0.45] (n=220)
AA	62.4 [56.5-68.3] (n=12)	6.84 [5.85-7.82] (n=12)	9.00 [5.78-12.2] (n=12)	0.42 [0.31-0.54] (n=12)
p-value	0.19	0.48	0.63	0.76
rs2066865				
CC	57.1 [56.4-57.9] (n=569)	6.33 [6.19-6.46] (n=842)	9.80 [9.49-10.1] (n=821)	0.43 [0.42-0.44] (n=813)
CT	57.5 [56.6-58.5] (n=569)	6.51 [6.35-6.68] (n=572)	9.78 [9.42-10.1] (n=565)	0.44 [0.42-0.45] (n=570)
TT	56.7 [54.7-58.7] (n=130)	6.39 [6.07-6.72] (n=131)	10.3 [9.46-11.1] (n=129)	0.43 [0.41-0.46] (n=130)
p-value	0.69	0.23	0.51	0.74
rs6050				
AA	57.2 [56.4-58.0] (n=747)	6.35 [6.21-6.49] (n=771)	9.73 [9.41-10.0] (n=752)	0.43 [0.42-0.44] (n=747)
AG	57.6 [56.7-58.6] (n=608)	6.46 [6.30-6.62] (n=616)	9.79 [9.43-10.2] (n=605)	0.43 [0.42-0.44] (n=609)
GG	56.6 [54.7-58.5] (n=155)	6.39 [6.10-6.68] (n=156)	10.3 [9.57-11.1] (n=155)	0.44 [0.42-0.47] (n=155)
p-value	0.55	0.58	0.29	0.64
rs2227385				
AA	57.3 [56.8-57.9] (n=1670)	6.45 [6.35-6.54] (n=1699)	9.70 [9.49-9.92] (n=1670)	0.43 [0.42-0.44] (n=1671)
AG	56.5 [54.1-59.0] (n=92)	6.66 [6.25-7.07] (n=95)	9.62 [8.72-10.5] (n=93)	0.44 [0.41-0.47] (n=92)
GG	(n=0)	(n=0)	(n=0)	(n=0)
p-value	0.50	0.30	0.76	0.63
rs2227388				
AA	57.3 [56.7-57.9] (n=1243)	6.45 [6.34-6.56] (n=1259)	9.73 [9.48-9.97] (n=1242)	0.43 [0.43-0.44] (n=1244)
AG	57.3 [56.2-58.4] (n=477)	6.52 [6.34-6.70] (n=492)	9.64 [9.23-10.0] (n=479)	0.42 [0.41-0.44] (n=477)
GG	55.0 [51.3-58.7] (n=44)	6.36 [5.83-6.89] (n=46)	9.62 [8.59-10.7] (n=45)	0.42 [0.39-0.46] (n=44)

p-value	0.41	0.76	0.69	0.44
rs1800791				
GG	57.3 [56.8-57.9] (n=1489)	6.46 [6.37-6.56] (n=1517)	9.68 [9.45-9.90] (n=1491)	0.43 [0.42-0.44)] (n=1490)
GA	57.0 [55.6-58.4] (n=278)	6.45 [6.21-6.68] (n=283)	9.85 [9.31-10.4] (n=278)	0.43 [0.41-0.45] (n=278)
AA	55.6 [51.3-59.9] (n=11)	7.04 [6.10-7.98] (n=11)	10.6 [8.06-13.2] (n=11)	0.49 [0.38-0.60] (n=11)
p-value	0.777205	0.619418	0.585311	0.467892
p-value after adjustment for fibrinogen	0.50	0.69	0.62	0.93
rs1800788				
CC	57.3 [56.8-57.9] (n=1605)	6.48 [6.38-6.57] (n=1635)	9.68 [9.46-9.90] (n=1607)	0.43 [0.42-0.44] (n=1606)
CT	56.8 [55.0-58.5] (n=170)	6.31 [6.04-6.58] (n=173)	10.0 [9.36-10.7] (n=170)	0.44 [0.42-0.47] (n=170)
TT	70.7 [32.4-109] (n=3)	7.63 [7.27-7.99] (n=3)	7.31 [2.84-11.8] (n=3)	0.44 [0.25-0.63] (n=3)
p-value	0.10	0.34	0.28	0.65
rs6003				
AA	56.5 [54.9-58.2] (n=190)	6.29 [6.00-6.59] (n=195)	10.0 [9.32-10.7] (n=190)	0.45 [0.42-0.48] (n=190)
AG	57.3 [56.5-58.1] (n=634)	6.39 [6.24-6.54] (n=648)	9.75 [9.42-10.1] (n=638)	0.43 [0.42-0.44] (n=634)
GG	57.5 [56.6-58.4] (n=690)	6.44 [6.29-6.59] (n=704)	9.81 [9.47-10.2] (n=688)	0.43 [0.42-0.44] (n=690)
p-value	0.60	0.66	0.75	0.33

Data reported as mean [95% confidence interval]. A, adenine; C, cytosine; G, guanine; SNP, single nucleotide polymorphism; T, thymine.

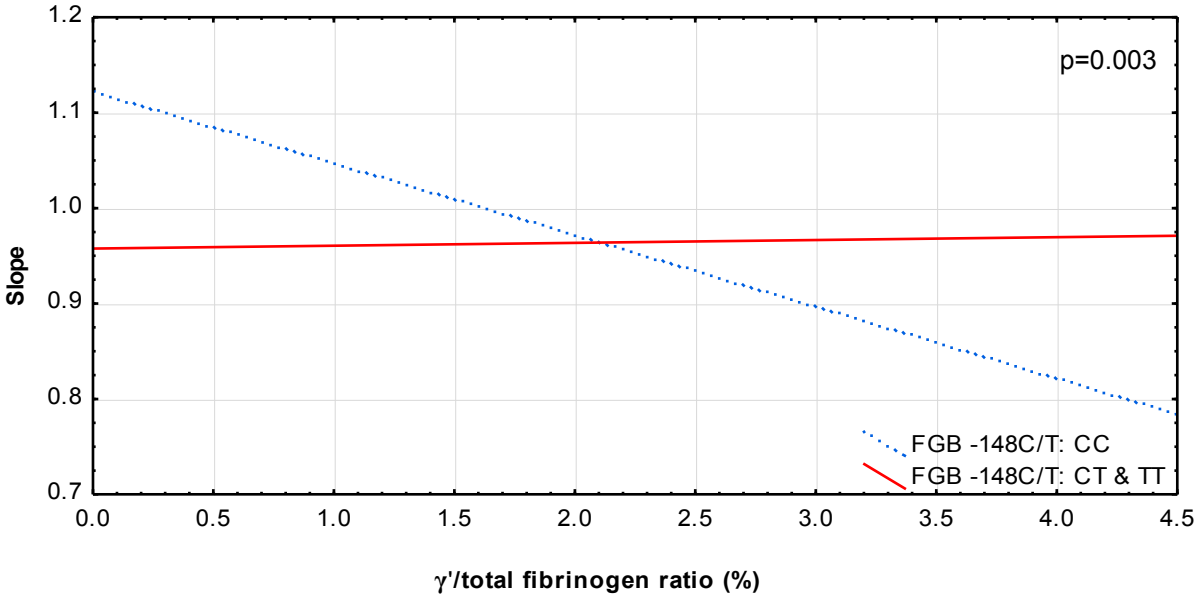
Additional Supplemental information

The graphs below depict the interactions between SNPs and total or γ' fibrinogen concentration in determining clot properties. The different lines in each graph represent the different genotypes of a particular SNP. For graphs where only two lines are presented, the homozygous minor allele group was combined with the heterozygous group due to the small sample number in the homozygous minor allele group.



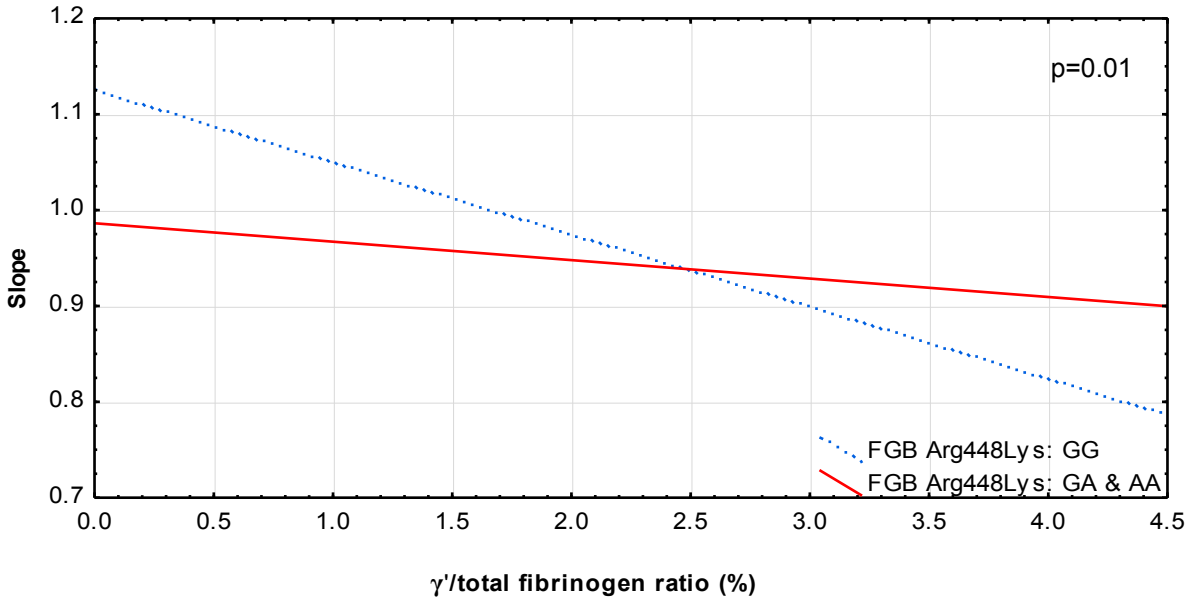
FGA 2224 G>A (rs2070011):
GG with total fibrinogen: y=0.7705+0.1474*x; r=0.4099, p=0.0000; r^2=0.1680
GA with total fibrinogen: y=0.8398+0.1101*x; r=0.3134, p=0.0000; r^2=0.0982
AA with total fibrinogen: y=0.9136+0.0139*x; r=0.0441, p=0.7986; r^2=0.0019

Additional Supplemental Figure 1: Interaction effect between FGA 2224G/A genotypes and total fibrinogen on slope



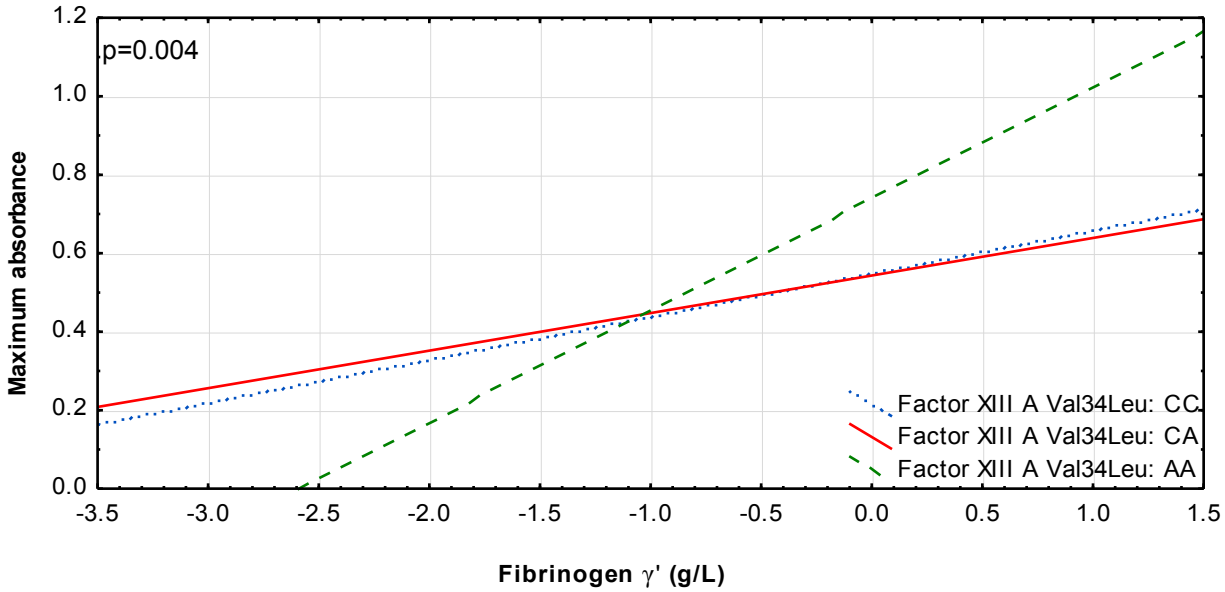
FGB -148C/T (rs1800787):
CC with γ'/total fibrinogen ratio: y=1.123-0.0753*x; r=-0.2214, p=0.0000; r^2=0.0490
CT & TT with γ'/total fibrinogen ratio: y=0.9576+0.003*x; r=0.0107, p=0.8939; r^2=0.0001

Additional Supplemental Figure 2: Interaction effect between FGB -148C/T genotypes and γ'/total fibrinogen ratio on slope



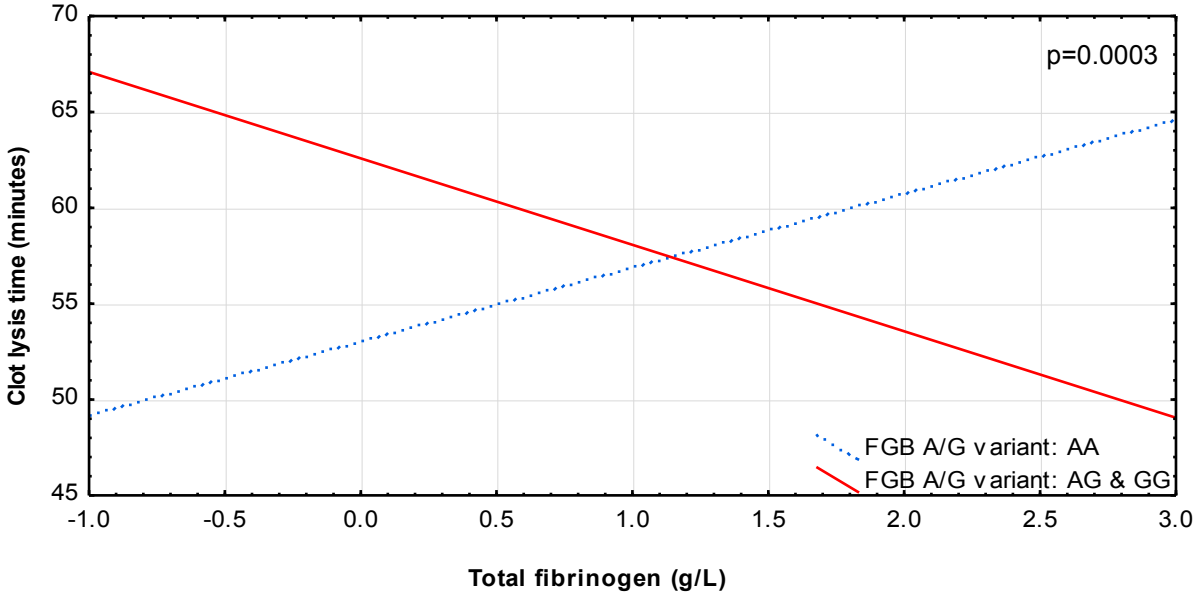
FGB Arg448Lys (rs4220):
GG with γ'/total fibrinogen ratio: y =1.1262-0.0758*x; r=-0.2278, p=0.0000; r^2=0.0519
GA & AA with γ'/total fibrinogen ratio: y=0.9863-0.0192*x; r=-0.0596, p=0.3771; r^2=0.0035

Additional Supplemental Figure 3: Interaction effect between FGB Arg448Lys genotypes and γ'/total fibrinogen ratio on slope



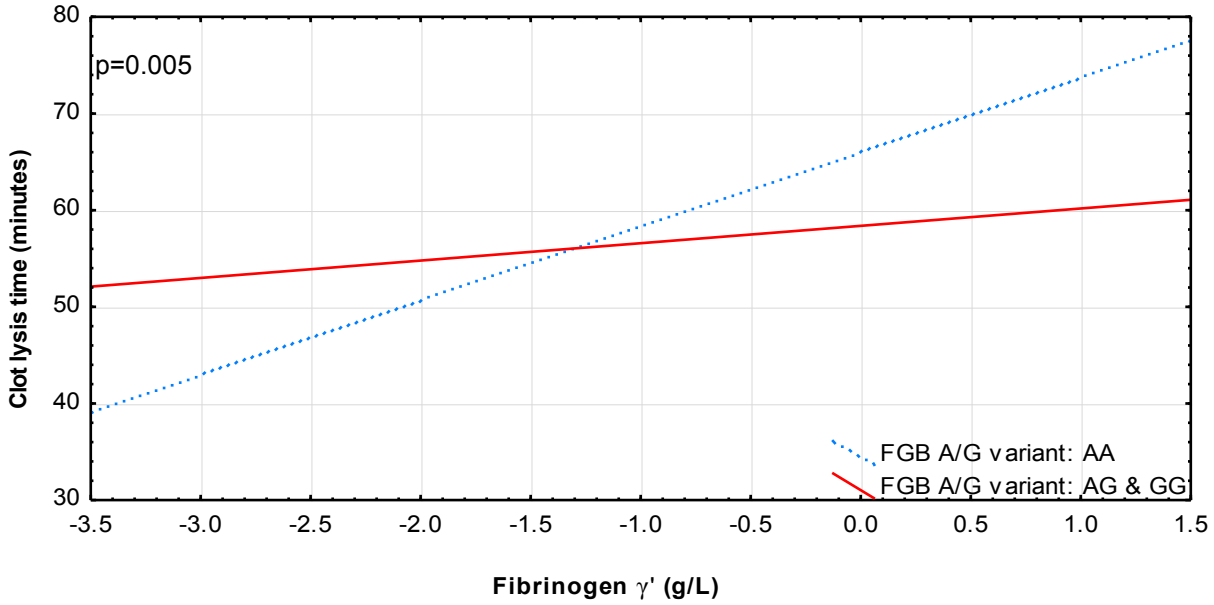
Factor XIII A Val34Leu (rs5985):
CC with fibrinogen γ': y=0.549+0.1096*x; r=0.3852, p=0.0000; r^2=0.1484
CA with fibrinogen γ': y=0.5434+0.0957*x; r=0.3103, p=0.00000; r^2=0.0963
AA with fibrinogen γ': y=0.7385+0.2853*x; r=0.6714, p=0.0001; r^2=0.4508

Additional Supplemental Figure 4: Interaction effect between Factor XIII A Val34Leu genotypes and fibrinogen γ' on maximum absorbance



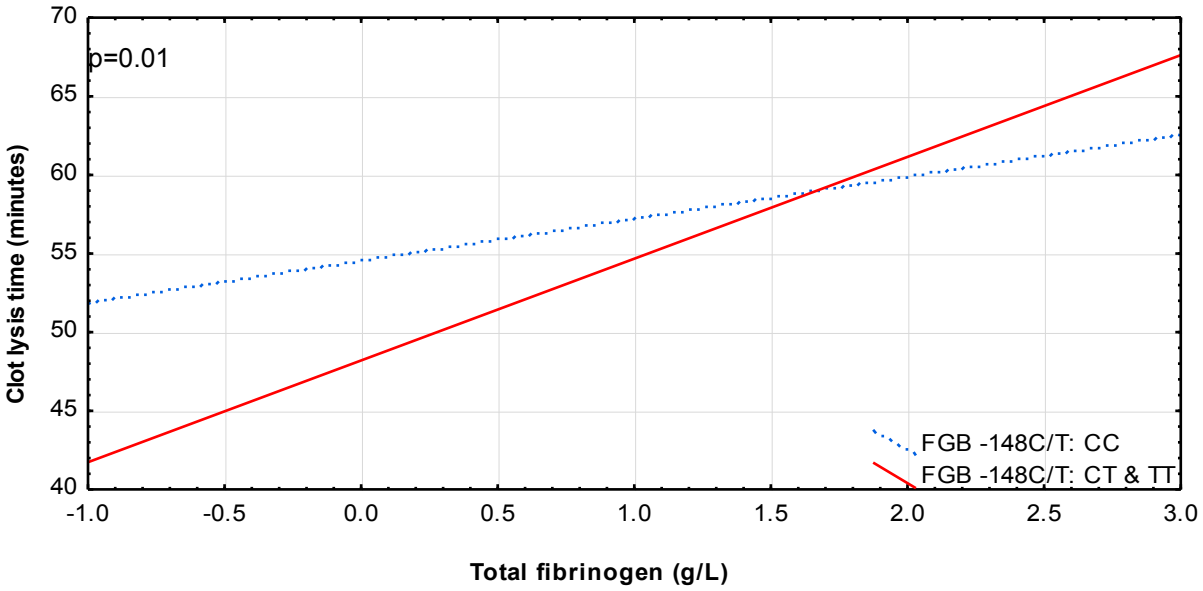
FGB A/G variant at rs2227385:
AA with total fibrinogen: y=53.0233+3.854*x; r=0.1877, p=0.0000; r^2=0.0352
AG & GG with total fibrinogen: y=62.5808-4.5098*x; r=-0.2137, p=0.0510; r^2=0.0457

Additional Supplemental Figure 5: Interaction effect between FGB A/G variant at rs2227385 genotypes and total fibrinogen on clot lysis time



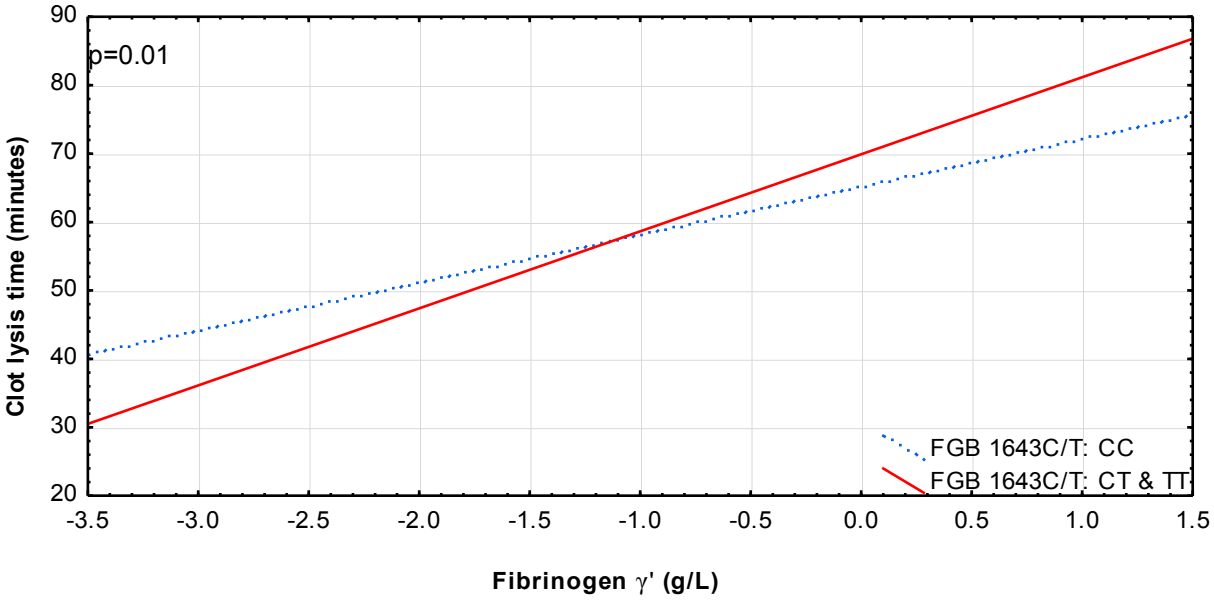
FGB A/G variant at rs2227385:
AA with fibrinogen γ': y=65.8867+7.6871*x; r=0.3641, p=0.0000; r^2=0.1326
AG & GG with fibrinogen γ': y=58.3843+1.7981*x; r=0.0824, p=0.4428; r^2=0.0068

Additional Supplemental Figure 6: Interaction effect between FGB A/G variant at rs2227385 genotypes and fibrinogen γ' on clot lysis time



FGB -148C/T (rs1800787):
CC with total fibrinogen: y=54.4732+2.6809*x; r=0.1322, p=0.00000; r^2=0.0175
CT & TT with total fibrinogen: y=48.2028+6.4715*x; r=0.3070, p=0.00008; r^2=0.0943

Additional Supplemental Figure 7: Interaction effect between FGB -148C/T genotypes and total fibrinogen on clot lysis time



FGB 1643C/T (rs1800788):
CC with fibrinogen γ': y=65.0215+6.9949*x; r=0.3349, p=0.0000; r^2=0.1121
CT & TT with fibrinogen γ': y=69.9065+11.2603*x; r=0.4726, p=0.0000; r^2=0.2233

Additional Supplemental Figure 8: Interaction effect between FGB 1643C/T genotypes and fibrinogen γ' on clot lysis time

CHAPTER 5:

CVD risk factors are related to plasma fibrin clot properties independent of total and or γ' fibrinogen concentration

Authors: Retha C.M. Kotzé, Robert A.S. Ariëns, Zelda de Lange and Marlien Pieters

This chapter includes:

- author instructions of the journal, *Thrombosis Research* (Impact factor 2.4)
- letter of acceptance of manuscript from *Thrombosis Research* (Aug 2014)
- the article titled "CVD risk factors are related to plasma fibrin clot properties independent of total and or γ' fibrinogen concentration", accepted for publication (Thromb Res. 2014 Aug 29. pii: S0049-3848(14)00454-X. doi: 10.1016/j.thromres.2014.08.018. [Epub ahead of print])

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[1] Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J Sci Commun 2010;163:51–9.
Reference to a book:
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ARTICLE

CVD risk factors are related to plasma fibrin clot properties independent of total and or γ' fibrinogen concentration

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Abstract

Introduction: Cardiovascular disease (CVD) risk factors are associated with total fibrinogen concentration and/or altered clot structure. It is unclear, however, whether such associations with clot structure can be ascribed to fibrinogen concentration or other independent mechanisms. We aimed to determine, firstly, whether CVD risk factors associated with increased total and/or γ' fibrinogen concentration were also associated with altered fibrin clot properties and, secondly, whether such associations were due to the fibrinogen concentration or to independent mechanisms.

Materials and methods: In a plasma setting, CVD risk factors (including total and γ' fibrinogen concentration) were cross-sectionally analysed in 2010 apparently healthy black South African participants. Kinetics of clot formation (lag time, slope and maximum absorbance) and clot lysis times were calculated from turbidity curves.

Results: Of the measured CVD risk factors, age, metabolic syndrome, C-reactive protein (CRP), high-density lipoprotein (HDL) cholesterol and homocysteine were significantly associated with altered fibrin clot properties after adjustment for total and or γ' fibrinogen concentration. Ageing was associated with thicker fibres (p=0.004) while both metabolic syndrome and low HDL-cholesterol levels were associated with lower rates of lateral aggregation (slope), (p=0.0004 and p=0.0009), and the formation of thinner fibres (p=0.007 and p=0.0004). Elevated CRP was associated with increased rates of lateral aggregation (p=0.002) and, consequently, thicker fibres (p<0.0001). Hyperhomocysteinaemia was associated with increased rates of lateral aggregation (p=0.0007) without affecting fibre thickness.

Conclusion: Final clot structure may contribute to increased CVD risk *in vivo* through associations with other CVD risk factors independently of total or γ' fibrinogen concentration.

Keywords: CVD risk factors, fibrin clot structure, fibrinogen, plasma

Abbreviations:

CVD, cardiovascular disease; CRP, C-reactive protein; HDL, high-density lipoprotein cholesterol; CLT, clot lysis time; γ', gamma prime; BMI, body mass index; MetS, metabolic syndrome; HbA1c, glycosylated haemoglobin; PURE, Prospective Urban and Rural Epidemiological; Hcy, homocysteine; $PAI\text{-}1_{act}$, plasminogen activator inhibitor-1 activity; hs-CRP, high-sensitivity CRP; tPA, tissue plasminogen activator; TF, tissue factor; CI, confidence interval; ANOVA, analysis of variance; ANCOVA, analysis of co-variance; IL-6, interleukin-6; WC, waist circumference; EC, endothelial cell; HTL, homocysteine thiolactone; Fbg, fibrinogen.

Introduction

Thrombosis, or fibrin clot formation obstructing critically situated blood vessels that cause loss of blood flow to vital organs, is considered the immediate underlying cause of CVD events [1]. Both arterial and venous CVD have been shown to be accompanied by altered clot structure [2-5]. Furthermore, the architecture of such a fibrin fibre network is important as it affects clot stability, *e.g.* viscoelastic or mechanical properties and fibrinolytic characteristics [6,7]. This eventually determines whether a clot will be lysed successfully, become occlusive, or embolise [6,8]. In particular, a clot structure which consists of tightly packed, thin fibres and which is characterised by reduced permeability and prolonged clot lysis time (CLT), has been associated with CVD events [9] such as stroke [3], myocardial infarction [2], acute coronary syndrome [10] and venous thromboembolism [4].

It is also known that clot structure can be influenced by several factors, such as plasma concentration of albumin [11], prothrombin [12], polyphosphates [13] and Factor XIIa [14]; alterations of pH and ionic strength [15]; cross-linking by Factor XIIIa [16,17]; and plasma total fibrinogen and fibrinogen gamma prime (γ') levels [18]. These factors influence not only the final clot structure but also the kinetics of clot formation [19,20]. Clot structure is to a large extent kinetically controlled and therefore information on clot formation will aid in the understanding of the final clot structure [21-23]. Increased fibrinogen levels have previously been associated with a shorter lag phase (associated with a tighter fibrin network) [19,21], an increased rate of lateral aggregation (slope) and increased maximum absorbance (indicative of thicker fibres) [19-21]. Fibrinogen γ' is associated with a heterogeneous, non-uniform clot structure with prolonged CLT [24,25]. Furthermore, total and γ' fibrinogen have been found to be associated with several known CVD risk factors in this African population [20,26]. Both total and γ' fibrinogen were associated with gender (increased levels in women), abdominal obesity, body mass index (BMI), metabolic syndrome (MetS), glycosylated haemoglobin (HbA1c), CRP and HDL-cholesterol (negatively) [20].

Fibrinogen is thought to contribute to CVD not only through altering the fibrin network structure, but also through several other mechanisms, such as increasing platelet

aggregation, plasma viscosity and red blood cell aggregation; compromising vascular or endothelial layer function and integrity and participating in the inflammatory process [27-32].

The main purpose of this study was to determine whether the CVD risk factors associated with increased total and/or γ' fibrinogen concentration were also associated with altered fibrin clot properties. The second aim was to investigate whether such associations were due to the fibrinogen concentration itself or whether the CVD risk factors influenced clot properties independently.

Materials and methods

Study population

Participants in the study were from the South African arm of the international Prospective Urban and Rural Epidemiological (PURE) study, which is a large cohort study tracking changing lifestyles, risk factors and chronic diseases over 10 years by using periodic standardised data collection in rural and urban areas of 21 countries in transition. Data reported in this study were from the baseline data of 2010 apparently healthy participants randomly selected from well-established rural (n = 1006, living under tribal law) and urban (n = 1004, living in informal and formal settlements surrounding cities) study sites in the North West Province of South Africa, collected over a twelve-week period in 2005. Details regarding the selection process and randomisation have been reported elsewhere [1,26]. Inclusion criteria were apparently healthy black South African men and women between the ages of 35 and 65 years. Exclusion criteria were the use of chronic medication for non-communicable diseases and/or any self-reported acute illness. Written informed consent was obtained from participants prior to commencement of the study. The study was approved by the Ethics Committee of the North-West University, South Africa.

Blood collection

Fasting blood samples were collected with minimum stasis from the antecubital veins of participants between 07:00 and 11:00 by qualified nurses, using sterile winged

infusion sets and syringes. Tubes without anticoagulant were used to collect serum for the analysis of lipids and CRP. To analyse homocysteine (Hcy) and HbA1c, blood samples were collected in EDTA tubes and, for glucose measurements, in fluoride tubes. Citrated tubes were used for the determination of plasminogen activator inhibitor-1 activity ($PAI\text{-}1_{act}$), CLT and total and γ' fibrinogen, as well as the turbidimetric measurements of clot formation. Samples were centrifuged at 2000 x g for 15 minutes at 10°C within 30 minutes of collection. Aliquots were frozen on dry ice, stored in the field at -18°C and then, after 2–4 days, at -82°C until analysis.

Laboratory analysis

The methods for lipids, high-sensitivity CRP (hs-CRP), Hcy, $PAI\text{-}1_{act}$, HbA1c and plasma glucose have been described previously [1,26]. Fibrinogen was measured by a modified Clauss method (Multifibrin U-test, BCS coagulation analyser, Dade Behring, Deerfield, IL, USA). The analysis of fibrinogen γ' was performed with an ELISA, using a 2.G2.H9 mouse monoclonal coating antibody against the human γ' sequence from Santa Cruz Biotechnology (Santa Cruz, USA) for antigen capture and a goat polyclonal horseradish peroxidise-conjugated antibody against human fibrinogen from Abcam for development (Cambridge, USA) [20,33]. Plasma fibrinolytic potential was determined by turbidimetric analysis (A405 nm), according to the method of Lisman et al. [34], and validated by Talens et al. [35]. Tissue-factor-induced plasma clots lysed by exogenous tissue plasminogen activator (tPA) were used, with slightly modified tissue factor (TF) and tPA concentrations in order to obtain comparable CLTs of about 60 min (intra-assay CV = 3.6%, between plate CV= 4.5%). The final concentrations used were as follows: TF (125x diluted – an estimated final concentration of 59 pM [36]; Dade Innovin, Siemens Healthcare Diagnostics Inc., Marburg, Germany), $CaCl_2$ (17 mmol/l), tPA (100 ng/ml; Actilyse, Boehringer Ingelheim, Ingelheim, Germany) and phospholipid vesicles (10 µmol/l; Rossix, Mölndal, Sweden). Clot lysis time was defined as the time from the midpoint of the transition from baseline to maximum turbidity, which is representative of clot formation, to the midpoint in transition from maximum turbidity back to baseline, which represents the lysis of the clot [34]. Kinetics of clot formation, *i.e.* lag time, slope, and maximum absorbance, were additionally calculated from these curves. Since plasma was clotted with TF, the lag time included the time required for

activation of the coagulation cascade as well as the time required for fibrin fibres to grow sufficiently to allow lateral aggregation. The rate of lateral aggregation (slope) was calculated at half maximum absorbance, and the maximum absorbance (indicator of fibre cross-sectional area) was calculated as the maximum absorbance at plateau minus the baseline. The CV for all assays was <10%.

Statistical analysis

Statistical analyses were performed using the computer software package Statistica (Statsoft, Tulsa, OK, USA). A p-value of <0.05 was regarded as statistically significant. Normally distributed data were reported as means [95% confidence interval (CI)] and nonparametric data as geometric means (95% CI). Nonparametric data were log transformed to improve normality. Independent t-tests and analysis of variance (ANOVA) with Tukey's Honest Significant Difference *post-hoc* tests were used to compare total and γ' fibrinogen and clot properties across CVD risk factor categories. In order to determine whether the association between clot properties and CVD risk factors was due to differences in total and or γ' fibrinogen, analysis of co-variance (ANCOVA) was performed by adjusting for total and or γ' fibrinogen when they differed significantly across the CVD risk factor categories. These results are reported as least square means (95%CI). Additionally, regression models were constructed, using continuous variables, to serve as trend tests to determine the existence of a significant linear trend in the association between the known CVD risk factors and total and γ' fibrinogen and clot properties. The method of Benjamini and Hochberg was used to account for multiple testing [37]. For all significant interactions, significance remained after accounting for multiple testing.

Results

Characteristics of the study population

Table 1 provides the descriptive characteristics of the study population. The geometric means for total and γ' fibrinogen concentrations were 3.16 (3.08-3.25) g/l and 0.33 (0.32-0.33) g/l, respectively. Means for measurements of clot formation were as follows: lag time 6.46 (6.37-6.55) minutes, maximum absorbance 0.43 (0.42-

0.44), and CLT 57.3 (56.8-57.8) minutes. The geometric mean for slope was 2.44 (2.38-2.49) x 10^{-3} absorbance units / s.

Effect of CVD risk factors on clot properties

Table 2 and 3 report the association of non-biochemical and biochemical CVD risk factors, respectively, with total and γ' fibrinogen and clot properties. For all of the CVD risk factors, except for HDL-cholesterol, differences in total and or γ' fibrinogen concentration across CVD risk factor categories resulted in altered rates of lateral aggregation and fibre thickness, as indicated by altered slope and maximum absorbance. However, for BMI, total cholesterol and HbA1c, the altered clot properties disappeared after adjusting for total and or γ' fibrinogen concentration, which differed across the CVD risk factor categories. For age, MetS, HDL-cholesterol, CRP and Hcy, the altered clot properties remained after adjustment for the total and or γ' fibrinogen concentration, indicating that these risk factors were associated with clot properties independently (at least in part) of total and or γ' fibrinogen concentration. Following adjustment for total fibrinogen, increased age was still associated with the formation of thicker fibres and, to a lesser extent, with an increased rate of lateral aggregation. Since only the total fibrinogen concentration but not γ' fibrinogen differed across the age categories, we separately adjusted for the γ' ratio, but this did not significantly alter the results. The presence of both the MetS and low HDL-cholesterol levels was associated with lower rates of lateral aggregation and, consequently, the formation of clots with thinner fibres. The association of increased CRP levels with increased rates of lateral aggregation and the presence of thicker fibres remained, while increased Hcy levels were associated with increased rates of lateral aggregation, which did not lead to a significantly altered fibre thickness. Differences in CLTs were also present across the CVD risk factor categories, but the differences remained after adjustment for total and or γ' fibrinogen concentration (data not shown). This indicates that the association of CLT with the CVD risk factors is largely independent of the fibrinogen concentration. A detailed discussion on the association of CLT with CVD risk factors is provided elsewhere [1].

Discussion

One of the mechanisms by which total fibrinogen and γ' fibrinogen concentration contribute to CVD is altered clot structure. Several CVD risk factors have previously been associated with total fibrinogen concentration and or altered clot structure. However, it is unclear whether such associations with altered clot structure can be ascribed to total fibrinogen concentration or to other independent mechanisms. In this study we demonstrated that although the association of some CVD risk factors such as BMI, total cholesterol and HbA1c with clot structure was attributable to differences in total and or γ' fibrinogen concentration, other CVD risk factors, such as age, MetS, HDL-cholesterol, CRP and Hcy, were associated with altered clot structure independently of total and or γ' fibrinogen concentration, suggesting that these associations are influenced by other mechanisms *in vivo.*

In our study ageing was associated with the formation of thicker fibres, regardless of the total fibrinogen concentration. Ageing is known to be associated with increased coagulation enzyme activity as well as enhanced fibrin formation [38], as seen by increased levels of various haemostatic factors such as fibrinogen, Factor VII, Factor VIII, Factor IX, Factor XIII, PAI-1 and platelet activity [39,40]. Many of these factors are associated with the formation of a clot structure consisting of thinner fibres [16,41-43]. Therefore, it does not seem likely that haemostatic factors *per se* explain our findings in this study. However, inflammatory markers, interleukin-6 (IL-6) and CRP also increase with ageing [39,44]. Interleukin-6 and CRP (in accordance with our results) have both been associated with increased fibre diameter [45] and this therefore indicates that the association of ageing with fibre thickness may possibly be mediated by the inflammatory process. See also the discussion on CRP below. Other factors that might also contribute to increased fibre thickness with ageing include the plasma proteins, albumin and fibronectin. Albumin levels have been shown to decrease with age, particularly in the elderly [46,47], and because albumin is normally associated with the formation of thinner fibres [11], one might suspect that lower albumin levels could result in thicker fibres. This, however, remains speculation only, as albumin levels were not measured in this study. Fibronectin, on the other hand, increases with ageing [48] and is associated with increased fibre thickness [49]. In contrast to our findings, a study by Pretorius et al. [50]

demonstrated by scanning electron microscopy that, in healthy individuals older than 75 years compared with young individuals, thick fibres were more sparsely arranged while thin fibres dominated, forming a fine netlike structure. It should be taken into account, however, that this study consisted of only a very small number of subjects and that the average age of our study population was much younger (48 years). Furthermore, owing to the limited data available on the impact of different age categories on fibrin clot structure, it is also not clear whether such changes in fibre thickness start to occur only above a certain age.

We further demonstrated that the presence of MetS was associated with lower rates of lateral aggregation and, consequently, the formation of clots with thinner fibres and, although MetS is related to haemostatic factors such as fibrinogen [51,52], the association we observed was independent of total and γ' fibrinogen concentration. In contrast to our findings, a study by Carter et al. [9] showed that MetS was associated with increased clot density or thicker fibres, but no adjustment was made for total and γ' fibrinogen concentration. Metabolic syndrome is characterised by a cluster of CVD risk factors such as hypertension, increased waist circumference (WC) (abdominal obesity), hypertriglyceridaemia, low HDL-cholesterol and increased fasting blood glucose [52,53]. However, it does not seem likely that central obesity and abnormal glucose metabolism explain the observed altered fibrin network structure in our study as both WC (data no shown) and BMI, as well as HbA1c, lost their association with altered fibrin network structure after adjusting for total and γ' fibrinogen concentration. The role of dyslipidaemia, however, in determining clot structure in MetS seems promising since, in our study, low HDL-cholesterol had effects similar to MetS on clot structure.

HDL-cholesterol is known to play a protective role in the development of CVD while low HDL-cholesterol levels are associated with increased CVD risk [54]. Altered fibrin network structure as a possible mechanism by which HDL-cholesterol can affect CVD development has been reported by a few studies, but the exact mechanism is still unclear. Recent evidence indicated that HDL has the potential to bind to fibrin clots as two-thirds of plasma clot components were found to be HDL proteins [49]. The presence of HDL-cholesterol in clots is supported by Kunz et al.

[55], who demonstrated that clots from patients with CVD had a reduced content of HDL-cholesterol. Furthermore, oxidised HDL has been shown to reduce clot firmness by affecting platelet aggregation through a mechanism that remains unknown [54]. Clots associated with CVD usually consist of tightly packed thin fibres; thus, in the light of our results, one could hypothesise that clots containing low levels of HDL-cholesterol may have thinner fibres, contributing to a more prothrombotic clot structure independently of the influence of total and γ' fibrinogen concentrations.

Regarding the effect of inflammatory markers on clot structure, our study showed an association between increased CRP levels and increased rates of lateral aggregation, as well as the presence of thicker fibres, independent of total and γ' fibrinogen concentration. The effect of CRP on fibrin network structure could be *via* its effect on TF. C-reactive protein has been shown to increase TF expression and activity in blood monocytes, vascular smooth muscle cells and endothelial cells (ECs) [56,57]. Recent evidence has associated CRP with faster fibrin polymerisation and consequently, greater maximum absorbance in ECs, and, in the presence of TF expression, CRP produced clots with thicker fibres that were more fibre dense [57]. CRP has additionally been shown to bind directly to fibrinogen [58] and in so doing may alter the structure of the ensuing fibrin network.

We further investigated the effect of Hcy levels on the kinetics of clot formation and fibrin network structure and showed an association between elevated Hcy levels and increased rates of lateral aggregation, but no association with altered fibre thickness. As hyperhomocysteinaemic participants in this study had mild to moderately increased levels (16-49.4 μM) [59] at most, the effect on plasma clot properties may not be as pronounced as with severe hyperhomocysteinaemia. Previous studies measuring plasma Hcy levels found decreased fibrin clot permeability and lysis as Hcy levels increase [60,61] while studies incubating plasma with Hcy *in vitro* showed conflicting results regarding the effect of Hcy on the process of clot formation [38,62-64]. It is well known that *in vivo* and *in vitro* effects may differ significantly as a result of the experimental design of *in vitro* studies; further research is needed to confirm the effect of Hcy *in vivo.* A possible mechanism suggested by which increased Hcy

may affect fibrin clot structure *in vivo* is homocysteinylation of plasma proteins (*e.g.* fibrinogen) by its highly active metabolite, homocysteine thiolactone (HTL), particularly homocysteinylation of lysine residues in fibrinogen [65,66]. Lysine sites are located in the Aα, Bβ and γ chains of fibrinogen and it has been suggested that homocysteinylation of the αC domain in the Aα chain may affect fibrin network structure owing to its involvement in the lateral association of fibrin protofibrils [65]. It has further been suggested that lysine homocysteinylation may reduce calcium binding by modifying the D domain of the γ chain, which contains the high-affinity binding sites for calcium, and consequently result in altered fibrin network structure [65].

Although this study, employing population data on an epidemiological level, confirms previous associations determined using *in vitro* experimental studies, causality could not be determined because of the cross-sectional nature of the study. While the use of plasma samples is of immense value in determining *in vivo* relationships, the interpretation of the results of an indirect technique such as turbidity is complicated because of the complex nature of plasma and the interplay of factors that determine maximum absorbancy. Because of the high throughput design of turbidity analysis and the time-consuming nature of more direct methods (such as microscopy), it can, however, play an important role in providing information on clot structure in an epidemiological study setting with large subject numbers. While every attempt has been made to prevent possible selection bias, it is not impossible that it may have occurred in some form.

In conclusion, our data suggest that kinetics of clot formation may be altered by several CVD risk factors, independently of their effects on total and or γ' fibrinogen concentration. This indicates that final clot structure is not simply a consequence of a specific fibrinogen concentration, but may contribute to increased CVD risk by independent associations with other CVD risk factors.

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R.A.S Ariëns – Interpretation of data, co-wrote the manuscript.
Z. de Lange – analysis and interpretation of data, final approval of version to be published.
M. Pieters - analysis and interpretation of data, critical writing of manuscript.

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Table 1: Characteristics of the study population

Variable	Total population (n=2010)	Population distribution
Age (years)	48.3 (47.9-48.7)	41.9-56.4
Gender: Men/Women n (%)	749 (37.3)/1261 (62.7)	
Body mass index (kg/m^2)	23.7 (23.5-24.0)	19.3-28.9
Waist circumference (cm)	78.7 (78.2-79.3)	70.2-87.7
Blood pressure (mm/Hg):		
Systolic	134 [132-135]	±24.5
Diastolic	87.7 [87.1-88.4]	±14.5
Fasting plasma glucose (mM)	4.82 (4.77-4.87)	4.30-5.30
Glycosylated haemoglobin A1c (%)	5.59 (5.56-5.62)	5.30-5.80
Metabolic syndrome:		
Yes n (%)	462 (24.7)	
No n (%)	1405 (75.3)	
Total cholesterol (mM)	4.82 (4.76-4.88)	4.01-5.87
High-density lipoprotein cholesterol (mM)	1.52 [1.49-1.55]	±0.63
Low-density lipoprotein cholesterol (mM)	2.67 (2.62-2.73)	2.08-3.65
Triglycerides (mM)	1.14 (1.11-1.16)	0.82-1.55
Plasminogen activator inhibitor-1 (U/ml)	4.55 (4.36-4.75)	1.27-7.92
hs-CRP (mg/l)	3.14 (2.93-3.37)	0.96-9.34
Homocysteine (μM)	9.59 (9.43-9.76)	7.45-12.1
Fibrinogen (g/l)	3.16 (3.08-3.25)	2.30-5.00
Fibrinogen γ' (g/l)	0.33 (0.32-0.33)	0.23-0.45
Lag time (minutes)	6.46 [6.37-6.55]	±1.97
Slope (x 10^{-3} au/s)	2.44 (2.38-2.49)	1.8-3.33
Maximum absorbance	0.43 [0.42-0.44]	±0.16
Clot lysis time (minutes)	57.3 [56.8-57.8]	±11.2

Parametric data reported as mean [95% confidence interval] and standard deviation. Non-parametric data reported as geometric mean (95% confidence interval) and 25th-75th percentiles. hs-CRP – high-sensitivity C-reactive protein; γ' - gamma prime; au - absorbance units.

Table 2: Effect of non-biochemical CVD risk factors on clot properties

Sub-division	Total fibrinogen (g/L)	Fibrinogen γ' (g/L)	Rate of lateral aggregation (x10^{-3} au/s)		Fibre thickness (Maximum absorbance)	
			Unadjusted means	Adjusted means	Unadjusted means	Adjusted means
Age (years):						
30-39	2.95 (2.79 - 3.12)[ab] n=334	0.32 (0.31 - 0.34) n=329	2.32 (2.22 - 2.44)[ab] n=733	2.35 (2.24 - 2.46)[ab] n=315	0.41 [0.39 - 0.42][ab] n=333	0.41 [0.40 - 0.43][ab] n=317
40-49	2.96 (2.85 - 3.09)[cd] n=700	0.31 (0.30 - 0.32) n=691	2.36 (2.28 - 2.45)[c] n=699	2.40 (2.32 - 2.48)[c] n=657	0.41 [0.40 - 0.43][cd] n=700	0.42 [0.41 - 0.43][cd] n=657
50-59	3.38 (3.21 - 3.56)[ac] n=461	0.34 (0.32 - 0.36) n=468	2.55 (2.46 - 2.65)[ac] n=484	2.54 (2.44 - 2.64)[ac] n=445	0.45 [0.44 - 0.47][ac] n=482	0.44 [0.43 - 0.45][ac] n=443
60+	3.60 (3.37 - 3.84)[bd] n=287	0.33 (0.31 - 0.35) n=283	2.56 (2.43 - 2.70)[b] n=291	2.47 (2.34 - 2.60)[b] n=264	0.47 [0.45 - 0.49][bd] n=288	0.44 [0.43 - 0.46][bd] n=262
p-value	<0.0001	0.28	0.0016	0.046 (total fbg)	<0.0001	0.004 (total fbg)
Trend test	<0.0001	0.16	<0.0001	0.01	<0.0001	0.0001 0.000006 (γ' ratio)
Body mass index (kg/m^2):						
<18.5	3.19 (3.00 - 3.39)[a] n=306	0.29 (0.28 - 0.31)[ab] n=303	2.53 (2.40 – 2.65) n=314	2.53 (2.40 - 2.65) n=284	0.44 [0.42 - 0.46][ab] n=314	0.43 [0.42 - 0.45] n=281
18.5-24.9	2.89 (2.78 - 3.01)[b] n=741	0.30 (0.29 - 0.31)[cd] n=732	2.37 (2.29 - 2.45) n=743	2.44 (2.36 - 2.52) n=691	0.41 [0.40 - 0.42][ac] n=740	0.42 [0.41 - 0.43] n=686
25.0 – 29.9	3.18 (2.99 - 3.38)[c] n=302	0.34 (0.32 - 0.36)[ace] n=298	2.48 (2.36 - 2.60) n=297	2.49 (2.37 - 2.62) n=278	0.43 [0.41 - 0.44][d] n=297	0.43 [0.41 - 0.44] n=277
≥ 30.0	3.90 (3.68 - 4.14)[abc] n=338	0.41 (0.38 - 0.43)[bde] n=345	2.52 (2.41 - 2.64) n=354	2.38 (2.27 - 2.49) n=322	0.47 [0.46 - 0.49][bcd] 354	0.43 [0.42 - 0.46] n=321
p-value	<0.0001	<0.0001	0.08	0.33 (total and γ' fbg)	<0.0001	0.39 (total and γ' fbg)
Trend test	<0.0001	<0.0001	0.51	0.09	<0.0001	0.96
Metabolic syndrome:						
Yes	3.54 (3.36 - 3.75)[a] n=415	0.39 (0.37 - 0.41)[a] n=425	2.36 (2.26 - 2.47) n=436	2.27 (2.17 - 2.36)[a] n=388	0.44 [0.43 - 0.46] n=437	0.41 [0.40 - 0.42][a] n=390
No	3.03 (2.94 - 3.12)[a] n=1309	0.31 (0.30 - 0.32)[a] n=1290	2.45 (2.39 - 2.51) n=1311	2.48 (2.42 - 2.54)[a] n=1222	0.43 [0.42 - 0.43] n=1309	0.43 [0.42 - 0.44][a] n=1212
p-value	<0.0001	<0.0001	0.13	0.0004 (total and γ' fbg)	0.057	0.007 (total and γ' fbg)

Parametric data reported as mean [95% confidence interval] and non-parametric data reported as geometric mean (95% confidence interval). [abcde] Means with the same letter differed significantly – as obtained from ANOVA (unadjusted models) and ANCOVA (adjusted models). au – absorbance units; fbg - fibrinogen; γ' - gamma prime. In the adjusted models, the variables adjusted for are indicated in brackets beneath the p-values.

Table 3: Effect of biochemical CVD risk factors on clot properties

Sub-division	Total fibrinogen (g/L)	Fibrinogen γ' (g/L)	Rate of lateral aggregation (x10^{-3} au/s)		Fibre thickness (Maximum absorbance)	
			Unadjusted means	Adjusted means	Unadjusted means	Adjusted means
Total cholesterol (mmol/L):						
<5.2	3.06 (2.96 - 3.17)[a] n=1046	0.32 (0.31 - 0.33) n=1030	2.41 (2.34 - 2.47) n=1059	2.42 (2.36 - 2.48) n=990	0.42 [0.41 - 0.43][a] n=1060	0.42 [0.41 - 0.43] n=990
≥5.2	3.31 (3.18 - 3.44)[a] n=703	0.32 (0.31 - 0.34) n=708	2.46 (2.38 - 2.54) n=713	2.44 (2.36 - 2.52) n=662	0.44 [0.43 - 0.45][a] n=710	0.43 [0.42 - 0.44] n=660
p-value	0.004	0.94	0.29	0.71 (total fbg)	0.003	0.11 (total fbg)
HDL-cholesterol (mmol/L):						
[¥]Normal	3.09 (2.99 - 3.18)[a] n=1228	0.31 (0.30 - 0.31)[a] n=1224	2.46 (2.40 - 2.52) n=1242	2.48 (2.42 - 2.54)[a] n=1149	0.43 [0.42 - 0.44) n=1234	0.43 [0.42 - 0.44][a] n=1137
[¥¥]Low	3.33 (3.17 - 3.50)[a] n=521	0.38 (0.36 - 0.39)[a] n=514	2.36 (2.26 - 2.46) n=530	2.30 (2.21 - 2.38)[a] n=485	0.43 [0.42 - 0.44) n=536	0.41 [0.40 - 0.42][a] n=488
p-value	0.009	<0.0001	0.08	0.0009 (total and γ' fbg)	0.89	0.0004 (total and γ' fbg)
HbA1c (mmol/L):						
Quartile 1 (<5.3)	2.83 (2.69 - 2.97)[a] n=443	0.29 (0.27 - 0.30)[ab] n=441	2.30 (2.21 - 2.40)[a] n=447	2.37 (2.28 - 2.47) n=414	0.39 [0.38 - 0.41][ab] n=443	0.41 [0.40 - 0.43] n=407
Quartile 2 (5.3-5.49)	2.96 (2.79 - 3.15)[b] n=292	0.29 (0.28 - 0.31)[c] n=291	2.47 (2.34 - 2.60) n=293	2.54 (2.41 - 2.67) n=273	0.43 [0.41 - 0.44][c] n=290	0.43 [0.42 - 0.45] n=269
Quartile 3 (5.5-5.79)	3.08 (2.94 - 3.24)[c] n=474	0.32 (0.31 - 0.34)[ad] n=471	2.42 (2.32 - 2.52) n=488	2.44 (2.35 - 2.54) n=443	0.43 [0.41 - 0.44][ad] n=490	0.43 [0.42 - 0.44] n=443
Quartile 4 (≥5.8)	3.67 (3.50 - 3.85)[abc] n=556	0.38 (0.37 - 0.40)[bcd] n=552	2.54 (2.45 - 2.64)[a] n=561	2.43 (2.34 - 2.52) n=518	0.47 [0.46 - 0.48][bcd] n=564	0.43 [0.42 - 0.44] n=520
p-value	<0.0001	<0.0001	0.007	0.22 (total and γ' fbg)	<0.0001	0.13 (total and γ' fbg)
Trend test	<0.0001	<0.0001	0.002	0.56	<0.0001	0.39
C-reactive protein (mg/L):						
≤3	2.60 (2.52 - 2.68)[a] n=832	0.28 (0.27 - 0.29)[a] n=822	2.22 (2.15 - 2.29)[a] n=831	2.34 (2.27 - 2.41)[a] n=777	0.38 [0.37 - 0.38][a] n=827	0.41 [0.40 - 0.42][a] n=770
>3	3.78 (3.64 - 3.93)[a] n=909	0.38 (0.36 - 0.39)[a] n=907	2.63 (2.56 - 2.71)[a] n=932	2.51 (2.43 - 2.58)[a] n=849	0.48 [0.47 - 0.49][a] n=935	0.44 [0.43 - 0.45][a] n=848
p-value	<0.0001	<0.0001	<0.0001	0.002 (total and γ' fbg)	<0.0001	<0.0001 (total and γ' fbg)

Homocysteine (µmol/L):						
Normal (3.0-14.9)*	3.17 (3.09 - 3.26) n=1538	0.33 (0.32 - 0.34)[a] n=1527	2.42 (2.37 - 2.48)[a] n=1557	2.40 (2.34 - 2.45)[a] n=1493	0.43 [0.43 - 0.44] n=1557	0.43 [0.42 - 0.44] n=1486
Hyperhomocysteinaemia (15.0-49.4)*	3.12 (2.87 - 3.39) n=191	0.28 (0.26 - 0.31)[a] n=190	2.64 (2.48 - 2.83)[a] n=197	2.69 (2.53 - 2.86)[a] n=188	0.42 [0.40 - 0.44] n=194	0.43 [0.41 - 0.46] n=185
p-value	0.67	0.0002	0.01	0.0007 (γ' fbg)	0.22	0.72 (γ' fbg)

Parametric data reported as means [95% confidence interval] and non-parametric data reported as geometric means (95% confidence interval). [abcd] Means with the same letter differed significantly – as obtained from ANOVA (unadjusted models) and ANCOVA (adjusted models). HDL-cholesterol: ¥Normal - Men >1, women >1.2 mmol/l; ¥¥Low - Men <1, women <1.2 mmol/l. au – absorbance units; HDL – high-density lipoprotein; HbA1c - glycosylated haemoglobin; fbg - fibrinogen; γ' - gamma prime. In the adjusted models, the variables adjusted for are indicated in brackets beneath the p-values. *Lowest and highest study participant values in each category.

CHAPTER 6:

Conclusion and recommendations

6.1 INTRODUCTION

This chapter provides a summary of the most important findings of the three articles presented in Chapters 3, 4 and 5 that form part of this thesis. The results have already been discussed, interpreted and compared with the relevant literature in the previous chapters. This Chapter draws conclusions based on the findings and provides recommendations for future research. The relevance of these results against a broader scientific background will also be highlighted. The main findings and conclusions drawn are aimed at addressing the objectives of this study. For ease of reference, the main aim and objectives are repeated below, followed by a general discussion and conclusion.

Aim and objectives

Aim:

The main aim of this study is to determine the relationship between fibrinogen functionality (total and γ' fibrinogen concentrations and fibrin clot properties) and CVD risk in the black South African PURE population in a plasma setting.

Objectives:

- To determine the relationship between plasma total fibrinogen, fibrinogen γ' concentration, γ'/total fibrinogen ratio and fibrin network properties, including clot lysis time (CLT), using the turbidimetric analysis method of Lisman *et al*. (2005).
- To determine the association of known CVD risk factors with plasma total and γ' fibrinogen concentrations and the γ'/total fibrinogen ratio.
- To investigate the influence of genetic polymorphisms (identified from the literature), located in both the fibrinogen and Factor XIII genes, on fibrinogen synthesis and fibrin network properties, as well as possible gene-environment interactions in determining clot properties.
- To investigate the association between known CVD risk factors and fibrin network properties.

6.2 THE RELATIONSHIP OF TOTAL AND γ' FIBRINOGEN CONCENTRATION WITH FIBRIN NETWORK PROPERTIES AND CVD RISK FACTORS IN AN AFRICAN POPULATION

Total fibrinogen and its isoform, fibrinogen γ', have previously been associated with cardiovascular disease (CVD) (arterial and venous thrombosis) (Danesh *et al*., 2005; De Moerloose *et al*., 2010; Uitte de Willige *et al*., 2005; Mannila *et al*., 2007b). The association of both total and γ' fibrinogen concentration with CVD can be partly related to their ability to alter the final fibrin network structure (Ajjan & Grant, 2005; Uitte de Willige *et al*., 2009b; Ariëns, 2011; Allan *et al*., 2012). Apart from the absolute amount of fibrinogen γ', its relative amount, represented by the γ'/total fibrinogen ratio, has also been associated with CVD (Uitte de Willige *et al*., 2005; Cheung *et al*., 2008b; Van den Herik *et al*., 2011).

It has recently become clear that the prevalence of CVD is increasing in the black South African population, a population known to have elevated fibrinogen levels, among other CVD risk factors (Pieters *et al*., 2011). Total fibrinogen concentration in black ethnicities has been reported to be higher than what is observed in white study populations. The main body of evidence regarding the association between total fibrinogen, fibrinogen γ' and CVD has hitherto been conducted in Caucasians. Only limited evidence is available on the relationship between fibrinogen and CVD in black ethnicities; moreover, no data exist on the role of fibrinogen γ' and γ'/total fibrinogen ratio in CVD risk in the African population. Furthermore, the effect of fibrinogen γ' on fibrin network structure has mostly been examined by *in vitro* study designs, making use of purified or recombinant fibrinogen, while little is known of its effect *in vivo*. We therefore determined clot properties of plasma samples in a cross-sectional epidemiological setting, employing turbidimetric analysis. We determined the time required for protofibrils to reach sufficient length to allow lateral aggregation (lag time), the rate of lateral aggregation and the fibre cross-sectional area, as well as CLTs. Total fibrinogen, fibrinogen γ' and γ'/total fibrinogen ratio were then related to these clot properties. In this plasma system no association was observed between lag time and any of the fibrinogen variables. Increasing total fibrinogen concentration was associated with an increase in slope (rate of lateral aggregation) and contributed to 14% of its variance, while fibrinogen γ' concentration did not significantly influence the rate of lateral aggregation. This latter finding is in contrast to findings in purified settings

demonstrating decreased rates of lateral aggregation in clots containing fibrinogen γ' (Gersh, 2009; Allan *et al*., 2012). This could possibly be explained by a smaller effect of fibrinogen γ' concentration in the plasma than in the purified setting in the presence of increased fibrinogen concentration. Both increased total fibrinogen (largest effect, explaining 33% of the variance) and fibrinogen γ' concentration (explaining 14.5% of its variance) resulted in increased maximum absorbance (indicative of thicker fibres), while an increased γ'/total fibrinogen ratio was associated with decreased maximum absorbance (indicative of thinner fibres). This indicates that the γ'/total fibrinogen ratio is an accurate indicator of the relative amount of γ' in the plasma clot, as clots made from purfied γ' fibrinogen have been shown to have thinner fibres. The influence of total fibrinogen concentration on maximum absorbance in our African population was smaller than the more than 40% contribution in subjects of white European descent in a study by Carter *et al*. (2007). This discrepancy may be ascribed to ethnic differences in the effect of fibrinogen on fibrin network structure. Furthermore, increased fibrinogen γ' concentration demonstrated a larger increase in CLT in comparison with total fibrinogen, contributing to 12% of CLT variance. Our data provide clear evidence for a role of γ' fibrinogen in the regulation of fibrinolysis rates in plasma obtained from a large number of subjects. This study demonstrated for the first time an association between absolute and relative γ' fibrinogen plasma concentration and fibrin network properties, using plasma samples on a population level with previous evidence being available only for *in vitro* studies.

It is known that several CVD risk factors are associated with fibrinogen concentration. The contribution of CVD risk factors to fibrinogen concentration in this African population seems to be similar to what has previously been described in Caucasians (Pieters *et al*., 2011). Much less is known, however, about the association of fibrinogen γ' and γ'/total fibrinogen ratio with CVD risk factors. In our study, increased total fibrinogen, fibrinogen γ' levels and γ'/total fibrinogen ratio were significantly associated with female gender, increased categories of waist circumference (WC), body mass index (BMI), C-reactive protein (CRP), glycosylated haemoglobin (HbA1c) and metabolic syndrome (MetS) and low levels of high-density lipoprotein (HDL) cholesterol. Furthermore, the association of fibrinogen γ' levels and γ'/total fibrinogen ratio with the above CVD risk factors was, at least in part, independent of total fibrinogen, suggesting that these associations do not merely reflect the association of the CVD risk factors with

total fibrinogen, but that they are likely independent relationships. However, compared with total fibrinogen, increased fibrinogen γ' levels were not associated with ageing and increased total cholesterol (TC). Both reduced fibrinogen γ' levels and γ'/total fibrinogen ratio were associated with hyperhomocysteinaemia. Of all the CVD risk factors (apart from total fibrinogen), CRP made the largest contribution to variance in both fibrinogen γ' levels and γ'/total fibrinogen ratio, suggesting an influence of the inflammatory process in regulating fibrinogen γ' levels. We provide, for the first time, evidence of an association between plasma fibrinogen γ' levels, γ'/total fibrinogen ratio and CVD risk factors in a black South African population. Our data show that physiological levels of fibrinogen γ' influence fibrin clot properties in plasma and that factors other than fibrinogen, such as the inflammatory response, is involved in the regulation of plasma γ' fibrinogen concentration. Our findings support the need for future studies on the role of fibrinogen γ' in thrombosis and further investigation into the role of fibrinogen γ' and γ'/total fibrinogen ratio in CVD development in African populations.

6.3 THE ROLE OF GENETICS IN DETERMINING TOTAL AND γ' FIBRINOGEN CONCENTRATION AND CLOT PROPERTIES IN AN AFRICAN POPULATION

Fibrinogen concentration has been shown to be influenced by several genetic factors (Standeven *et al*., 2009) in Caucasians. The contribution of genetic polymorphisms to fibrinogen synthesis seems to be lower in African Americans (Reiner *et al*., 2006; Wassel *et al*., 2011) than in Caucasians (Reed *et al*., 1994; Friedlander *et al*., 1995). There is, however, a lack of data on the role of genetics in determining total as well as γ' fibrinogen concentration in the black South African population. Sub-Saharan Africa is considered to have the greatest genetic variation in the world (Huang *et al*., 2011) and genetic data of other ethnicities can thus not simply be extrapolated to the black ethnicities. We therefore investigated the effect of single nucleotide polymorphisms (SNPs) in both the fibrinogen and Factor XIII genes (as identified from the literature) in determining total fibrinogen and fibrinogen γ' concentration in the PURE population. Compared with data reported for participants of European descent, different genetic variation was observed in this study. Regarding minor allele frequencies (MAFs), we observed overall, except for two SNPs (rs6050 and rs2066865), much lower MAFs compared with what has been reported for white study populations and found no variation in the well-studied SNP rs1800790. Differences were also observed regarding linkage disequilibrium (LD) patterns. No LD was found between rs1800791, rs1800878,

rs4220, rs1049636 and rs2070011, as previously reported (Behague *et al*., 1996; Mannila *et al*., 2005; Mannila *et al*., 2006b), while LD existed between rs1800791, rs2227385 and rs2227388. The low levels of LD observed in our study support evidence that African populations usually have low levels of LD (Huang *et al*., 2011).

Genetic variation has also been found in the association between SNPs and fibrinogen levels. Various SNPs in the fibrinogen β (FGB), fibrinogen α (FGA) and fibrinogen γ (FGG) genes, as well as in the Factor XIII gene, which have previously been associated with total fibrinogen levels in Caucasians, did not show any association in our study, except for SNP rs1049636 (FGG gene), which was associated with increased fibrinogen levels. Regarding associations between SNPs and fibrinogen γ' levels, we also demonstrated different findings compared with what has been reported in the literature. In our study only rs2070011 (FGA gene) was associated with increased fibrinogen γ' levels. These findings are not entirely unexpected since LD patterns of Africans are considered to be different from those of other ethnicities (Teo *et al*., 2010). Furthermore, the low level of LD observed in our study may also reduce the chances of finding significant genotype-phenotype associations seen in other studies (Teo *et al*., 2010).

In addition to regulating fibrinogen concentration, genetic polymorphisms have also been shown to alter fibrin clot properties (Scott *et al*., 2004). In a study by Mannila *et al*. (2006b), rs1049636 and rs2070011 were associated with clot structure, independent of fibrinogen concentration. In our study, on the other hand, significant associations between rs1049636, rs2070011 and clot properties did not remain after adjustment for total and γ' fibrinogen concentration, respectively, indicating that the observed associations were probably mediated by differences in total and γ' fibrinogen concentration between the genotypes. However, we did find a new association between rs1800787 and increased maximum absorbance. Also, in the case of rs1800787, the observed increased maximum absorbance may be a result of increased fibrinogen concentration, although this could not be conclusively proven in our study, owing to the small sample of homozygous minor allele carriers.

Only one study previously investigated possible gene-environment interactions between SNPs in the fibrinogen gene and total fibrinogen concentration in determining clot properites (Lim *et al*., 2003). No such interactions have been investigated between

SNPs and fibrinogen γ' concentration. Upon investigating gene-environment interactions between SNPs in the fibrinogen and Factor XIII genes and total and γ' fibrinogen in determining clot structure in Africans, we found several significant interactions in our study. A novel finding from our study is that the SNPs influenced the relationship between total and γ' fibrinogen concentration and clot structure in opposing directions, *i.e*. prothrombotic or antithrombotic. The presence of the minor alleles of rs1800787 and rs1800788, for example, enhanced the effect of total and γ' fibrinogen on CLT respectively, while the presence of the minor allele of rs2227385 reduced the effects of both total and γ' fibrinogen on CLT. This is also the first study providing evidence on SNP rs2227385 (showing variation in black ethnicities only) and its involvement in gene-environment interactions in determining clot structure.

Furthermore, we could not find any interactions between rs5985, rs6050, and fibrinogen concentration in determining clot structure, as previously demonstrated by Lim *et al*. (2003). However, we did find a significant interaction between rs5985 and fibrinogen γ' concentration on clot structure (increased maximum absorbance or thicker fibres with increased fibrinogen γ' concentration), suggesting that the results of the study mentioned above may reflect an association with fibrinogen γ' rather than total fibrinogen. An important consequence of our findings is that our results could potentially explain why Mendelian randomisation studies could not conclusively link fibrinogen concentration to CVD. The data further suggest that increased total or γ' fibrinogen concentration would not by default increase thrombosis risk, but, depending on the effect of SNPs on its functionality, may either have no effect or could even potentially decrease the risk attributed to/dependent on clot structure and lysis.

Results from our study in part contribute to the role genetics may play in CVD development in black South Africans and highlight the importance of focusing on fibrinogen function and not only its concentration in investigating its role in CVD.

6.4 THE RELATIONSHIP BETWEEN CVD RISK FACTORS AND TOTAL AND γ' FIBRINOGEN CONCENTRATION IN DETERMINING CLOT PROPERTIES IN AN AFRICAN POPULATION

Fibrin network structure plays an important role in CVD. In particular, a clot structure consisting of tightly packed thin fibres has previously been associated with CVD events

(Carter e*t al*., 2007). The final clot structure, however, can be altered by several factors (Scott *et al*., 2004), including total and γ' fibrinogen concentration (Ariëns, 2013). Furthermore, total and γ' fibrinogen have each been associated with several CVD risk factors such as age, BMI, WC, MetS, CRP, and HDL-cholesterol in this black South African population (Pieters *et al*., 2011; Pieters *et al*., 2013). It is not clear, however, whether the association of these factors with total and γ' fibrinogen will result in an altered clot structure or whether associations of these factors with clot structure are mediated solely by total and/or γ' fibrinogen concentration or whether independent mechanisms exist. In this study we observed that the association of some of the CVD risk factors such as BMI, TC and HbA1c with clot properties was primarily mediated through total and or γ' fibrinogen concentration while significant associations with age, MetS, CRP, HDL-cholesterol and homocysteine (Hcy) concentrations were independent of total and/or γ' fibrinogen concentration, suggesting that these associations are influenced by other mechanisms *in vivo*. Although direct relationships between CVD risk factors and clot properties have been investigated *in vitro*, this data provide evidence of such relationships *in vivo* in plasma samples on a population level.

Despite the prothrombotic risk associated with ageing, we observed thicker fibres in our population (average age of 48 years) in contrast to a study observing thinner fibres in much older subjects (>75 years) (Pretorius *et al*., 2010a). This suggests that prothrombotic changes in clot structure may occur at a later age, but further research is needed to clarify the change in clot structure with age. It should also be noted that ethnic differences may influence such changes as more than half of CVD mortality in Africa occurs at ages approximately five to ten years younger than in European countries (Mbewu, 2009). It also seems from our results that the inflammatory process, as indicated by an association between CRP and clot properties, may also mediate the association between ageing and clot structure as CRP has previously been shown to increase with age. Regarding MetS, our results point toward a possible role for low HDL-cholesterol levels in determining clot properties. Most components of the MetS measured in our study did not alter fibrin network properties individually while both MetS and low HDL-cholesterol were associated with decreased rates of lateral aggregation and, consequently, the formation of thinner fibres. Carter *et al*. (2007) found thicker fibres in the presence of MetS, but without adjustment for total and or γ' fibrinogen concentration. Our findings further support the known association between low HDL-cholesterol levels and increased CVD risk and suggest that clots with a low HDL-

cholesterol content, as observed in CVD, are associated with a network consisting of thinner fibres.

C-reactive protein is the CVD risk factor with the largest independent contribution to variation in total and γ' fibrinogen concentration in this black South African population (Pieters *et al*., 2013). Additionally in our study, CRP also significantly altered clot properties (increased rate of lateral aggregation and thicker fibres) regardless of the total and γ' fibrinogen concentration. Although most data on CRP and clot structure are from *in vitro* studies, we found comparable results *in vivo*. Potential mechanistic links between CRP and its effect on clot structure could be *via* increasing tissue factor (TF) expression (Fay, 2010) as well as through the ability of CRP to bind directly to fibrinogen (Salonen *et al*., 1984). However, owing to limited data regarding the effect of CRP on clot properties *in vivo*, further research is needed to clarify its role in determining clot properties. Lastly, we observed increased rates of lateral aggregation with hyperhomocysteinaemia. Most studies on Hcy and its association with clot properties have been done *in vitro* and have conflicting results. The role of Hcy in determining clot properties *in vivo* is still unclear and warrants further investigation.

In conclusion, our data suggest that kinetics of clot formation may be altered by several CVD risk factors, independently of their effects on total and/or γ' fibrinogen concentration. This suggests that final clot structure is not simply a consequence of a specific fibrinogen concentration, but may contribute to increased CVD risk by independent associations with other CVD risk factors.

6.5 RECOMMENDATIONS FOR FUTURE RESEARCH

1. In order to aid in preventative and treatment strategies, research is needed regarding the relationship of total and γ' fibrinogen concentration and altered clot structure with the onset/prevalence of CVD events in Africans. Prospective data from the PURE population (collected in 2010 and again in 2015) providing information on CVD event incidence will help in determining the above. While more direct measures of clot properties, such as scanning electron microscopy (SEM), will provide supporting information for the indirect method of turbidimetry, its use in population studies is limited owing to the time-consuming nature of the analysis.

2. Novel polymorphisms that can influence total and or γ' fibrinogen concentration and function should be identified in Africans, as it is clear from the above results that differences were observed in comparison with what has previously been reported in populations of European descent. Studies investigating genetic factors influencing total and γ' fibrinogen should also include genes outside the fibrinogen and Factor XIII genes, located in other regulatory sites.

3. Black South Africans are exposed to rapid urbanisation that is accompanied by an increase in CVD risk factors, resulting in the increased prevalence of CVD. It is therefore important to investigate other possible independent mechanisms mediating the association between individual CVD risk factors and altered clot structure *in vivo* in order to help with treatment and prevention strategies for CVD in the black South African population.

Findings from this study contribute to a better understanding of the role total and γ' fibrinogen, clot properties, and related genetics play in the development of CVD in Africans. Our results also provide novel insights into fibrinogen functionality (total and γ' fibrinogen, and clot properties) in plasma and the role thereof in CVD risk. This study indeed leads to new questions in this field of research.

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ADDENDA

ADDENDUM A

Ethical approval 2005

YUNIBESITI YA BOKONE-BOPHIRIMA
NORTH WEST UNIVERSITY
NOORDWES UNIVERSITEIT

Etiekkomitee
Tel (018) 299 2558
Faks (018) 297 5308
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2 September 2004

Dr A Kruger
Bussie 594
Noordwes-Universiteit
(Potchefstroomkampus)

Geagte dr Kruger

GOEDKEURING VIR EKSPERIMENTERING MET MENSE

Hiermee wens ek u in kennis te stel dat u projek getiteld *"PURE study (Prospective Urban and Rural Epidemiology study)"* deur die Etiekkomitee goedgekeur is met nommer 04M10.

Gebruik asseblief die nommer genoem in paragraaf 1 in alle korrespondensie rakende bogenoemde projek en let daarop dat daar van projekleiers verwag word om jaarliks in Junie aan die Etiekkomitee verslag te doen insake etiese aspekte van hulle projekte asook van publikasies wat daaruit voortgespruit het. U sal in Mei 2005 die dokumentasie hieroor ontvang.

Goedkeuring van die Etiekkomitee is vir 'n termyn van hoogstens 5 jaar geldig (volgens Senaatsbesluit van 4 November 1992, art 9.13.2). Vir die voortsetting van projekte na verstryking van hierdie tydperk moet opnuut goedkeuring verkry word.

Die Etiekkomitee wens u alle voorspoed met u werk toe.

Vriendelike groete

N.T. Malan

PROF. NT MALAN
VOORSITTER: ETIEKKOMITEE

POTCHEFSTROOMKAMPUS
Privaatsak X6001, Potchefstroom, Suid-Afrika, 2520
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ADDENDUM B

Informed consent form 2005-phase-1

PURE-SA Project

INFORMED CONSENT FORM (PHASE 1)

I, the undersigned .....(full names) understand that the only information that will be asked from me is the family census and household questionnaires. I understand that a field worker from the PURE-study will ask me the questions and that all the information gained from me will be kept confidential.

I indemnify the University, also any employee or student of the University, of any liability against myself, which may arise during the course of the project.

I will not submit any claims against the University regarding personal detrimental effects due to the project, due to negligence by the University, its employees or students, or any other subjects.

.....
(Signature of the subject)

Signed at ..... on .....

Witnesses

1. .....

2. .....

Signed at ..... on .....

ADDENDUM C

Informed consent form 2005-phase-2

PURE-SA Project

INFORMED CONSENT FORM (Phase 2)

I, the undersigned .....(full names) read / listened to the information on the project in PART 1 and PART 2 of this document and I declare that I understand the information. I had the opportunity to discuss aspects of the project with the project leader and I declare that I participate in the project as a volunteer. I hereby give my consent to be a subject in this project.

I agree to be tested for HIV	Yes	No
I want to know my HIV-status	Yes	No
I agree to give a blood sample	Yes	No

I hereby also declare that I am aware that:

1. this blood sample will be used for the purpose of
   a. Isolating DNA to look at genetic factors that are currently associated with Type 2 Diabetes (i.e. the Calpain10, Adiponectin, Leptin and Leptin Receptor genes), or genetic factors that may be associated with non-communicable diseases in the future. We give the assurance that all genetic tests and experiments will only focus on genotypes suspected to contribute to an increased risk of non-communicable diseases of lifestyle.
   b. Testing for liver function by determining liver enzymes such as AST, GGT,
   c. Analyses of other than genetic parameters for Diabetes Mellitus such as HbA_1C, Blood glucose and Insulin
   d. Analyses of clotting factors and hypertension markers
   e. Analyses of bone health, iron and nutrition status
   f. And may be stored until such time as the above measurements/analyses will be done.
2. A two hour glucose tolerance test will be done
3. Body measurements such as height, weight, skinfold thicknesses, arm and leg circumferences will be taken
4. Electrocardiograph be taken
5. Blood pressure to be taken
6. Pulse wave velocity measurements will be made
7. A urine sample to be collected to analyse for the presence of heavy metals such as lead and mercury,
8. A Spirometer test to be performed to determine lung function
9. A handgrip test to be performed to test muscle strength
10. A hair sample to be taken to test for fumonisin mycotoxins.

.....

(Signature of the subject)

Signed at ... **Potchefstroom / Ganyesa** ... (delete not applicable option) on ...../...../ **2005**

Witnesses

1. ..... **2.** .....

Signed at ... **Potchefstroom / Ganyesa** ... (delete not applicable option) on ...../...../ **2005**

PART 1

1. School/Institute:
Faculty of Health Sciences, North-West University

2. **Title of project/trial:**
PURE: Prospective Urban and Rural Epidemiological study

3. **Full names, surname and qualifications of project leader:**
Dr. Annamarie Kruger, Ph.D. (Nutrition)

4. **Rank/position of project leader:**
Research Manager

5.. Aim of this project

PURE's aim is that understanding the different lifestyle and health transitions of individuals in response to societal changes will elucidate societal and individual adaptive strategies that could diminish the adverse health effects of industrialisation and urbanisation on health, while retaining its benefits.

6. Explanation of the nature of all procedures, including identification of new procedures:

Each participant will have to fill in a number of questionnaires (Adult questionnaire, Physical activity questionnaire, Food frequency questionnaire, Health questionnaire) with the help of field workers. A blood and urine sample will be taken. Physical measures will be performed, including anthropometric measures (such as weight, height, and waist circumference), blood pressure, lung capacity and lung volume and an ECG will be performed.

7. Description of the nature of discomfort or hazards of probable permanent consequences for the subjects which may be associated with the project: (Including possible side-effects of and interactions between drugs or radio-active isotopes which may be used.)

It will take each participant quite a while (about two hours) to complete all the tests and discomfort may be experienced with the taking of blood samples. No measures will have permanent damage or consequences for the participants.

8. Precautions taken to protect the subjects:

The research nurse will be present at all times, and will be responsible for the blood sampling. She is very experienced and has performed these procedures numerous times in previous studies.

9. Description of the benefits which may be expected from this project:

When measures with immediate results are taken, such as blood glucose levels or blood pressure, the information will be communicated to the individual to seek professional help. Since this study is a longitudinal study, subjects that are high at risk will be identified from the dataset and personal feedback will be given.

10. Alternative procedures which may be beneficial to the subjects:

There will be tested for HIV/AIDS, therefore pre-test counselling will be given. If the subject wants to know his/her status and he/she tests positive, post counselling will also be given.

PART 2

To the subject signing the consent:
You are invited to participate in a research project. It is important that you read/listen to and understand the following general principles, which apply to all participants in our research project:

1. Participation in this project is voluntary.

2. It is possible that you personally will not derive any benefit from participation in this project, although the knowledge obtained from the results may be beneficial to other people.

3. You will be free to withdraw from the project at any stage without having to explain the reasons for your withdrawal. However, we would like to request that you would rather not withdraw without a thorough consideration of your decision, since it may have an effect on the statistical reliability of the results of the project.

4. The nature of the project, possible risk factors, factors which may cause discomfort, the expected benefits to the subjects and the known and the most probable permanent consequences which may follow from your participation in this project, are discussed in Part 1 of this document.

5. We encourage you to ask questions at any stage about the project and procedures to the project leader or the personnel, who will readily give more information. They will discuss all procedures with you.

6. The University staff will use standardised procedures and take all possible precaution to protect the subject from risks.

7. All information will be kept CONFIDENTIAL and no personal information will be published without my consent.

Dr ANNAMARIE KRUGER
Contact details: 082 7715778 / 018 2994037(W) / 018 2907024(H)

ADDENDUM D

Fibrinogen γ' analytical protocol

Prepare the following reagents:

- **5 M NaCl**
  - Place 116.9 g NaCl in container
  - Add double distilled water (ddH_2O) to make 400 ml

- **0.5 M EDTA**
  - Place 37.23 g EDTA in container
  - Add ddH_2O to make 200 ml
  - Adjust the pH with NaOH until the EDTA completely dissolved

- **M benzamidine**
  - Add 500 ml ddH_2O to 6 g benzamidine

- **Coating buffer**
  - Pour 900 ml ddH_2O in a container
  - Add 8.4 g $NaHCO_3$ and 29.22 g NaCl to the ddH_2O
  - Adjust the pH to 8.99 with NaOH
  - Add ddH_2O to make 1 L

- **Washing buffer**
  - Pour 45.85 ml ddH_2O in a container
  - Add 16.65 ml triethanolamine (TEA), 50 ml of 5M NaCl, 50 ml of 0.5M EDTA and 2.5 ml of Tween-20 to the ddH_2O
  - Adjust the pH to 7.55
  - Add ddH_2O to make 2.5 L

- **Blocking buffer**
  - Pour 500 ml washing buffer in a container
  - Add 5 g bovine serum albumin (BSA) to the washing buffer
  - Aliquot the blocking buffer in 15 ml units and store it at -20°C

- **Dilution buffer**
  - Pour 45.85 ml ddH_2O in a container
  - Add 16.65 ml TEA, 50 ml of 5M NaCl, 50 ml of 0.5M EDTA, 2.5 ml of Tween-20 and 250 ml of 0.1M benzamidine to the ddH_2O
  - Adjust the pH to 7.58
  - Add ddH_2O to make 2.5 L

- **Stop buffer**
  - Pour 473.3 ml ddH_2O in a container
  - Add 26.7 ml H_2SO_4

The ELISA procedure worked as described below:

1. A plastic 96-well microtiter plate was coated by 110 µl of a preparation of monoclonal mouse anti-human fibrinogen γ' (CT, clone 2.G2.H9; Santa Cruz Biotechnology, Santa Cruz, USA) and coating buffer.
2. The 96-well microtiter plate was then incubated overnight at 4-10 °C.
3. The plate was then washed four times with 200 µl washing buffer in each well.
4. After this the plate was blocked by 110 µl blocking buffer in each well and then incubated for one hour.
5. At the end of the incubation period the plate was again washed four times with 200 µl washing buffer in each well.
6. Seventy-four wells were then filled with a 100 µl plasma sample diluted by dilution buffer in a ratio of 1:5000.
7. Four other wells which were separated over the plate were filled with 100 µl control pooled plasma at a ratio of 1:5000 dilution.
8. Eighteen other wells were filled with 100 µl of six different dilutions of pooled plasma with a known fibrinogen γ' concentration (three wells each diluted by ratios 1:1000, 1:2000, 1:5000, 1:8000, 1:16000 and 1:32000) which were used to calculate the calibration or standard curve. The γ' fibrinogen concentration of the pooled plasma was measured using purified γ' fibrinogen.
9. The plate was again incubated for one hour and after this the plate was again washed by the same washing procedure.
10. After this 100 µl conjugate (HRP-conjugated polyclonal goat anti-human fibrinogen; Abcam Cambridge, USA) was added to each well for development and then the plate was again incubated for one hour.
11. The washing procedure then followed again and 100 µl substrate (TMB and UP; Boxtel, Netherlands) were added to each well.
12. The incubation period was then 5 minutes and the ELISA procedure was stopped by adding 100 µl stop buffer to each well.

13. After a time period of 5 minutes the results of the ELISA procedure were determined by a Thermo Scientific (Multiscan FC) spectrophotometer.

14. A second pair of results was determined by a second reading after 3 minutes by the Thermo Scientific (Multiscan FC) spectrophotometer.

15. This complete ELISA was repeated for 18 plastic 96-well microtiter plates to analyse fibrinogen γ' in 1260 plasma samples.

16. A linear calibration curve was used to determine the concentration of each fibrinogen γ' sample.

ADDENDUM E

Published article: Evidence that fibrinogen γ' regulates plasma clot structure and lysis and relationship to cardiovascular risk factors in black Africans (Chapter 3)

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Evidence that fibrinogen γ′ regulates plasma clot structure and lysis and relationship to cardiovascular risk factors in black Africans

Marlien Pieters,[1] Retha C. Kotze,[1] Johann C. Jerling,[1] Annamarie Kruger,[2] and Robert A. S. Ariëns[3]

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Key Points

- This paper describes the effect of fibrinogen γ′ on clot structure in plasma (previously shown in purified systems).
- This paper also describes the respective roles of total fibrinogen, fibrinogen γ′ concentration, and ratio on clot structure and lysis rates.

Fibrinogen γ′ is known to influence fibrin clot structure in purified experimental models, but little is known regarding its influence on clot structure in plasma. Furthermore, the environmental and biological factors that affect its concentration are poorly described. We analyzed fibrinogen γ′, total fibrinogen concentration, and fibrin clot structure in 2010 apparently healthy black South Africans and related them to traditional cardiovascular disease (CVD) risk factors. Fibrinogen γ′ generally increased with increasing fibrinogen concentration, but a decreased γ′/total fibrinogen ratio was found at the highest total fibrinogen concentrations. Clot maximum absorbance increased with total fibrinogen and fibrinogen γ′, but decreased with γ′/total fibrinogen ratio. Clot lysis time showed a stronger relationship with fibrinogen γ′ than with total fibrinogen, whereby increased fibrinogen γ′ delayed clot lysis. CVD risk factors (excluding fibrinogen) explained 20% and 3%, respectively, of the variance in fibrinogen γ′ and the γ′/total fibrinogen ratio, with C-reactive protein making the biggest contribution. More than 50% of the variance in fibrinogen γ′ and γ′/total fibrinogen ratio is explained by factors other than total fibrinogen or other traditional CVD risk factors. Our data show that fibrinogen γ′ modulates plasma clot structure and fibrinolysis and is also influenced by factors other than fibrinogen. (*Blood*. 2013;121(16):3254-3260)

Introduction

Fibrinogen γ prime (γ′; previously also called γB or γ57.5) arises from a splice variant of the γ-chain messenger RNA resulting from an alternative polyadenylation signal in intron 9.[1,2] The alternative polyadenylation leads to the translation of a unique 20-amino-acid C-terminal extension encoded by intron 9, which substitutes the 4 γA amino acids of exon 10.[2-4] Approximately 8% to 15% of total fibrinogen is composed of γ′ fibrinogen, of which the majority is in the heterodimeric γA/γ′ form.[3] Fibrinogen γ′ is associated with both venous[5] and arterial thrombosis.[6-10] This association with different thrombotic disorders has been ascribed in part to the effects of γ′ on clot structure, crosslinking by factor XIIIa, thrombin activity, or fibrinolysis.[11]

The relationship between γ′ fibrinogen and clot structure/function has been investigated mainly using in vitro experimental models with purified fibrinogen.[12-14] Although all of these studies agree that γ′ fibrinogen influences clot structure, some differences were observed in the type of changes, possibly due to differences in experimental designs, source material (ie, fibrinogen purified from plasma or recombinant fibrinogen), and selective copurification of other plasma proteins such as FXIII with plasma-derived fibrinogen. However, some of these discrepancies may also be the result of a heterogeneous, nonuniform clot structure that was observed in clots containing γ′ fibrinogen in subsequent in vitro studies.[15,16] Although the use of purified fibrinogen and fixed protein concentrations allows for the detailed study of mechanisms underpinning the effect of γ′ fibrinogen on clot structure, data on the effect of γ′ fibrinogen in varying plasma concentrations in the presence of varying total fibrinogen concentrations on clot structure are needed to provide insight into the in vivo relationships between these variables.

Furthermore, the factors that contribute to γ′ fibrinogen formation and to γ′/total fibrinogen ratio in vivo are poorly understood. Although a constituent of total fibrinogen, variation in γ′ fibrinogen concentration is not merely a reflection of changes in total fibrinogen levels but is also a result of independent control mechanisms.[17,18] For example, in a study comparing ischemic stroke patients in the acute phase with healthy controls, both γ′ fibrinogen and γ′/total fibrinogen ratio were increased,[9] whereas absolute γ′ fibrinogen levels, but not the γ′/total fibrinogen ratio, were increased in intracerebral hemorrhage patients.[19] Fibrinogen γ′ is thought to be related to cardiovascular disease (CVD) risk independent of total fibrinogen concentration.[11,20] Therefore, not only factors that affect γ′ fibrinogen levels but also factors that could potentially influence the γ′/total fibrinogen ratio should be determined.

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Table 1. Correlation between γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen and measures of clot structure

	γ′ fibrinogen		γ′/total fibrinogen ratio		Total fibrinogen	
Variable	r	P	r	P	r	P
Lag time (min)	0.0517	.04	−0.0245	.32	0.08	.002
Slope	0.1712	<.0001	−0.1829	<.0001	0.3651	<.0001
Maximum absorbance	0.3623	<.0001	−0.2091	<.0001	0.5802	<.0001
Clot lysis time	0.3568	<.0001	0.1720	<.0001	0.1631	<.0001
γ′ fibrinogen	—	—	0.5319	<.0001	0.403	<.0001
γ′/total fibrinogen ratio	0.5319	<.0001	—	—	−0.561	<.0001
Total fibrinogen	0.403	<.0001	−0.561	<.0001	—	—

Our aim was to analyze whether γ′ fibrinogen, total fibrinogen, and γ′/total fibrinogen ratio relate to plasma clot structure in a large population-based study of apparently healthy black South Africans. For this, we used the South African arm of the international Prospective Urban and Rural Epidemiology (PURE) study.[21,22] We also aimed to analyze the relationship between γ′ fibrinogen and cardiovascular risk factors in this well-characterized epidemiological study.

Materials and methods

Study cohort

PURE is a large-scale cohort study that tracks changing lifestyles, risk factors, and chronic disease in rural and urban areas of 17 countries in transition over 12 years. The data reported here are from the baseline data of 2010 randomly selected participants (1260 women and 750 men) from well-established rural (n = 1006, living under tribal law) and urban (n = 1004, living in informal and formal settlements surrounding cities) communities in the North West Province of South Africa collected over a 12-week period in 2005. These participants were recruited from 6000 randomly selected households from the two communities based on representativeness and feasibility for long-term follow-up, according to the guidelines stipulated in the overarching PURE study.[21,22] Apparently healthy black South Africans between the ages of 35 and 65 years were eligible to participate. Exclusion criteria were use of chronic medication for noncommunicable diseases and/or any self-reported acute illness. The study was approved by the ethics committee of the North-West University, South Africa, and subjects signed informed consent before taking part in the study in accordance with the Declaration of Helsinki. All data were treated confidentially and all analyses were performed with coded data.

Blood processing

Fasting blood samples were collected with minimum stasis from the antecubital veins of participants between 7:00 and 11:00 AM. For the analysis of lipids and C-reactive protein (CRP), blood was collected in tubes without anticoagulant. Blood was collected in EDTA tubes for the determination of homocysteine and glycosylated hemoglobin (hemoglobin A1c [HbA_{1c}]) and in fluoride tubes for glucose measurements. For the analysis of γ′ fibrinogen, total fibrinogen, and turbidimetric measurement of clot formation and lysis, blood was collected into citrate tubes and kept on ice until centrifugation (<30 minutes). This procedure did not significantly influence plasma fibrinogen level (data not shown). Samples were centrifuged at 2000*g* for 15 minutes at 10°C within 30 minutes of collection. Aliquots were frozen on dry ice, stored in the field at −18°C, and then after 2 to 4 days stored at −82°C until analysis.

Laboratory and clot structure/fibrinolysis analysis

Details regarding methods used to analyze CRP, serum lipids, total homocysteine, glucose, and HbA_{1c} were published previously.[23] Fibrinogen was measured using a modified Clauss method (Multifibrin U-test, BCS coagulation analyzer; Dade Behring, Deerfield, IL). Turbidimetric analysis (A405 nm) was used to determine plasma fibrinolytic potential of tissue-factor–induced clots and lysed by exogenous tissue plasminogen activator (tPA) with the method of Lisman et al[24] with slightly modified tissue factor and tPA concentrations in order to obtain comparable clot lysis times (CLTs) of about 60 minutes (intra-assay coefficient of variance [CV] = 3.6%, between-plate CV = 4.5%). Final concentrations were tissue factor (125× diluted to an estimated final concentration of 59 pM according to Duckers et al[25]; Dade Innovin, Siemens, Marburg, Germany), $CaCl_2$ (17 mmol/L), tPA (100 ng/mL; Actilyse, Boehringer Ingelheim, Ingelheim, Germany), and phospholipid vesicles (10 μmol/L; Rossix, Mölndal, Sweden). CLT was defined as the time from the midpoint in the transition from the initial baseline to maximum turbidity, which is representative of clot formation, to the midpoint in the transition from maximum turbidity to the final baseline turbidity, which represents the lysis of the clot.[24] Kinetics of clot formation, lag time, slope, and maximum absorbance were additionally calculated from the turbidity curves (supplemental Figure 1). When clotting plasma with thrombin, lag time represents the time required for fibrin fibers to grow sufficiently to allow lateral aggregation. Because we clotted our plasma with tissue factor, lag time additionally included the time it took for activation of the coagulation cascade and was taken at the point where absorbance increased 0.015 from baseline. The slope, calculated at half maximum absorbance, represents the rate of lateral aggregation and the maximum absorbance, calculated as the maximum absorbance at plateau minus the baseline, is an indication of average fiber size. Fibrinogen γ′ was measured according to the method of Uitte de Willige,[5] with an enzyme-linked immunosorbent assay using a 2.G2.H9 mouse monoclonal coating antibody against the human γ′ sequence from Santa Cruz Biotechnology (Santa Cruz, CA) for antigen capture and a goat polyclonal horseradish peroxidase–conjugated antibody against human fibrinogen from Abcam for development (Cambridge, MA). The CV for all assays was <10%.

Statistical analysis

The computer software package Statistica (Statsoft, Tulsa, OK) was used for statistical analyses. A P value ≤ .05 was regarded as statistically significant. Normally distributed variables are reported as mean (95% confidence interval). Nonparametric data were log transformed to improve normality and reported as median (25^{th}-75^{th} percentile). Student t tests for independent samples for parametric data and the Mann-Whitney U test for nonparametric data were used for comparison between 2 groups. Analysis of variance with Tukey honest significant difference post hoc test was used for comparison among 3 or more groups. Analysis of covariance was used when comparison between groups required adjustment for confounders. Pearson correlations and univariate regression were used to determine associations between γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen and markers of clot structure using normally distributed data or log-transformed data for nonparametric variables. Forward stepwise multiple regression analysis was used to determine the main contributors to the variance in γ′ fibrinogen and γ′/total fibrinogen ratio using parametric and log-transformed data.

Results

The mean total fibrinogen concentration of the population was 3.7 g/L (±2.18). The mean γ′ fibrinogen and γ′/total fibrinogen ratio of the population was 0.38 g/L (±0.27) and 12.1% (±8.11), respectively. Table 1 presents the correlation between γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen and markers of clot formation and structure in plasma. Lag time did not show noteworthy correlations with any of the fibrinogen variables. Slope showed the strongest correlation with total fibrinogen (r = 0.37). Maximum absorbance showed a positive correlation with total fibrinogen (r = 0.58) and γ′ fibrinogen (r = 0.36) and a negative correlation with γ′/total fibrinogen ratio (r = −0.21). The correlation between

Table 2. Univariate regression results of γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen with measures of clot structure

	Lag time (min)			Slope			Maximum absorbance			Clot lysis time (min)		
Univariate regression	β	95% CI	%*	β	95% CI	%*	β	95% CI	%*	β	95% CI	%*
γ′ fibrinogen	0.25	0.08; 0.42	1	0.06	0.04; 0.08	2.5	0.11	0.09; 0.12	13.5	7.30	6.37; 8.24	12
γ′/total fibrinogen ratio	−0.11	−0.26; 0.05	<1	−0.06	−0.08; −0.05	3.5	−0.06	−0.07; −0.04	4.5	3.35	2.45; 4.25	3
Total fibrinogen	0.32	0.16; 0.49	1	0.13	0.12; 0.15	14	0.17	0.15; 0.18	33	3.28	2.31; 4.25	2.5

CI, confidence interval.
*Percent variance explained ($R^2 \times 100$).

γ′ fibrinogen and maximum absorbance, although less strong, remained significant after adjustment for total fibrinogen ($r = 0.17$, $P < .0001$; data not shown). CLT showed the strongest correlation with γ′ fibrinogen ($r = 0.36$). Fibrinogen γ′ correlated positively with both γ′/total fibrinogen ratio ($r = 0.53$) and total fibrinogen ($r = 0.40$) whereas γ′/total fibrinogen ratio correlated negatively with total fibrinogen ($r = -0.56$).

The contribution of fibrinogen variables to the variance in the markers of clot formation and structure was analyzed using univariate regression models (Table 2). The results of the regression models are in agreement with the correlations observed. None of the fibrinogen variables contributed significantly to the variance in lag time (all 1% or less). Total fibrinogen was the largest contributor to variance in slope, explaining 14% of the variance. Total fibrinogen was also the largest contributor to the variance in maximum absorbance, explaining 33% of the variance, with γ′ fibrinogen explaining 13.5%. The γ′/total fibrinogen ratio additionally explained 4.5% of the variance in maximum absorbance, with an increase in the ratio being associated with a decreased maximum absorbance. Fibrinogen γ′ was the largest contributor to CLT, explaining 12% of its variance.

The association of γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen with CVD risk markers was analyzed by stratification of the CVD risk markers and comparison of the distribution of the fibrinogen variables between the strata (Table 3 and supplemental Table 1). If total fibrinogen differed between the strata, then an analysis of covariance was performed for γ′ fibrinogen and γ′/total fibrinogen ratio adjusting for total fibrinogen. All 3 fibrinogen variables increased significantly with abdominal obesity and increasing body mass index (BMI) and CRP categories, and the significance for γ′ fibrinogen and γ′/total fibrinogen ratio remained after adjustment for total fibrinogen (Table 3). Individuals with low high-density lipoprotein (HDL) cholesterol had higher levels of all fibrinogen variables, and the significance for γ′ fibrinogen and γ′/total fibrinogen ratio also remained after adjustment for total fibrinogen. Participants with the metabolic syndrome (using the criteria recommended by Alberti et al[26]) also had higher levels of all fibrinogen variables than those without metabolic syndrome, and the significance for γ′ fibrinogen and γ′/total fibrinogen ratio remained after adjustment for total fibrinogen. The same pattern was observed for increasing HbA_{1c} categories (supplemental Table 1). Women had higher γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen

Table 3. The association between γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen and traditional CVD risk factors

CVD risk factor	γ′ fibrinogen (mg/mL)	γ′/total fibrinogen ratio (%)	Total fibrinogen (mg/mL)
BMI, kg/m²			
<18.5	0.28 (0.20-0.42) (n = 303)	9.24 (9.06-13.4) (n = 288)	2.95 (2.20-4.80) (n = 306)
18.5-24.9	0.28 (0.21-0.41) (n = 732)	10.3 (7.19-14.7) (n = 712)	2.60 (2.10-4.10) (n = 741)
25-29.9	0.35 (0.24-0.46) (n = 298)	10.6 (7.39-15.3) (n = 285)	2.90 (2.30-5.00) (n = 302)
≥30	0.38 (.029-0.56) (n = 345)	10.4 (7.33-15.1) (n = 327)	3.70 (2.60-6.20) (n = 338)
Unadjusted *P* value	<.0001	.001	<.0001
P value adjusted for Fbg	<.0001	<.0001	—
Waist circumference, cm			
Normal	0.28 (0.21-0.40) (n = 1150)	9.98 (7.12-14.3) (n = 1112)	2.60 (2.10-4.10) (n = 1167)
Abdominal obesity	0.36 (0.28-0.53) (n = 605)	10.4 (7.28-15.2) (n = 574)	3.30 (2.50-5.90) (n = 559)
Unadjusted *P* value	<.0001	.067	<.0001
P value adjusted for Fbg	<.0001	<.0001	—
HDL cholesterol, mmol/L			
Normal (≥1.0)	0.29 (0.22-0.43) (n = 1224)	9.76 (6.84-14.2) (n = 1175)	2.80 (2.20-4.80) (n = 1228)
Low (<1.0)	0.35 (0.26-0.51) (n = 514)	11.1 (8.03-15.3) (n = 496)	3.00 (2.30-5.50) (n = 521)
Unadjusted *P* value	<.0001	<.0001	.009
P value adjusted for Fbg	<.0001	<.0001	—
Metabolic syndrome			
Yes	0.38 (0.27-0.52) (n = 425)	10.4 (7.54-15.1) (n = 397)	3.30 (2.50-5.80) (n = 415)
No	0.29 (0.22-0.42) (n = 1290)	10.0 (7.08-14.5) (n = 1251)	2.80 (2.20-4.50) (n = 1309)
Unadjusted *P* value	<.0001	.06	<.0001
P value adjusted for Fbg	<.0001	<.0001	—
CRP, mg/L			
≤3	0.27 (0.21-0.37) (n = 822)	10.5 (7.77-14.8) (n = 797)	2.50 (2.00-3.20) (n = 832)
>3	0.36 (0.26-0.52) (n = 907)	9.73 (6.77-14.3) (n = 866)	3.60 (2.50-6.10) (n = 909)
Unadjusted *P* value	<.0001	.007	<.0001
P value adjusted for Fbg	<.0001	<.0001	—

Fbg, fibrinogen.

Table 4. Multiple regression results for γ′ fibrinogen, γ′/total fibrinogen ratio, and total fibrinogen and traditional CVD risk factors

	Percent variance explained		
Variable	Model 1: CVD risk factors plus total fibrinogen	Model 2: CVD risk factors plus γ′ fibrinogen	Model 3: CVD risk factors
γ′ fibrinogen			
Total variance explained	26.3		19.9
Risk factors			
Total fibrinogen	16		Not included
CRP	5		13.5
HDL cholesterol	3		3
Gender	2		2
HbA_{1c}	1		1
Homocysteine	1		0.5
γ′/total fibrinogen ratio			
Total variance explained	39.3	37	3
Risk factors			
γ′ fibrinogen	Not included	28.8	Not included
Total fibrinogen	31	Not included	Not included
CRP	4	7	0.5
HDL cholesterol	2	—	2
Gender	1	0.5	—
Homocysteine	0.5	—	1
HbA_{1c}	0.5	—	—
Age	—	1	0.5

Variables that did not enter forward stepwise model are indicated by —.

than men. Total fibrinogen increased with 10-year age categories, whereas γ′ fibrinogen did not. Individuals with increased total cholesterol had increased total fibrinogen but not γ′ fibrinogen. Individuals with hyperhomocysteinemia had lower γ′ fibrinogen and γ′/total fibrinogen ratio but similar total fibrinogen compared with individuals with normal homocysteine levels. Total fibrinogen was moderately increased in individuals with hypertension whereas no difference was observed for γ′ fibrinogen.

To determine the respective contribution of the CVD risk factors to the variance in γ′ fibrinogen and the γ′/total fibrinogen ratio, the CVD risk factors presented in Table 3 and supplemental Table 1 with and without total fibrinogen and or γ′ fibrinogen were entered into forward stepwise regression models (Table 4). The CVD risk factors together with fibrinogen explained 26.3% of the variance in γ′ fibrinogen, with total fibrinogen explaining 16%, followed by CRP (5%), HDL cholesterol (3%), gender (2%), HbA_{1c} (1%), and homocysteine (1%). When fibrinogen was excluded from the model, the CVD risk factors alone explained 19.9% of the variance in γ′ fibrinogen, with CRP now explaining 13.5% and little change in the contribution of the other CVD risk factors. When total fibrinogen or γ′ fibrinogen was entered together with the CVD risk factors, the models explained 39.3% and 37%, respectively, of the variance in γ′/total fibrinogen ratio. The CVD risk factors alone explained only 3% of the variance, of which CRP and HDL cholesterol made the largest contribution.

Discussion

Our data provide evidence that γ′ fibrinogen influences fibrin clot structure in plasma. It has been shown that γ′ fibrinogen changes fibrin structure in vitro in purified systems at levels of 100%[12-16]; however, whether such effects would also occur at physiological levels of 10% to 15% γ′ fibrinogen and in the presence of other plasma proteins was hitherto unknown. We used a large, well-characterized, population-based study to show that this is indeed the case. We found associations of γ′/total fibrinogen ratio with lower maximum absorbency and prolongation of lysis time in agreement with previously published in vitro effects. We also found that γ′ fibrinogen levels associate with other cardiovascular risk factors and not only with total fibrinogen levels.

Fibrinogen γ′ levels correlated positively with total fibrinogen, although the γ′/total fibrinogen ratio decreased with increasing fibrinogen concentration, suggesting that while γ′ fibrinogen increases as total fibrinogen increases, it does so to a relatively lesser extent (ie, γ′ levels were not a constant fraction of total fibrinogen levels throughout the entire range of fibrinogen concentrations), resulting in the decreased ratio at higher total fibrinogen levels. The median γ′ fibrinogen concentration in this black African population is higher to what was previously reported for whites from the Framingham Offspring study (0.38 vs 0.23 g/L). This is in agreement with the relatively high total fibrinogen concentration (3.7 g/L) found in this and other studies investigating fibrinogen concentration in black Africans.[23,27]

Turbidity curves are often used as a measure of clot formation and structure in large data sets due to its high-throughput methodology.[28] Its relevance is further supported by the fact that final clot structure is to a large extent kinetically controlled and information on clot formation is therefore critical in determining clot structure.[29,30] None of the fibrinogen variables showed noteworthy associations with lag time. This is likely due to the fact that the samples were clotted with tissue factor, so lag time represented the time required not only for the formation and growth of protofibrils from monomers but also for activation of the coagulation cascade. Tissue factor rather than thrombin was used to clot the samples because it allows for comprehensive analysis of plasma fibrinolytic potential (reported as CLT), including all coagulation and lysis components in plasma. Additionally, it allows for better comparison of the different variables because clot formation variables and lysis times were determined in the same experiment. In vitro experiments using purified fibrinogen also found no difference in lag time between clots made from γ′ or γA fibrinogen.[12,15]

Of the fibrinogen variables, total fibrinogen correlated best with slope (rate of lateral aggregation), although the fibrinogen variables explained only a small percentage of the variance observed in the slope. These results indicate an increased rate of lateral aggregation with increasing fibrinogen concentration. This is in agreement with a kinetic model developed by Weisel et al[29] that indicates an increase in the maximum rate of protofibril addition per fiber with increasing fibrinogen concentration. An increase in γ′ fibrinogen was associated with a small increase in rate of lateral aggregation, while an increase in the γ′/total fibrinogen ratio was associated with a small decrease. When adjusting for difference in total fibrinogen, the association between γ′ fibrinogen and slope disappeared, suggesting that in vivo (at least in the plasma setting) γ′ fibrinogen does not significantly affect rate of lateral aggregation despite decreased rates observed in purified models for clots made of γ′ compared with γA fibrinogen.[12,15,16] It may be that the effect of γ′ fibrinogen in plasma is relatively small compared with the effect of increasing fibrinogen concentration in this setting.

The variance in maximum absorbance was explained to a much larger extent (>30%) by the fibrinogen variables than by either lag time or slope. An increase in both γ′ fibrinogen and total fibrinogen

was associated with an increased maximum absorbance, with total fibrinogen contributing to a larger extent than γ′ fibrinogen. An increase in the γ′/total fibrinogen ratio was, however, associated with a decrease in maximum absorbance. In agreement with this, turbidity curves obtained from purified fibrinogen models showed decreased maximum absorbance in clots containing γ′ compared with γA fibrinogen, indicating that the γ′/total fibrinogen ratio is indeed an accurate indicator for the relative amount of γ′ in the plasma clot.[12,15,16] Reduced maximum absorbance from turbidity analysis is an indicator of thinner fibrin fibers at constant fibrinogen concentration. The effect of γ′ fibrinogen on fiber diameter as measured by scanning electron microscopy in vitro is generally in agreement with this. Several studies showed that clots containing γ′ fibrinogen have thinner fibers.[12,14-16] Others, however, using a recombinant homodimer form of fibrinogen γ′, found no difference in fiber diameter.[13] Several studies showed that fibrinogen γ′ fibrinogen produced clots with increased branching.[14,16] Additionally it was found that clots containing γ′ fibrinogen were nonhomogenously arranged into tight interconnecting bundles with tighter pores with bundled fibers and large open pores in other areas of the clot.[15,16] Although the use of plasma samples is of immense value in determining the in vivo relationship among γ′ fibrinogen, total fibrinogen, and clot structure, the interpretation of experimental results, especially from an indirect technique such as turbidity, is complicated due to the complex nature of plasma and the interplay of factors that determine maximum absorbency, including fibrinogen concentration and average fibrin fiber diameter. Because of the high-throughput design of turbidity analysis and the time-consuming nature of more direct methods (such as microscopy), it can, however, play a significant role in providing information regarding clot structure in an epidemiological study setting with large subject numbers.

There has been only 1 previous study that investigated the effect of γ′ fibrinogen concentration on clot structure in plasma. Mannila et al[8] investigated the effect of γ′ fibrinogen concentration on plasma permeability (Ks) and fiber mass-length ratio (μ, calculated from Ks) in 60 control patients from the Stockholm Coronary Artery Risk Factor study. Fibrinogen γ′ concentration was not found to be associated with Ks or μ. However, the authors did not analyze γ′/total fibrinogen ratio and the overall study size was probably too small to detect the relatively moderate effects of physiological levels of γ′ fibrinogen on clot structure. Our current study is much larger in size and showed significant effects of γ′ fibrinogen on plasma clot structure. In particular, the γ′/total fibrinogen ratio showed similar effects on clot structure as previous in vitro studies, with an association of increased γ′ fibrinogen content with thinner fibers (lower maximum absorbance) and prolonged fibrinolysis.

Although CLT correlated positively with both γ′ fibrinogen and total fibrinogen, fibrinogen γ′ had the strongest association with CLT, explaining 12% of its variance. This suggests that γ′ fibrinogen has a larger effect on CLT than total fibrinogen. An increased CLT indicates a decreased lysis rate, which is in agreement with results from studies using purified fibrinogen that showed decreased lysis rates for clots containing γ′ compared with γA fibrinogen.[13,14,31] Falls and Farrell[31] additionally found decreased lysis rates in afibrinogenemic plasma to which γA/γ′ fibrinogen was added compared with plasma containing an equal concentration of γA/γA fibrinogen. They ascribed the decreased fibrinolysis rate to increased FXIII binding to γ′ fibrinogen and subsequent increased crosslinking. Allan et al,[16] however, found no difference in γ-chain crosslinking between γA/γA- and γA/γ′-containing clots and decreased α-chain crosslinking in γA/γ′ clots, indicating that other factors likely play a role in the decrease of fibrinolysis rates by γ′ fibrinogen. Our current data provide clear evidence for a role of γ′ fibrinogen in the regulation of fibrinolysis rates also in plasma obtained from a large number of subjects with varying γ′ fibrinogen levels. As γ′ fibrinogen level increases in individuals, CLT also increases, and γ′ fibrinogen has a larger effect on CLT than total fibrinogen. The results furthermore suggest that this association between γ′ fibrinogen and CLT is not only the result of a denser clot network formed in the presence of higher γ′ fibrinogen concentration because total fibrinogen had a larger impact on maximum absorbance than γ′ fibrinogen and maximum absorbance did not correlate with CLT (data not shown). The mechanism by which γ′ fibrinogen influences fibrinolysis rates should be further investigated using experimental models of clot formation and fibrinolysis.

In general, the association of γ′ fibrinogen with CVD risk factors followed the same trend as that of fibrinogen. Increased levels were observed in women compared with men, subjects with increased BMI, increased waist circumference, elevated CRP, and HbA_{1c} categories and in individuals with metabolic syndrome as well as in individuals with low HDL cholesterol. The association between γ′ fibrinogen and these CVD risk factors remained significant after adjustment for total fibrinogen and is also accompanied by an increase in the γ′/total fibrinogen ratio, suggesting that these associations are not merely reflecting the association of the CVD risk factors with total fibrinogen and that they are likely independent relationships. These results are in agreement with the study of Lovely et al[32] who also found significant associations between γ′ fibrinogen and BMI, (decreased) HDL cholesterol, diabetes, blood glucose, and gender in whites. In two other studies, γ′ fibrinogen did not, however, differ between genders,[7,33] and while we and others[7,33] found no association between γ′ fibrinogen and age, a positive association was found by another group.[32] In this study population, total fibrinogen increased over 10-year age categories and in individuals with increased total cholesterol (>5.2 mmol/L) while no increase was observed for γ′ fibrinogen, resulting in a decreased γ′/total fibrinogen ratio. In hyperhomocysteinemic individuals, on the other hand, decreased γ′ fibrinogen was observed with no change in total fibrinogen consequently also decreasing the γ′/total fibrinogen ratio.

Multiple regression analysis indicated that fibrinogen concentration explained the largest percentage of the variation in γ′ fibrinogen and that the CVD risk factors alone, excluding total fibrinogen, explained 20% and 3% of the variances of γ′ fibrinogen and γ′/total fibrinogen ratio, respectively, suggesting that the γ′/total fibrinogen ratio in apparently healthy black Africans is not strongly affected by CVD risk factors other than γ′ fibrinogen and total fibrinogen. The γ′/total fibrinogen ratio was, however, found to be different between several CVD patient groups and white controls.[5,9,34] Of the other CVD risk factors, CRP was found to be the biggest determinant of both γ′ fibrinogen and γ′/total fibrinogen ratio. These results are in agreement with a case-control study by Cheung et al[34] who found a significant association between CRP and γ′/total fibrinogen ratio in the acute phase of ischemic stroke. These authors hypothesized that messenger RNA processing of γ′ fibrinogen may be altered during the acute phase reaction.

In conclusion, γ′ fibrinogen levels increase as total fibrinogen increases, although to a lesser extent, resulting in a decrease in γ′/total fibrinogen ratio at high fibrinogen concentrations in this apparently healthy black African population. Increases in both γ′ fibrinogen and total fibrinogen were associated with increased maximum absorbance, in agreement with the formation of clots composed of increased fibrin material. However, the γ′/total fibrinogen ratio was associated with decreased maximum absorbance, in agreement with clots made of thinner fibrin fibers. Increased γ′ fibrinogen levels

were associated with prolonged clot lysis. Traditional CVD risk factors (excluding fibrinogen) explained 20% and 3%, respectively, of the variance in γ′ fibrinogen and γ′/total fibrinogen ratio, with CRP making the largest contribution. These data show that physiological levels of γ′ fibrinogen influence fibrin clot structure in plasma and that factors other than fibrinogen, likely involved in the inflammatory response, regulate plasma γ′ fibrinogen concentration. Our findings support future studies of the role of γ′ fibrinogen in thrombosis.

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Authorship

Contribution: M.P. performed research, collected data, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript; R.C.K. analyzed and interpreted data and performed statistical analysis; J.C.J. and A.K. designed the research and collected data; and R.A.S.A. analyzed and interpreted data and critically reviewed the manuscript.

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