Global fibrinolytic potential of black South Africans in the North West Province

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Opgedra aan my ouers, Hennie en Irma de Lange, met lof en dank aan God ons Vader vir die geleenthede en krag wat Hy aan ons skenk.

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ABSTRACT

INTRODUCTION AND AIM

The prevalence of cardiovascular disease (CVD) has increased significantly in the black South African population in recent years. Early in the development of CVD, atherosclerotic plaques form in the vessel wall. When this plaque becomes unstable and ruptures, the coagulation cascade is activated and a blood clot forms. The function of this clot is to stop bleeding. However, it cannot remain in the vasculature indefinitely and has to be lysed again. The ability of the body to lyse clots can be measured with global fibrinolytic potential (GFP) assays and expressed as lysis time. Increased clot lysis time (CLT) has been shown to be significantly associated with various CVD risk factors and CVD events in Caucasian populations while very little information is available for other ethnicities. In this study we investigated plasma GFP and its relation to CVD risk factors in a large black African population. We also determined the effect of three polymorphisms in the promoter area of the plasminogen activator inhibitor-1 (PAI-1) gene on PAI-1_{act} (activity) levels (a main determinant of CLT) and CLT, together with gene–environment interactions and the effect of urbanisation on these interactions.

PARTICIPANTS AND METHODS

Apparently healthy men and women between the ages of 35 and 65 years were recruited to take part in the South African arm of the Prospective Urban and Rural Epidemiology (PURE) study. Approximately 1000 rural and 1000 urban black African individuals participated. Data and samples were collected during a 12-week collection period in 2005 for cross-sectional analysis.

RESULTS

Increased PAI-1_{act} levels, body mass index (BMI), glycosylated haemoglobin (HbA1c), triglycerides, fibrinogen concentration, C-reactive protein, female sex, positive HIV-status and the metabolic syndrome were all associated with prolonged CLTs, while increased habitual alcohol consumption was associated with shorter

CLTs. Urban-rural differences for CLT existed in women only. This is likely due to the larger extent of rural-urban differences in other CVD risk factors observed in women compared to what was observed in men. Of the CVD risk factors measured, PAI-1 explained the largest proportion of the variance in CLT (27%). Owing to the important role PAI-1_{act} plays in CLT, we investigated three polymorphisms in the PAI-1 gene promoter area (the 4G/5G polymorphism, the novel SNP C428T and SNP G429A (previously identified)), and the influence of these polymorphisms on PAI-1_{act} levels and CLT. The frequency of the 5G allele was high (0.85) in comparison with previously reported literature. PAI-1_{act} increased significantly across genotypes in the urban (5G/5G: 3.84 U/ml; 4G/5G: 4.85 U/ml; 4G/4G: 5.96 U/ml p=0.009) but not the rural subgroup, while CLT did not differ. We found significant interactions between the 4G/5G polymorphism and BMI, waist circumference and triglycerides in determining PAI-1_{act}, and between the 4G/5G polymorphism and fibrinogen and fibrinogen gamma prime in determining CLT. Direct relationships with PAI-1_{act} or CLT were not found for the C428T and G429A polymorphisms; they did, however, influence associations of other environmental factors with PAI-1_{act} and CLT. Several of these interactions differed significantly between rural and urban subgroups, particularly in individuals harbouring the mutant alleles.

CONCLUSION

CLT associated with many of the same CVD risk factors described in the literature for Caucasian populations, but also with other risk factors. Rural-urban differences in CLT are dependent on the association of CLT with other CVD risk factors in the rural-urban setting. Genetic polymorphisms of the PAI-1 gene did not directly influence CLT, despite influencing PAI-1_{act}. The main contributor to PAI-1_{act} variance, however, was (central) obesity. The effect of the 4G/5G polymorphism on PAI-1_{act}, as well as gene–environment interactions for the C428T and G429A genotypes in determining PAI-1_{act} and CLT, were significantly influenced by urbanisation.

CVD, PAI-1, fibrinolysis, clot lysis time, polymorphisms

OPSOMMING - Globale fibrinolitiese potensiaal van swart Suid Afrikaners in die Noordwes Provinsie

INLEIDING EN DOEL

Die voorkoms van kardiovaskulêre siekte (KVS) in die swart Suid-Afrikaanse bevolking het in onlangse jare betekenisvol verhoog. Vroeg in die ontwikkeling van KVS vorm plaak in die vaskulêre wand. Wanneer hierdie plaak onstabiel raak en skeur, word die stollingskaskade geaktiveer en ‘n bloedklont vorm. Die funksie van hierdie klont is om bloeding te stop. Dit kan egter nie onbepaald in die sirkulasie bly nie en moet afgebreek word. Die vermoë van die liggaam om klonte af te breek kan met globale fibrinolitiese potensiaal (GFP)-metings bepaal word en as lisetyd uitgedruk word. Verhoogde klontlisetyd (KLT) is volgens waarnemings betekenisvol geassosieer met verskeie KVS-risikofaktore en KVS-voorvalle in Kaukasiese bevolkings hoewel baie minder inligting beskikbaar is vir ander etniese groepe. In hierdie studie het ons plasma-GFP en die verband met KVS-risikofaktore in ‘n groot, swart Afrikapopulasie ondersoek. Ons het ook die effek bepaal van drie polimorfismes in die promoter-area van die plasminogeen-aktiveerderinhibeerder-1 (PAI-1)-geen op PAI-1_{akt} (aktiwiteit) (‘n hoofdeterminant van KLT) en KLT, tesame met geen-omgewingsinteraksies en die effek van verstedeliking op hierdie interaksies.

DEELNEMERS EN METODES

Oënskynlik gesonde mans en vroue tussen die ouderdomme van 35 en 65 jaar is gewerf om deel te neem aan die Suid-Afrikaanse arm van die *Prospective Urban and Rural Epidemiology* (PURE)-studie. Data en monsters is gedurende ‘n 12-week-versamelingsperiode in 2005 gekollekteer vir dwarssnit-analise.

RESULTATE

Verhoogde PAI-1_{akt}-vlakke, liggaamsmassa-indeks (LMI), geglikosileerde hemoglobien (HbA1c), trigliseriede, fibrinogeenkonsentrasie, C-reaktiewe proteïen, vroulike geslag, positiewe MIV-status en metaboliese sindroom was almal met

verlengde klontlisetye (KLTe) geassosieer, terwyl verhoogde gebruiklike alkoholinname met korter KLTe geassosieer was. Stedelik-plattelandse verskille vir KLT het net in vroue bestaan, waarskynlik as gevolg van die groter mate van platteland-stedelike verskille in ander KVS-risikofaktore, in vergelyking met mans. Van die KVS-risikofaktore gemeet, het PAI-1 die grootste gedeelte van die variansie in KLT (27%) verklaar. Omrede die belangrike rol wat PAI-1_{akt} in KLT speel, het ons drie polimorfismes ondersoek in die PAI-1-geenpromotorarea (die 4G/5G-polimorfisme, die nuwe SNP C428T en SNP G429A (vantevore geïdentifiseer)), en die invloed van hierdie polimorfismes op PAI-1_{akt}-vlakke en KLT. Die frekwensie van die 5G allele was hoog (0.85) in vergelyking met vantevore gerapporteer in die literatuur. PAI-1_{akt} was betekenisvol verhoog oor genotipes heen in die stedelike (5G/5G: 3.84 E/ml; 4G/5G: 4.85 E/ml; 4G/4G: 5.96 E/ml p=0.009) maar nie in die plattelandse subgroep nie, terwyl KLT nie verskil het nie. Ons het betekenisvolle interaksies gevind tussen die 4G/5G-polimorfisme en LMI, middelomtrek en trigliseriede in bepaling van PAI-1_{akt} en tussen die 4G/5G-polimorfisme en fibrinogeen en fibrinogeen-gamma-*prime* in die bepaling van KLT. Direkte verwantskappe met PAI-1_{akt} of KLT is nie vir die C428T en G429A-polimorfismes gevind nie; hulle het egter assosiasies van ander omgewingsfaktore met PAI-1_{akt} en KLT beïnvloed. Verskeie van hierdie interaksies het betekenisvol tussen plattelandse en stedelike subgroepe verskil, veral in individue met die mutante allele.

GEVOLGTREKKING

KLT was geassosieer met baie van dieselfde, maar ook ander KVS-risikofaktore as wat in die literatuur vir Kaukasiese populasies beskryf is. Platteland-stedelike verskille in KLT is afhanklik van die assosiasie van KLT met ander KVS-risikofaktore in die platteland-stedelike opset. Genetiese polimorfismes van die PAI-1-geen het nie KLT direk beïnvloed nie, ten spyte van die invloed op PAI-1_{akt}. Die hoofbydraer tot PAI-1_{akt}-variansie was egter (sentrale) vetsug. Die effek van die 4G/5G-polimorfisme op PAI-1_{akt}, asook geen-omgewingsinteraksies vir die C428T en G429A-genotipes in die bepaling van PAI-1_{akt} en KLT, is betekenisvol deur verstedeliking beïnvloed.

KVS, PAI-1, fibrinolise, KLT, polimorfismes

CONTENTS

		Page
	ABSTRACT	iii
	OPSOMMING	v
	CONTENTS	vii
	LIST OF TABLES	xi
	LIST OF FIGURES	xiii
	LIST OF ADDENDA	xiv
	LIST OF ABBREVIATIONS	xv
	CHAPTER 1:	
	Introduction	1
1.1.	BACKGROUND	2
1.2.	AIM AND OBJECTIVES	3
1.3.	STRUCTURE OF THIS THESIS	4
1.4.	RESEARCH TEAM AND CONTRIBUTIONS TO ARTICLES PRESENTED AS PART OF THIS THESIS	6
	CHAPTER 2: Global fibrinolytic potential in black South Africans	8
2.1.	INTRODUCTION	9
2.2.	OVERVIEW OF THE COAGULATION AND FIBRINOLYSIS PROCESSES	10
2.2.1.	Coagulation	10
2.2.2.	Fibrinolysis	15

2.3. DIFFERENT METHODS OF MEASUREMENT OF FIBRINOLYSIS..... 19
2.4. FIBRINOLYSIS AND CVD..... 27
2.4.1. CVD prevalence in black South Africans..... 27
2.4.2. Involvement of fibrinolysis in CVD risk..... 27
2.4.3. Associations between fibrinolysis and individual (traditional) CVD risk factors..... 30
2.5. CLOT LYSIS AND HAEMOSTATIC VARIABLES..... 40
2.5.1. Plasminogen activator inhibitor type-1 and its influence on clot lysis..... 41
2.5.2. PAI-1 genotypes and their influence on PAI-1 levels..... 41
2.5.3. Plasminogen and its influence on clot lysis..... 43
2.5.4. Thrombin-activatable fibrinolysis inhibitor (TAFI) and its influence on clot lysis..... 43
2.5.5. Prothrombin and its influence on clot lysis..... 45
2.5.6. Alpha 2-antiplasmin and its influence on clot lysis..... 45
2.5.7. Fibrinogen and its influence on clot structure and lysis..... 46
2.6. CONCLUSION..... 47

CHAPTER 3: Plasma clot lysis time and its association with cardiovascular risk factors in black South Africans..... 48
INSTRUCTIONS TO AUTHORS: THE PUBLIC LIBRARY OF SCIENCE (PLOS ONE)..... 49
ARTICLE..... 58
Abstract..... 59
Introduction..... 60
Materials and methods..... 62

Results..... 66

Discussion..... 69

Acknowledgements..... 75

References..... 76

Legends to Tables..... 78

Table 1..... 80

Table 2..... 82

Table 3..... 84

CHAPTER 4: The effect of genetic polymorphisms of the PAI-1 gene on PAI-1 activity levels and plasma clot lysis time in black South Africans: the influence of urbanisation..... 85

INTRUCTIONS TO AUTHORS FOR THROMBOSIS AND HAEMOSTASIS..... 86

Proof of submission for publication..... 95

MANUSCRIPT..... 96

Abstract..... 97

Introduction..... 98

Methods..... 100

Results..... 104

Discussion..... 108

Acknowledgements..... 115

Reference list..... 117

Legends to Tables..... 122

Table 1..... 123

Table 2..... 125

Table 3..... 126

Supplemental material..... 127

CHAPTER 5: Conclusion and recommendations..... 137

5.1. INTRODUCTION..... 138

5.2. THE RELATIONSHIP BETWEEN GFP AND CVD RISK FACTORS IN BLACK SOUTH AFRICANS 139

5.3. THE EFFECT OF URBANISATION ON GFP IN BLACK SOUTH AFRICANS…… 141

5.4. GENETIC POLYMORPHISMS IN THE PROMOTER AREA OF THE PAI-1 GENE IN THIS STUDY POPULATION..... 142

5.5. THE EFFECT OF THE PAI-1 PROMOTER AREA POLYMORPHISMS ON PAI-1_{ACT} AND GFP IN THIS BLACK SOUTH AFRICAN POPULATION…….. 145

5.6. RECOMMENDATIONS FOR FUTURE RESEARCH..... 147

6. REFERENCE LIST..... 149

7. ADDENDA..... 164

ADDENDUM A..... 165

ADDENDUM B..... 169

ADDENDUM C..... 172

LIST OF TABLES

		Page
Chapter 2		
Table 2.1.	Concentrations of coagulation and lysis agents used in assays discussed in section 2.3.....	24
Table 2.2.	CVD risk factors and their associations with fibrinolysis.....	30
Chapter 3		
Table 1.	Characteristics of the South African PURE study population....	80
Table 2.	Non-biochemical cardiovascular disease risk factors and their association with CLT.....	82
Table 3.	Biochemical cardiovascular disease risk factors and their association with CLT.....	84
Chapter 4		
Table 1	$PAI\text{-}1_{act}$ levels and clot lysis time of PAI-1 promoter area polymorphisms.....	123
Table 2	$PAI\text{-}1_{act}$ and CLT across BMI and waist circumference categories for 5G/5G participants and those harbouring the 4G genotype.....	125
Table 3	4G/5G genotype gene-environment interactions for $PAI\text{-}1_{act}$ and CLT.....	126
Supplemental table 1	Sequences used for PAI-1 promoter area.....	127
Supplemental table 2	PAI-1 promoter area polymorphisms.....	128
Supplemental table 3	Primers and probes used for genotyping of PAI-1 polymorphisms.....	129
Supplemental table 4	Characteristics of total study population, urban and rural participants (Pieters *et al.,* 2011)……....	130

Supplemental table 5 — Significant gene-environment interactions for the C428T and G429A genotypes in determining PAI-1_{act} and CLT - the effect of urbanisation..... 134

LIST OF FIGURES

		Page
Figure 2.1.	Model for the causal association of haemostatic factors and cardiovascular disease (adapted from Pearson *et al*., 1997).....	10
Figure 2.2.	Three phases of coagulation (Compiled from Ajjan & Grant, 2006; Monroe & Hoffman, 2006; Hoffman & Monroe, 2007)……..	12
Figure 2.3.	Formation of protofibrils (Mosesson, 2003).....	14
Figure 2.4.	The fibrinolytic process (adapted from Ajjan & Grant, 2006; Rijken & Lijnen, 2009).....	18
Figure 2.5.	Determination of CLT from turbidity curves.....	26

LIST OF ADDENDA

		Page
ADDENDUM A	PURE informed consent form.....	165
ADDENDUM B	Plasma fibrinolytic potential protocol.....	169
ADDENDUM C	Chapter 3 – Published article.....	172

LIST OF ABBREVIATIONS

ANCOVA	Analysis of co-variance
ANOVA	Analysis of variance
AP-1	Activator protein-1
ARNTL	Aryl hydrocarbon receptor nuclear translocator-like
ATTAC	The arterial thrombosis at young age: the role of TAFI and other coagulation factors study
BAEC	Bovine aorta endothelial cells
BMI	Body mass index
CAD	Coronary artery disease
CI	Confidence interval
CloFAL	Clot Formation and Lysis
CLT	Clot lysis time
cm	Centimeters
CRABIS	Prevention Program Krakow–Atherosclerosis Bio-imaging Study
CRP	C-reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
°C	Degrees Celsius
DBP	Diastolic blood pressure
DCLT	Dilute clot lysis time
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DVT	Deep vein thrombosis
DWBCLT	Dilute whole-blood clot lysis time
ECLT	Euglobulin Clot Lysis Time
ELA	Euglobulin lysis area
F	Female
FA	Fibrinolytic activity
FnDP	Fibrin degradation products
g	Gravitational force
GFC	Global fibrinolytic capacity
GFP	Global fibrinolytic potential

Glu	Glutamic acid
g/day	Gram per day
g/l	Gram per litre
GWAS	Genome-wide association study
HbA1c	Glycosylated haemoglobin
HDL	High-density lipoprotein
HDL-cholesterol	High-density lipoprotein cholesterol
HIV	Human immunodeficiency virus
IU/ml	International units per millilitre
kd	Kilo dalton
LDL	Low-density lipoprotein
LETS	Leiden Thrombophilia Study
Lys	Lysine
M	Male
MEGA	Multiple Environmental and Genetic Assessment study
MI	Myocardial infarction
µg/ml	Microgram per millilitre
µg/l	Microgram per litre
µM	Micromolar
µmol/L	Micromol per litre
min	Minutes
mg/l	Milligram per litre
mmol/l	Millimol per litre
N	Size of study population
Ng/ml	Nanogram per millilitre
nm	Nanometer
NPHS	Northwick Park Heart Study
OCP	Overall coagulation potential
OFP	Overall fibrinolysis potential
OHP	Overall haemostatic potential
PAI-1	Plasminogen activator inhibitor type-1
PAR-4	Protease-activated receptor 4
pM	Picomolar

PPARG	Peroxisome proliferator-activated receptor gamma
PURE study	Prospective Urban and Rural Epidemiological study
R	Rural
SBP	Systolic blood pressure
SD	Standard deviation
SERPINS	Serine protease inhibitors superfamily
SMAC	Sequential multiple analyser computer
SMILE	Study of Myocardial Infarctions Leiden
SNP	Single nucleotide polymorphism
TAFI	Thrombin-activatable fibrinolysis inhibitor
TBS	Tris-buffered saline
TC	Total cholesterol
TF	Tissue factor
TFBS	Transcription factor binding site
TFPI	Tissue factor pathway inhibitor
THUSA	Transition and Health during Urbanisation in South Africa
TM	Thrombomodulin
t-Hcy	Total homocysteine
t-PA	Tissue plasminogen activator
U/ml	Units per millilitre
u-PA	Urokinase-type plasminogen activator
U	Urban
VLDL	Very-low-density lipoprotein
VTE	Venous thromboembolism
vWF	Von Willebrand factor
WC	Waist circumference

CHAPTER 1: Introduction

1.1. BACKGROUND

In recent years the prevalence of cardiovascular disease (CVD) has increased considerably in the black population of South Africa. This increase in CVD prevalence may be attributed to urbanisation, which has been shown to be associated with changes in diet and physical activity that may negatively influence body composition and lipid concentrations (Vorster, 2002). Early in the development of CVD, plaques form in the vessel wall and, when a plaque becomes unstable and ruptures, the coagulation cascade is activated (Ajjan & Grant, 2005). In the final steps of coagulation, fibrin fibres are formed, which branch into a three-dimensional network known as a clot (Ryan *et al*., 1999). The ability of the body to dissolve such clots plays an important role in CVD. The longer the clot is present in the vasculature, the higher the likelihood of partial or complete occlusion of the blood vessel, resulting in a CVD event such as stroke, myocardial infarction (MI) or deep vein thrombosis (DVT). Incomplete dissolution of clots may also result in smaller pieces breaking off and travelling down the vasculature, resulting in an embolism when it blocks a smaller blood vessel downstream.

The ability of the body to lyse clots can be measured in different ways. Activity and concentration of the individual components of the fibrinolytic system, such as tissue plasminogen activator (t-PA) or plasminogen activator inhibitor type-1 (PAI-1), can be measured. Alternatively, one can measure the global ability of blood or plasma to lyse clots with the use of global fibrinolytic assays. These assays give an indication of the speed with which the body can break down clots, often reported as lysis time, but they do not provide information on the individual components of the fibrinolytic system. Several global fibrinolytic assays exist but not all of them reflect the true global potential of the body to lyse existing clots. Recently, a method which is thought to be a reliable reflection of global plasma fibrinolytic potential was developed and, for the purpose of this study, we propose to use this global fibrinolytic assay of Lisman *et al.* (2005).

Most of the existing literature regarding the role of fibrinolysis in CVD was obtained by analysis of activity and concentration of the individual components of the fibrinolytic and coagulation systems (Mertens & van Gaal, 2002; Hawkins, 2004). To

date, only a small number of studies have employed the use of global assays to determine the role of fibrinolysis in CVD (Anand *et al*., 2003; Carter *et al*., 2007; Meltzer *et al*., 2009a). As previously mentioned, however, many of these assays were not a reflection of the true potential of plasma clot lysis, limiting the interpretation and extrapolation of their results. Only a few large epidemiological studies on the use of these assays are available (Meade *et* al., 1993; Guimarães *et al.,* 2009; Meltzer *et* al., 2008; Meltzer *et al*., 2009a; Siegerink *et al.,* 2011) and there are no data on Africans.

The assay of Lisman *et al.* (2005) will be used to measure the global plasma fibrinolytic potential in 2 000 Tswana-speaking black South Africans who form part of the Prospective Urban and Rural Epidemiological (PURE) study. The role in CVD of global plasma fibrinolytic potential (GFP), expressed as clot lysis time (CLT), will be investigated in this population by determining its relation to other CVD risk factors. Because a large portion of the variance of CLT is attributed to PAI-1 (Meltzer *et al.,* 2010a), we will also investigate the effect of three polymorphisms in the promoter region of the PAI-1 gene on PAI-1_{act} levels and whether changes in PAI-1_{act} levels from these polymorphisms translate to shorter or longer clot lysis times.

1.2. AIM AND OBJECTIVES

Aim: To investigate global plasma fibrinolytic potential (GFP) and its relation with CVD risk factors in apparently healthy black South Africans.

Objectives:

- To analyse global fibrinolytic potential using a turbidimetric clot lysis assay (Lisman *et al.,* 2005) in the South African PURE study population and to determine the association between global fibrinolytic potential and CVD risk factors.

- To determine the effect of urbanisation on global fibrinolytic potential.

- To identify genetic polymorphisms in the promoter area of the PAI-1 gene in this study population and to measure the prevalence of selected polymorphisms.
- To determine both the independent effect of these polymorphisms as well as gene-environment interactions on PAI-1_{act} and global fibrinolytic potential in this study population and whether urbanisation influenced this.

1.3. STRUCTURE OF THIS THESIS

This thesis is presented in article format. The technical aspects follow the guidelines of the North-West University, and the document has been edited by a competent language editor. References for Chapters 1, 2 and 5 are provided at the end of the thesis, while references for Chapters 3 and 4 are provided at the end of the respective chapters as it forms part of the individual manuscripts. This introductory chapter includes a background to the study and the motivation for undertaking it, as well as the main aim and objectives of the study, the research team involved and the structure of the thesis.

Chapter 2 gives a review of the relevant literature, including literature on the process of blood coagulation and fibrinolysis and the various factors that play different roles in these two processes; the 4G/5G polymorphism in the promoter area of the PAI-1 gene is also reviewed. The literature reviewed in this chapter gives the background necessary for interpretation of data in Chapters 3 and 4.

Chapter 3 consists of an article published in 2012, titled “Plasma clot lysis time and its association with cardiovascular risk factors in black Africans” (*PLOS ONE*, vol. 7(11):e48881) and was prepared according to the journal specifications of *PLOS ONE*. In this article, clot lysis time and its association with various traditional and novel CVD risk factors was investigated.

Chapter 4 presents an article pertaining to genetic polymorphisms of the PAI-1 gene and the effect of these polymorphisms on PAI-1_{act} and clot lysis time. This

manuscript was prepared according to the authors' guidelines of the journal *Thrombosis and Haemostasis* and was submitted for publication in April 2013.

In Chapter 5, we will give a summative assessment of the data generated in the study by means of revisiting the main aim and objectives and highlighting the contribution of the thesis to the greater scientific knowledge base. The main focus of this chapter is the conclusions that can be drawn from the results of this research topic and recommendations for future research.

1.4. RESEARCH TEAM AND CONTRIBUTIONS TO ARTICLES PRESENTED AS PART OF THIS THESIS

Initials, surname and signature*	Affiliation	Role in the study
Miss Z. de Lange 	Centre of Excellence for Nutrition, North-West University, Potchefstroom Campus, South Africa	Full-time Ph.D. student, protocol writing, setting up of the clot lysis time assay method in the North-West University laboratory, analysis of samples, statistical analysis, interpretation of results and writing up of the literature and data (first author Chapters 3 and 4).
Prof. M Pieters 	Centre of Excellence for Nutrition, North-West University, Potchefstroom Campus, South Africa	Promoter of Ph.D. thesis, guidance regarding protocol writing, statistical analysis, interpretation of results and co-author of Chapters 3 and 4.
Prof. J.C. Jerling 	Centre of Excellence for Nutrition, North-West University, Potchefstroom Campus, South Africa	Co-promoter of Ph.D. thesis, guidance regarding protocol writing, and co-author of Chapters 3 and 4.
Dr D.C. Rijken 	Department of Haematology, Erasmus University Medical Centre, Rotterdam, The Netherlands	Co-promoter of Ph.D. thesis, guidance regarding protocol writing and sample analysis, interpretation of results and co-author of Chapters 3 and 4.
Dr T.Hoekstra 	Department of Clinical Epidemiology, Leiden University Medical Centre, Leiden, The Netherlands	Co-author of Chapter 4, laboratory analysis of polymorphisms and writing pertaining to the genetic aspects of the paper.
Dr K. Conradie 	Centre of Excellence for Nutrition, North-West University, Potchefstroom Campus, South Africa	Co-author of Chapter 4, laboratory analysis of polymorphisms and writing pertaining to the genetic aspects of the paper.
Prof. A. Kruger 	Africa Unit for Transdisciplinary Health Research, North-West University, Potchefstroom Campus, South Africa	South African PURE study coordinator.
*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 Zelda de Lange.		

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CHAPTER 2:

Global fibrinolytic potential in black South Africans

2.1. INTRODUCTION

Cardiovascular disease (CVD) has become a major health problem, with myocardial infarction and cerebrovascular disease ranked as the first and second leading causes of mortality in the world (Mathers & Loncar, 2006). In recent years the prevalence of CVD morbidity and mortality has also increased in black South Africans (Amira *et al*., 2006; Steyn, 2007; Stewart *et al*., 2011). This increase in CVD prevalence is attributed to the urbanisation of the black South African population, as the massive migration of rural populations to urban areas is associated with changes in diet and physical activity which may negatively influence body composition and lipid values (Vorster, 2002). In the very early stages of CVD, fatty streaks form in the vessel wall and later develop into plaques (Ajjan & Grant, 2006). Later in life this plaque may become unstable and rupture, resulting in the activation of the coagulation cascade and the formation of a three-dimensional network structure known as a clot (Ryan *et al*., 1999; Ajjan & Grant, 2006). The purpose of this clot is to prevent further blood loss in the event of damage to the vessel wall. However, this clot may not grow and occlude the vessel, as it can cause a CVD event, and needs to be broken down. This process of clot breakdown is called fibrinolysis; the balance between coagulation and fibrinolysis is very delicate and when this balance is swayed in either direction the result may be detrimental. Figure 2.1 shows the effect of this balance being swayed in favour of coagulation.

In this literature review the processes of coagulation and fibrinolysis are described, as well as the roles these processes play in CVD. We investigate different methods of determining the rate of clot lysis and discuss how these methods compare with each other, and with clot formation and breakdown *in vivo*. Also investigated are the associations between traditional CVD risk factors and clot lysis, and various haemostatic variables playing important roles in the variance of clot lysis.

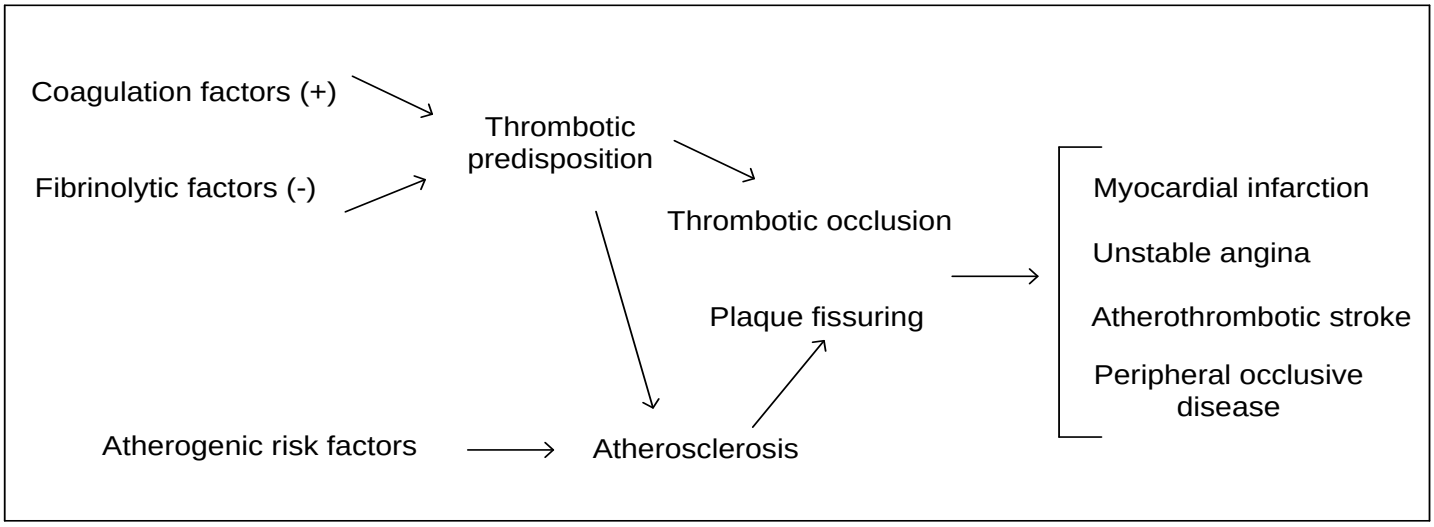


Figure 2.1. Model for the causal association of haemostatic factors and cardiovascular disease. Coagulation factors and fibrinolytic factors can act either by promoting atherosclerosis or by causing thrombotic occlusion on a plaque fissure (Pearson *et al*., 1997).

2.2. OVERVIEW OF THE COAGULATION AND FIBRINOLYSIS PROCESSES

2.2.1. Coagulation

The haemostatic system of the human body is intricately designed to maintain the fluid state of blood under normal physiological conditions, but also to limit blood loss by sealing the damaged vessel wall in the case of vascular injury (Colman, 2000:3). After injury to a vessel wall, blood is exposed to structures and proteins outside the vasculature and coagulation is initiated (Figure 2.2) (Hoffman & Monroe, 2007). The process of coagulation, also known as the coagulation cascade, can be viewed as occurring in three phases. In the first phase of the coagulation cascade, namely the initiation phase, coagulation is initiated on tissue factor (TF)-bearing cells (Hoffman & Monroe, 2007). Circulating factor VII binds to TF on the surface of a TF-bearing cell to form the FVII/TF complex (Monroe & Hoffman, 2006). FVII/TF complexes then activate FIX and FX. The activated FX (FXa) binds to factor Va on the cell surface and converts a small amount of prothrombin to thrombin (Monroe & Hoffman, 2006). FXa is localised to the cell surface by tissue factor pathway inhibitor (TFPI) and anti-thrombin (Monroe & Hoffman, 2006). Activated FIX will later be involved in additional thrombin generation (Wolberg, 2010). FIXa can move away from the TF-bearing cell to nearby platelets because it is inhibited at a slower rate than FXa by anti-thrombin and not inhibited by TFPI (Monroe & Hoffman, 2006). FIXa will interact

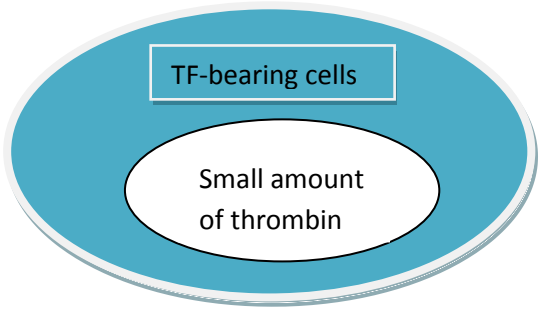
with its cofactor FVIIIa and activate FX directly on the platelet surface (Hoffman & Monroe, 2007).

The second phase of the coagulation cascade involves the amplification of the pro-coagulant signal of thrombin generated on the TF-bearing cell (Hoffman & Monroe, 2007). Large components of the coagulation cascade include platelets and FVIII bound to von Willebrand factor (vWF) (Hoffman & Monroe, 2007). These components cannot leave the vascular compartment because of their size. Von Willebrand factor is responsible for platelet adhesion to the damaged endothelium and stabilises FVIII via the formation of non-covalent complexes (Ajjan & Grant, 2006). These large components are kept separate from the extravascular compartment, but when injury disrupts the vessel wall, platelets and FVIII-vWF spill over from the vascular space into the extravascular space and bind to collagen and other matrix components at the site of damage. Thrombin cleaves FVIII, releasing it from vWf to bind to the surface of platelets that start to adhere to the site of injury, where FVIII will play a role in the activation of FX, together with FIX (Lenting *et al*., 1998; Monroe & Hoffman, 2006). The binding of platelets to collagen and vWF partially activates platelets (Ajjan & Grant, 2006; Hoffman & Monroe, 2007). Only when enough thrombin is generated in the amplification phase on or near TF-bearing cells, is full activation of platelets and activation of coagulation cofactors on the platelet surface triggered (Hoffman & Monroe, 2007). Even though the amount of thrombin generated is not enough to clot fibrinogen, it is sufficient to activate platelets and enhance the initial pro-coagulant signal and, in so doing, set the stage for generation of larger amounts of thrombin (Monroe & Hoffman, 2006).

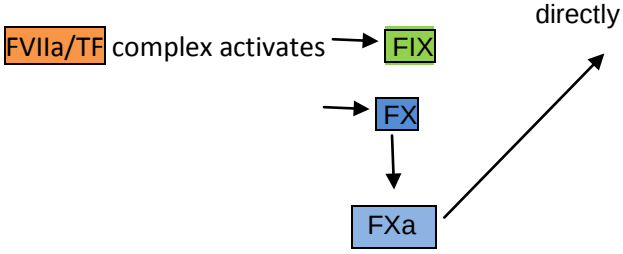
During the propagation phase of the coagulation process, enough thrombin for effective haemostasis is generated on platelet surfaces (Hoffman & Monroe, 2007). FIXa, activated during the initiation phase, can diffuse through the fluid phase and bind to FVIIIa on the platelet surface; additionally, more FIXa is supplied by platelet-bound FXIa. FIXa activates FX in the presence of calcium, phospholipids and FVIIIa. As mentioned earlier, FXa is localised to the cell surface by TFPI and anti-thrombin and forms a complex with FVa on the platelet surface to produce enough thrombin to clot fibrinogen (Hoffman & Monroe, 2007).

Initiation

After injury to the vessel wall coagulation proteins leave the vasculature by percolating through tissues found in the lymph roughly in proportion to their molecular size. FVII is probably bound to extravascular TF, even in the absence of an injury, and extravascular factors IX and X can be activated as they pass through the tissues. Large components of the coagulation system, such as platelets and FVIII complexed with vWF, are kept apart in the vascular space. Damage to the vasculature then causes platelet and FVIII/vWF to spill into extravascular tissues, thereby activating the coagulation cascade.



FXa binds to factor Va on the cell surface and converts a small amount of prothrombin to thrombin. FXa is localised to the cell surface by TFPI and anti-thrombin. FIXa can move away from the TF-bearing cell to nearby platelets. FIXa interacts with its cofactor FVIIIa and activates FX directly on the platelet surface.



Amplification

During the amplification phase, the small amount of thrombin generated cleaves FVIII, releasing it from vWf to bind to the platelet surface.
The binding of platelets to collagen and vWF partially activates platelets.
Only when enough thrombin is generated in the amplification phase on or near TF-bearing cells is full activation of platelets and activation of coagulation cofactors on the platelet surface triggered.
Even though the amount of thrombin generated is not enough to clot fibrinogen, it is sufficient to activate platelets and enhance the initial pro-coagulant signal and, in so doing, set the stage for generation of large amounts of thrombin.

Propagation

FX/FV complexes formed during the propagation phase of coagulation produce a burst of thrombin of sufficient magnitude to clot fibrinogen and stabilise the clot by:
1. activating FXIII
2. activating TAFI
3. cleaving the platelet PAR-4 receptor
4. being incorporated into clot structure.

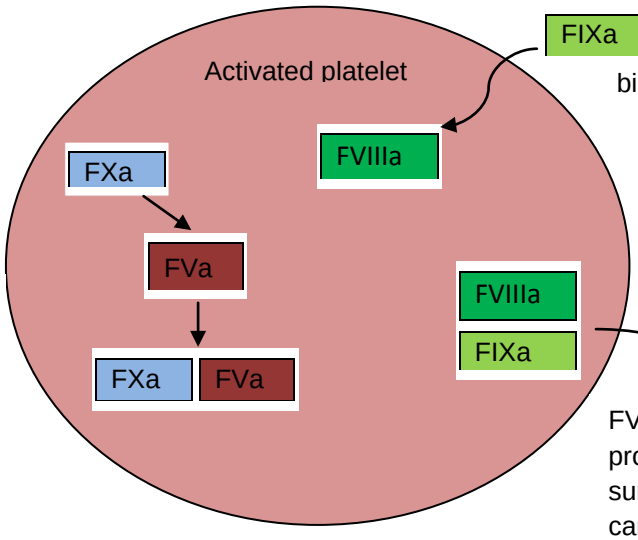


Figure 2.2. Three phases of coagulation (Compiled from Ajjan & Grant, 2006; Monroe & Hoffman, 2006; Hoffman & Monroe, 2007). TF: Tissue factor; TFPI: Tissue factor pathway inhibitor; vWF: von Willebrand factor; PAR-4: Protease-activated receptor 4.

In essence, activation of the coagulation cascade generates thrombin, which converts fibrinogen to fibrin (Colman 2000:11; Cesarman-Maus & Hajjar, 2005). Fibrinogen is a 340-kd glycoprotein with a trinodular structure, of which two D-domains are connected through a coiled-coil segment to a central E-domain (reviewed by Mosesson, 2003; Weisel, 2005). These coiled coils consist of two sets of three polypeptide chains known as the Aα-, Bβ- and γ-chains (reviewed by Mosesson, 2003). Located on the central E-domain are two pairs of A and B fibrinopeptides (Weisel, 2005). Fibrin is formed when thrombin catalyses the removal of fibrinopeptides A and B from fibrinogen, thus converting the fibrinogen molecule to a fibrin monomer. During this process, binding sites at the central domain of the molecule are revealed, which interact with complementary sites on the α- and β-chains at the end domains of other fibrin monomers (Figure 2.3; Mosesson, 2003). Fibrin monomers assemble in a half-staggered manner into two-stranded protofibrils; these protofibrils will continue to assemble until a certain length is reached and will then start to aggregate laterally to form fibrin fibres that branch into a three-dimensional network (Hantgan & Hermans, 1979). This network traps red blood cells, white blood cells and platelets to form a blood clot (Ryan *et al*., 1999). The structure of the clot is further stabilised by thrombin and calcium which activate factor XIII to cross-link fibrin fibres by a transglutaminase reaction (Ariëns *et al*., 2002).

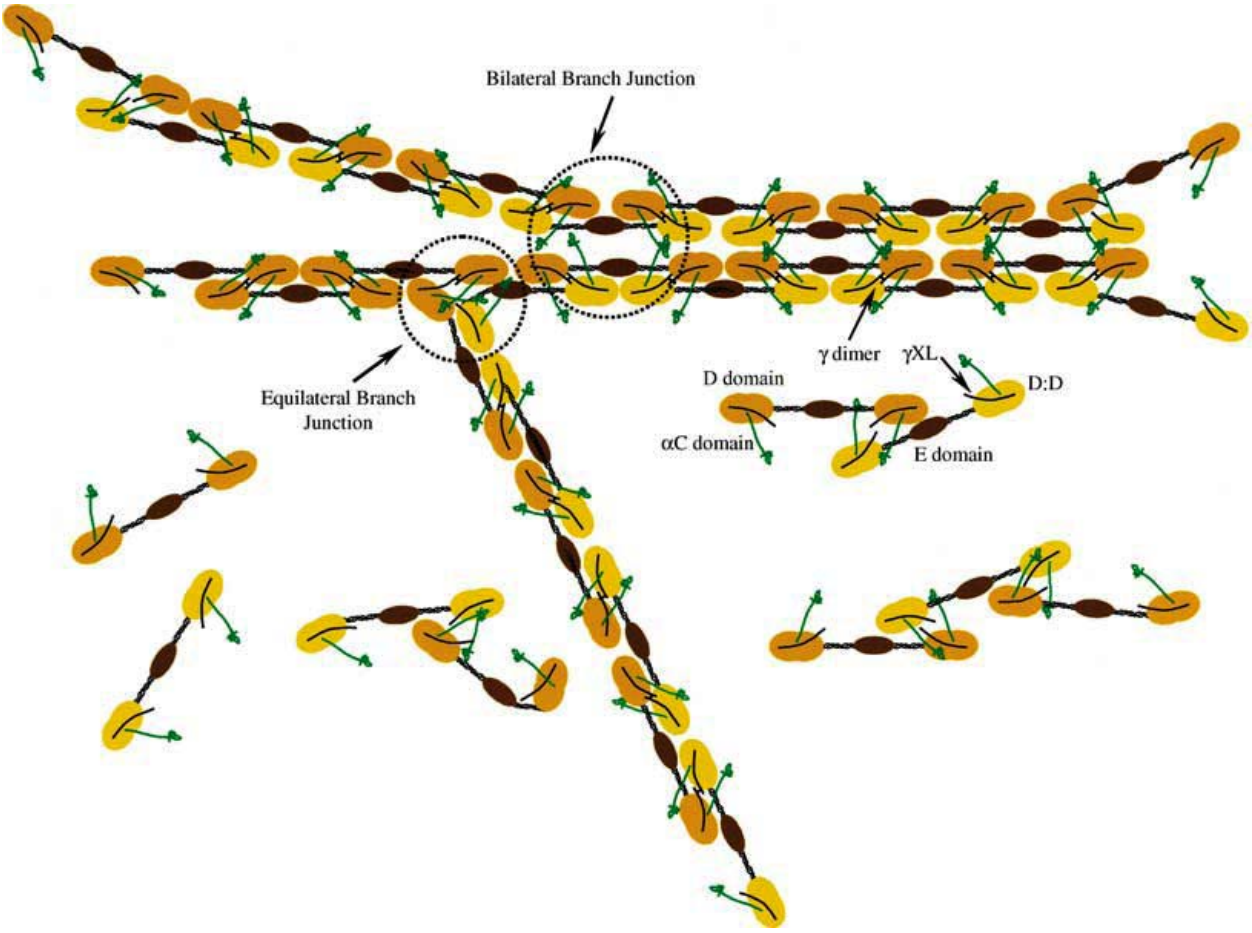


Figure 2.3. Formation of protofibrils (Mosesson, 2003). After fibrinogen is converted to fibrin through the removal of fibrinopeptides A and B, the central domain of each molecule can interact with sites on end domains of other fibrin monomers to form protofibrils.

The powerful pro-coagulant substances activated and the clot that forms must remain at the site of injury in order to prevent the coagulation cascade from being initiated elsewhere. The clot remains at the site of injury for wound healing to occur; it must also remain at the site of injury to prevent it from obstructing blood flow further in the vasculature. This is done by the pro-coagulant reactions of specific cell surfaces being localised through tissue factor pathway inhibitor and anti-thrombin (Ajjan & Grant, 2006, Monroe & Hoffman, 2006). Several mechanisms exist for the control and localisation of haemostasis (Colman, 2000:12). These mechanisms include: 1) The disruptive pull of blood flow to which the clot is exposed, which will wash away in the blood small clumps of platelets that are inadequately attached to the main body of platelets or vessel wall; 2) thrombin, which has many roles and is present inside the clot, will bind with thrombomodulin (TM) on endothelial cells. The

thrombin-thrombomodulin complex converts protein C into activated protein C, which reacts with Factors V and VIII to destroy the coagulant properties of these factors and thereby limit the effect of thrombin (Colman, 2000:13). This action inhibits further growth of the fibrin-platelet clot. 3) The third mechanism is the diffusion of activated coagulant proteins, such as Factor Xa or thrombin, away from the clot. These factors are then bound to inhibitory plasma proteins that destroy or diminish their coagulant potential (Colman, 2000:13).

The clot forms the framework on which wound healing, including endothelial cell regrowth and vessel recanalisation, occurs and will be removed during the process of healing (Colman, 2000:13; Monroe & Hoffman, 2006). The major mechanism for the breakdown of existing clots is the process of fibrinolysis, which will be discussed in the following section.

2.2.2. Fibrinolysis

The formation of fibrin triggers the activation of fibrinolysis on the surface of fibres of existing clots (Medved & Nieuwenhuizen, 2003). The process of fibrinolysis is depicted in Figure 2.4. Plasminogen, a single-chain glycoprotein produced in the liver, is activated to plasmin by tissue-type plasminogen activator (t-PA), which is the most important plasminogen activator in the circulation (Hoylaerts *et al*., 1982; Medved & Nieuwenhuizen, 2003). Another plasminogen activator, namely urokinase-type plasminogen activator (u-PA), is also present in the circulation. Both t-PA and u-PA can convert plasminogen to plasmin and are secreted as single-chain proteins; t-PA is active as a single-chain protein whereas u-PA has to be cleaved by either plasmin or FXIIa via kallikrein to become active (Vasalli et al., 1991; Rijken & Sakharov, 2001; Cesarman-Maus & Hajjar, 2005). The circulating form of plasminogen, amino-terminal glutamic acid (Glu) plasminogen, is converted by proteolysis through plasmin to a collection of modified forms, known as amino-terminal lysine (Lys) plasminogen, (Mosnier & Bouma, 2006). The modified Lys-plasminogen has a higher avidity for cellular receptors and is activated 10−20 times faster than Glu-plasminogen (reviewed by Cesarman-Maus & Hajjar, 2005). Once the plasmin is formed, it cleaves a polypeptide chain on fibrin in the cross-linked fibrin clot, preferentially just after a lysine residue, and new lysine residues are

formed for further binding of plasminogen and t-PA, which enables the formation of more plasmin and more fibrin being cleaved (Rijken & Sakharov, 2001). Fibrin fibres are cleaved transversely, as plasminogen binding sites in adjacent protofibrils spaced vertically from the cleaved fibrin molecules are closer than binding sites along the length of a protofibril (Veklich *et al*., 1998).

Several inhibitors of fibrinolysis exist in the system (Figure 2.4). Plasminogen activator inhibitor type-1 (PAI-1) inhibits both t-PA and u-PA by binding the proteins to form inert, covalent complexes and thus prevents their binding to and activation of plasminogen on fibrin (Vaughan, 2005). Plasmin is inhibited mostly by α2-antiplasmin and to a lesser extent by α2-macroglobulin (Rijken & Sakharov, 2001). Another inhibitor of fibrinolysis is thrombin-activatable fibrinolysis inhibitor (TAFI); this single-chain plasma glycoprotein is activated by thrombin in the presence of thrombomodulin and inhibits fibrinolysis by removing C-terminal lysines on partially degraded fibrin, thereby inhibiting binding of plasminogen and t-PA to fibrin and the subsequent activation of plasminogen to plasmin (Colucci & Semeraro, 2012). In the fibrinogen molecule, t-PA and plasminogen binding sites are cryptic; this prevents binding and the subsequent degradation of fibrinogen and other proteins in the blood, whereas exposure of t-PA and plasminogen binding sites on fibrin ensures restriction of fibrinolysis to places of fibrin deposition or injury (Medved & Nieuwenhuizen, 2003). The presence of fibrin increases the activation rate of plasminogen to plasmin by t-PA and the initial cleavage of fibrin by plasmin at specific binding sites further increase the rate of conversion of plasminogen to plasmin by forming new carboxyterminal lysine residues where plasminogen can bind (Hoylaerts *et al*., 1982; Suenson *et al*., 1984; Higgins & Vehar, 1987; Rijken & Sakharov, 2001). This process of binding, degradation and further binding continues, and subsequently the clot is degraded into small soluble fibrin fragments which are carried away in the circulation (Mosnier & Bouma, 2006) (Figure 2.4).

The presence of a clot in the vasculature may be a risk for partial or complete occlusion of a blood vessel, which may result in a CVD event such as stroke, myocardial infarction (MI) or deep vein thrombosis (DVT). The incomplete dissolution of clots may also lead to smaller pieces of the clot breaking off and

travelling down the vasculature, which can result in an embolism when a smaller blood vessel downstream is blocked. Therefore, the ability of the body to dissolve or break down such a clot plays an important role in CVD. In the following section, different methods which are used to measure the ability of the body to dissolve clots will be discussed.

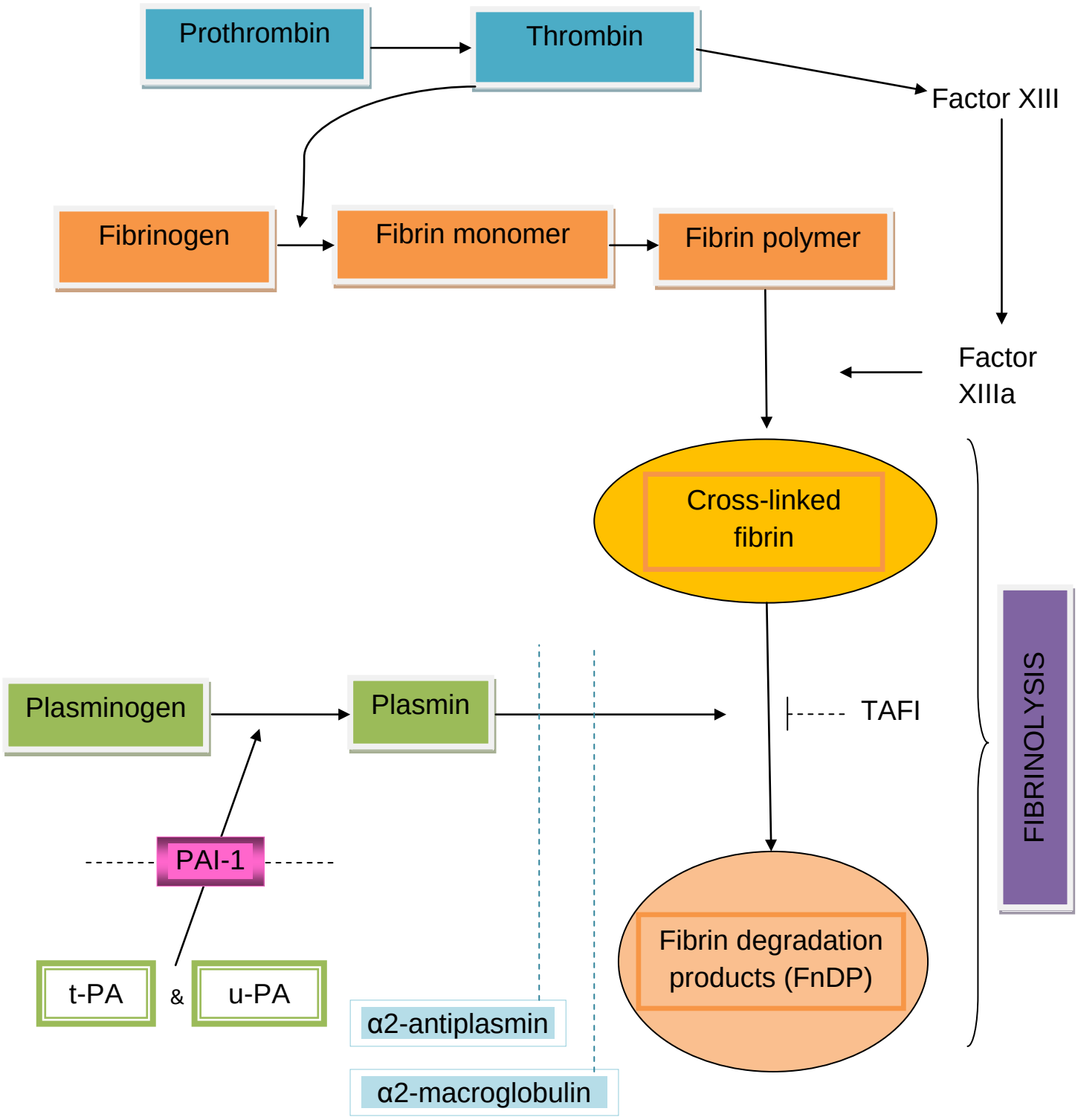


Figure 2.4. The fibrinolytic process (Adapted from Ajjan & Grant, 2006; Rijken & Lijnen, 2009). t-PA: tissue plasminogen activator; u-PA: urokinase plasminogen activator; PAI-1: Plasminogen activator inhibitor type-1; TAFI: Thrombin activatable fibrinolysis inhibitor.

2.3. DIFFERENT METHODS OF MEASUREMENT OF FIBRINOLYSIS

The ability of the body to dissolve clots can be measured in different ways. Some methods measure the activity and concentrations of individual factors involved in the coagulation and fibrinolytic processes, such as t-PA or PAI-1, while other methods aim to measure the global ability of blood or plasma to lyse clots. The latter methods are called global fibrinolytic assays and give an indication of the speed with which the body breaks down clots, often reported as lysis rate. Global fibrinolytic assays do not, however, provide information on the individual components of the fibrinolytic system. These types of assays aim to give an indication of the global ability of plasma or whole blood to break down clots; however, owing to differences in the design of these assays, not all are true reflections of an individual's fibrinolytic potential as many assays do not include all components of coagulation and clot breakdown. An assay that measures true fibrinolytic potential should be the most physiologic possible measure of an individuals' ability to lyse clots. Lisman *et al.* (2005) defines this as the potential of the body to lyse clots, taking into account the interplay between coagulation and fibrinolysis, therefore including proteins involved in both thrombin generation and fibrinolysis, such as plasminogen, α2-antiplasmin, PAI-1, TAFI and antithrombin. Therefore, such an assay should take coagulation and fibrinolysis into account as these are not two separate but highly interlinked systems. For example, thrombin that is generated at the start of coagulation, also regulates the inhibitor TAFI, which plays an important regulatory role in lysis.

Even though differences in the design of assays make it difficult to compare clot lysis times across studies, it is possible to compare the clot lysis times of participants from the same study.

In the following section, different global fibrinolytic assays available in the literature will be discussed and compared. Most of these assays are based on a clotting model in which citrated plasma is clotted using thrombin or tissue factor, and in which subsequent clot lysis takes place through exogenously added t-PA. In most assays absorbance is measured over time. Methods investigating the fibrinolytic potential in whole blood will also be discussed.

The Euglobulin clot lysis time (ECLT) method, which for many years was the most extensively used method of determining fibrinolysis until it was realised that PAI-1 is partially precipitated, is not included in this section. The euglobulin fraction used does not really contain inhibitors and the interplay between coagulation and fibrinolysis through TAFI and also FXIII is excluded. It seems that it is only the interaction of fibrinogen, plasminogen and plasminogen activators which is being measured (Kowalski *et al*., 1959; Lisman *et al*., 2005).

Firstly, turbidity methods in which citrated plasma were used will be described. A turbidimetric clot lysis assay used by Undas *et al*. (2006) to investigate the influence of total homocysteine levels on fibrinolytic potential will now be described. In this assay, citrated plasma is combined with a buffer which contains calcium chloride, thrombin to initiate coagulation, and t-PA (Undas *et al*., 2006). Clots are formed and absorbance measured at 405 nm in duplicate aliquots. Lysis time is defined as the time that is required for gel turbidity to decrease by 50% from the maximum optical density, as described by Bajzar *et al*., (1990). This may mean that the clotting time of clots is not taken into consideration with this assay. However, a very high concentration of t-PA is used in this assay, which may lead to shorter lysis times.

A simple and rapid method for the determination of overall haemostatic potential in plasma (OHP) was developed by He *et al*. (1999). Citrated plasma is diluted with a mixture of Tris, NaCl, $CaCl_2$, thrombin and t-PA. Absorbance is then measured at 405 nm every minute for 30 minutes. A fibrin time curve is drawn and the area under the curve expressed by a summation of absorbance values (He *et al*., 1999). As fibrinogen and plasminogen are present in the plasma, when the fibrin aggregation curve is created fibrinogen is converted to fibrin by generated thrombin. The exogenous t-PA added in the assay, together with plasminogen, generates plasmin which digests fibrin (He *et al*., 1999). Thus, each absorbance value represents the fibrin level at that time, and the area under the curve (the absorbance sum) provides information regarding fibrin generation and lysis throughout the 30 minutes of measurement (He *et al*., 1999).

He *et al.* (1999), who developed the OHP assay, went on to modify their method in such a way that it can be used for routine laboratory and research work. In the

preliminary study (He *et al*., 1999), OHP was found to be higher in coagulation-deficient plasma than was expected; a possible reason for these high values could have been the relatively high concentration of thrombin (0.2 IU/ml) used in the assay (He *et al*., 2001). In the new assay a smaller amount of thrombin (final concentration 0.09 IU/ml) is used and two fibrin aggregation curves are generated. One curve is the overall haemostatic potential (OHP) and the other curve the overall coagulation potential (OCP), to which no t-PA is added. Two assay mixtures are prepared, one containing $CaCl_2$, thrombin and a Tris buffer, pH 7 for the OCP, and another including the above reagents as well as t-PA for OHP (He *et al*., 2001). Each assay mix is then added to plasma and absorbance measured every minute for 40 minutes. Again the area under the curve is expressed as a summation of the absorbance values. The difference between the OHP curve and the OCP curve reflects the overall fibrinolysis potential (OFP), calculated as OFP= ((OCP-OHP)/OCP) x100% (He *et al*., 2001). The researchers suggest using OCP and OFP as supplementary parameters to OHP, providing information regarding underlying changes in the coagulation and/ or fibrinolytic system (He *et al*., 2001). However, because thrombin is used to initiate clotting in this assay, part of the coagulation cascade and factors involved are not represented.

Carter *et al*. (2007) published clot lysis time results by combining data from a turbidimetric clotting assay without t-PA and the same assay with t-PA added to facilitate fibrinolysis. In this assay, citrated plasma is added to a Tris buffer activation mix containing thrombin and calcium. For the turbidimetric lysis assay, t-PA is added to the assay buffer before adding the activation mix (Carter *et al*., 2007). Clot formation and lysis is measured in a plate reader at 340 nm every 12 seconds for an hour and after that every two minutes for nine hours (Carter *et al*., 2007). Fifty percent lysis time and time to complete lysis was determined from the turbidity curves (Carter *et al*., 2007). Time to complete lysis was defined as the time from maximum absorbance to the time absorbance returns to baseline (Carter *et al*., 2007). In this assay, however, coagulation is initiated with thrombin and not tissue factor, thus excluding the interplay of various factors in the coagulation cascade from clot formation and lysis.

Up to now, the studies discussed used thrombin to initiate clotting. The assays discussed in the following section are also turbidimetric assays performed with citrated plasma, but tissue factor, which is considered to be more physiologically relevant in these assays, is used for initiation of coagulation.

In the study of Leander *et al.* (2012) the turbidimetric assay of He *et al.* (2001) was modified by replacing thrombin with tissue factor. In this assay, citrated plasma is mixed with a t-PA solution in a 96-well plate, after which a coagulation trigger solution containing tissue factor, a purified phospholipid mixture and $CaCl_2$ is added. Optical density was recorded every 30 seconds for 300 minutes to a turbidity curve (Leander *et al.,* 2012). Clot lysis time (CLT) was determined by using this curve and was defined as the clotting maximum value to become reduced by 50%. The CLT is expressed in seconds (Leander *et al.,* 2012). This assay is sensitive to fibrinolysis inhibitors PAI-1 and TAFI (He *et al*., 2007).

Colucci *et al*. (2008) use a turbidimetric fibrinolysis assay in which citrated plasma is combined with thromboplastin (a mixture of tissue factor and phospholipids to initiate coagulation), phospholipid vesicles, t-PA, Tris-buffered saline and $CaCl_2$. Absorbance is measured at 405 nm and a temperature of 37°C every five minutes for up to three hours, and a curve representing clot formation and subsequent clot breakdown is plotted (Colucci *et al*., 2008). The researchers defined fibrinolysis time as the time from the midpoint of clear-to-maximum-turbid transition to the midpoint of maximum-turbid-to-clear transition (Colucci *et al*., 2008). Compared with other studies, a very small concentration of t-PA is used in this assay in order to keep the t-PA concentration close to physiological t-PA concentrations (Colucci *et al*., 2008). These researchers suggest that the presence of high t-PA concentrations in the other assays may inhibit the role that TAFI plays in the inhibition of fibrinolysis. In this model, however, TAFI did not emerge as an independent predictor of lysis time when using low concentrations of t-PA (Colucci *et al*., 2008).

The Clot formation and lysis (CloFAL) assay (Goldenberg *et al*., 2005) is also an assay that makes use of citrated plasma and uses tissue factor for clotting initiation. In this method, citrated platelet-poor plasma is diluted 50% with reactant solution consisting of lipidated tissue factor, t-PA, and Tris-buffered saline containing $CaCl_2$ in

a 96-well plate (Goldenberg *et al*., 2005). Three wells with reactant solution and one blank well (containing only Tris-buffered saline) are included for each participant (Goldenberg *et al*., 2005). Absorbance is measured at 405 and 630 nm at 45-second intervals for three hours, and the values plotted on a graph. In order to eliminate artefact in baseline absorbance values, absorbance data are blanked by subtracting 630 nm values from the 405 nm value and also by subtracting the TBS blank well data from reagent well data (Goldenberg *et al*., 2005). This reflects the dynamic contributions of ongoing coagulation activation and fibrinolysis reactions during the process of clot formation and fibrinolysis (Goldenberg *et al*., 2005). The researchers, reported, however, that despite shortened lysis time in PAI-1 deficient plasma, the expected brisk decline in absorbance was not observed and this might be an indication that the CloFAL assay is not sensitive to PAI-1 (Goldenberg *et al*., 2005).

In some assays the fibrinolytic potential of whole blood is determined. The following methods discussed are not turbidimetric methods but are based on observation of clot formation and breakdown. One method described in the literature is the dilute whole-blood clot lysis time (DWBCLT) assay, with some studies describing minor modifications to the method (Spittle *et al*., 1968; Meade *et al.,* 1993; Gadbut *et al.,* 1999). In the paper by Meade *et al.* (1993) fibrinolytic activity of participants from the Northwick Park Heart Study was determined with the DWBCLT. For this method, whole blood is diluted in a test tube with a thrombin-phosphate buffer mixture (in some variations the blood is diluted in a phosphate buffer and the thrombin added later). The tube is kept on ice until a clot has formed, after which the tube is placed in a water bath at 37°C. Clots are observed at regular intervals and the time of complete dissolution is noted (Meade *et al.,* 1993). Fibrinolytic activity is expressed as 100/lysis time in hours, with a low reading indicating low fibrinolytic activity and slow clot dissolution (Meade *et al.,* 1993). An advantage of the DWBCLT assay is that lysis time depends on endogenous plasminogen activators; however, because the assay is performed in the absence of calcium, and the clot is formed by the addition of thrombin instead of tissue factor, the lysis time is independent of thrombin generation and, as a consequence, independent of TAFI and should be interpreted with caution (Lisman *et al*., 2001; Cellai *et al*., 2010).

Rijken *et al*. (2007) also developed a method for measuring the global fibrinolytic capacity (GFC) in whole blood. Blood is collected in tubes with and without aprotinin (an inhibitor of proteolytic enzymes including plasmin). After three hours of incubation the clotted blood is centrifuged and the serum collected. This is supplemented with aprotinin and analysed for fibrin degradation products (FnDP) with the use of an enzyme immunoassay (Rijken *et al*., 2007). The GFC (expressed in µg/ml) was determined by calculating the difference between the FnDP level obtained in the blood sample which was incubated in the absence of aprotinin, and the FnDP level from the sample incubated in the presence of aprotinin (Rijken *et al*., 2007). A possible limitation in using this assay is that it is not always possible to perform laboratory analyses directly after blood collection, especially in large studies. Concentrations of thrombin, tissue factor and t-PA used the studies discussed above are presented in Table 2.1.

Table 2.1. Concentrations of coagulation and lysis agents used in assays discussed in section2.3

Reference	**Coagulation agent and concentration**	**Lysis agent and concentration**
Undas *et al*., 2006	Thrombin [0.5 U/ml]	tPA [500 ng/ml]
He *et al*., 1999	Thrombin [0.2 IU/ml]	tPA [700 ng/ml]
He *et al*., 2001	Thrombin [0.04 IU/ml]	tPA [300 ng/ml]
Carter *et al*., 2007	Thrombin [0.03 U/ml]	tPA [83 ng/ml]
Leander *et al*., 2012	Tissue factor [2.1 pM]	tPA [133 ng/ml]
Colucci *et al*., 2008	Thromboplastin (tissue factor and phospholipid mix) 1000x diluted	tPA [15 ng/ml]
Goldenberg *et al*., 2005	Tissue factor [5 pM]	tPA [450 ng/ml]
Meade *et al*., 1993	Thrombin [2.5 U/ml]	-
Rijken *et al*., 2007	0.3 NIH U/ml	-

As can be seen from the literature, several global fibrinolytic assays exist, but not all of them reflect the true global potential of the body to lyse existing clots. First of all, most of the assays are performed using plasma rather than whole blood (Prins & Hirsh, 1991). These assays therefore do not reflect true *in vivo* clot lysis, as the cellular component present in whole blood is not present in the plasma system. Such assays therefore have the potential to reflect the global ability of plasma only to lyse clots. As discussed, the experimental design of many of the assays also does not allow for a true reflection of global plasma clot lysis as not all of them include all the coagulation and fibrinolysis components in plasma.

For the purpose of this study we propose to use the global fibrinolytic assay of Lisman *et al.* (2001; 2005), which is thought to be a true reflection of global plasma fibrinolytic potential. This is a turbidity assay in which citrated plasma is diluted with an activation mix containing tissue factor, phospholipids, $CaCl_2$ and t-PA. With this method, coagulation is initiated by the addition of tissue factor, which means that coagulation is dependent on the body's own coagulation cascade. Clots are lysed by exogenously added t-PA, which corresponds to a massive release of t-PA from the endothelium *in vivo* after vessel wall injury (Lisman *et al*., 2001). Also, the outcome of the assay has been shown to be influenced by levels of plasminogen, alpha2-antiplasmin, PAI-1 and TAFI (Meltzer *et al*., 2009a). The time it takes for the formed clot to lyse is known as the clot lysis time (CLT) and is defined as the time from the midpoint of clear-to-maximum-turbid transition (50% clotting time), representing clot formation, and the midpoint of maximum-turbid-to-clear transition (50% lysis time), representing clot breakdown (Lisman *et al*., 2001) (Figure 2.5).

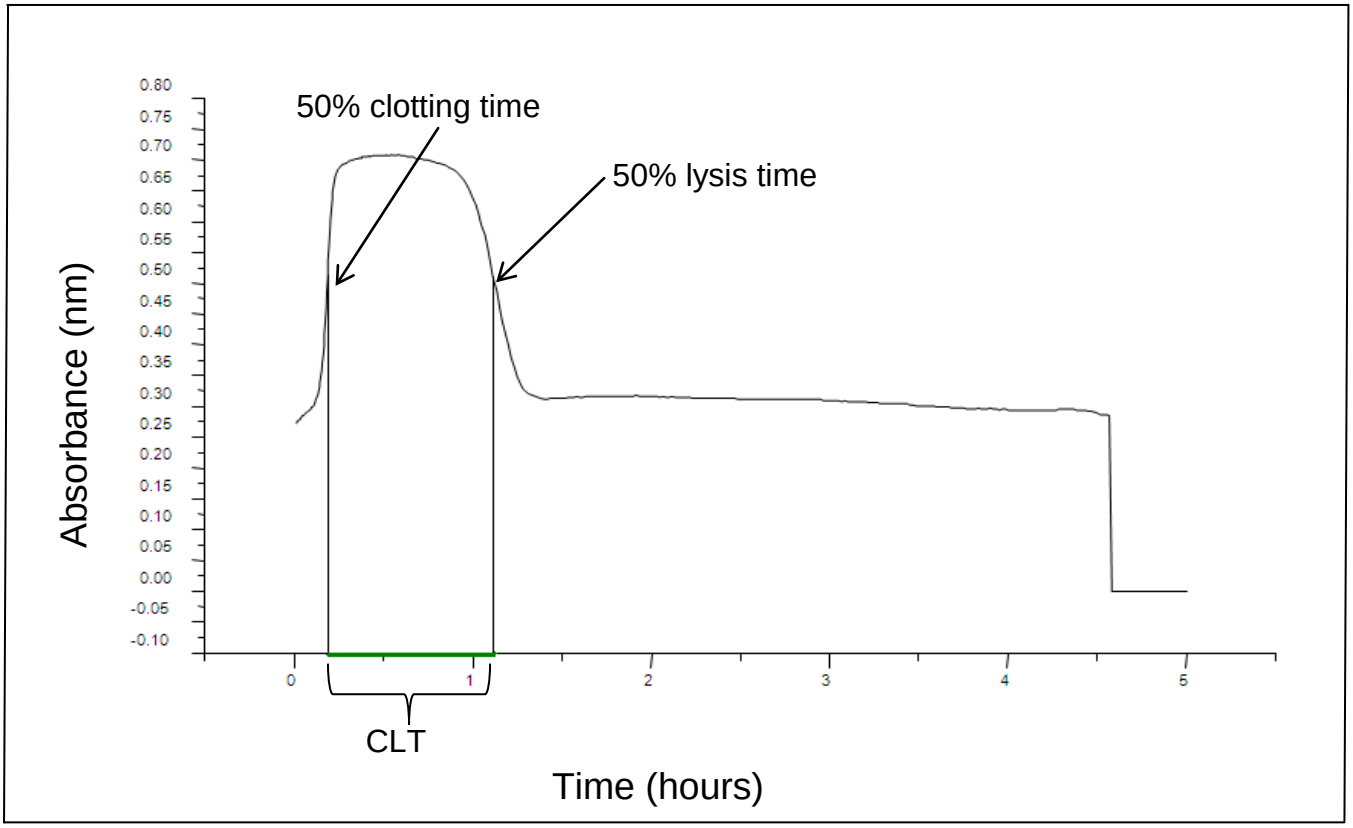


Figure 2.5. Determination of CLT from turbidity curves

Individual components at play in the fibrinolytic and coagulation systems and the activities and concentrations of these components have mainly been used to describe the role that fibrinolysis play in CVD (Mertens & van Gaal, 2002; Hawkins, 2004). Until now, only a small number of studies have reported on the use of global assays and the role of fibrinolysis in CVD (Anand *et al*., 2003; Carter *et al*., 2007; Meltzer *et al*., 2009a; Leander *et al*., 2012). However, as mentioned earlier, many of these assays were not a reflection of the true potential of plasma clot lysis, thus, limiting the interpretation and extrapolation of their results. To date, only a few large epidemiological studies have reported on the use of these assays in CVD-risk prediction (Meade *et* al., 1993; Meltzer *et* al., 2008; Meltzer *et al*., 2009a; Leander *et al*., 2012) and no data are available on Africans, a little-studied population in CVD aetiology.

2.4. FIBRINOLYSIS AND CVD

In this section the prevalence of CVD in black South Africans as well as the role played by fibrinolysis in CVD will be discussed.

2.4.1. CVD prevalence in black South Africans

The prevalence of CVD in the black South African population has increased significantly in recent years (Amira *et al*., 2006). A comparison of mortality figures from 1984 to 1986 shows that mortality from CVD in urban black Africans results mainly from stroke (96.4 per 100 000), followed by hypertension, diabetes and ischaemic heart disease (Bradshaw *et al*., 2000). More recently, Norman *et al*. (2006) reported age-standardised mortality rates for stroke in the black South African population to be double (145 for men and 160 for women per 100 000 deaths) that of the white population (72 for men and 84 for women per 100 000). This increase in prevalence of CVD may be due to epidemiological transition in this population, as the rapid migration of black South Africans to urban centres has been associated with increased poverty, obesity, hypertension and cholesterol levels, decreased physical activity and the Westernisation of diets consumed (Yusuf *et al*., 2001b; Vorster, 2002). Results of a study on CVD in Soweto, which has one of the largest black urban populations in South Africa, showed that the complexity and spectrum of CVD in black South Africans has broadened over the past years (Sliwa *et al.*, 2008). The combinations of hypertension with smoking and hypertension with obesity appear to specifically favour the development of CVD in black South African men and women (Seedat *et al*., 1992). In fact, these combinations, together with the increased fibrinogen concentrations in Black South Africans, may increase even further the risk for developing CVD (Voster, 2002).

2.4.2. Involvement of fibrinolysis in CVD risk

Recent developments in the measurement of fibrinolytic activity have shown reduced fibrinolytic activity or hypofibrinolysis in subjects with venous thrombosis (Lisman *et al*., 2005; Meltzer *et al.,* 2008). A few studies have also shown associations between prolonged CLT and arterial thrombosis in younger individuals (Meade *et al.,* 1993; Guimarães *et al*., 2009; Meltzer *et al*., 2009a; Siegerink *et al*., 2011). Thus, hypofibrinolysis may increase the risk of CVD. In fact, some studies have found that

the association between CLT and venous and arterial thrombosis remains, even after adjustment for individual fibrinolytic factors (Meltzer *et al*., 2010a; Leander *et al.,* 2012). This may suggest that CLT is not only a reflection of concentrations of the various fibrinolytic factors involved in clot breakdown, but that it could be a CVD risk factor on its own.

Arterial and venous thrombi may differ owing to differences such as blood flow velocity, blood pressure and valves in the veins of lower limbs in the environments where the thrombi are formed (Rosendaal, 2003). It seems that there may also be differences in the way venous and arterial thrombi are formed. Damage to the vessel wall initiates the coagulation cascade and the formation of thrombi in arteries, the process of which is described in detail in section 2.2.1. It has, come to light through pathological studies, however, that vessel wall injury is not always present in the formation of venous thrombi, and that most venous thrombi consist of two different regions (Sevitt, 1974). One region, namely the red thrombus, consists mostly of fibrin and erythrocytes, and seems to attach the thrombus to the vessel wall (Sevitt, 1974). The region found away from the site of attachment and composed mainly of aggregated platelets is called the white thrombus (Sevitt, 1974). These two types of regions of the thrombi suggest that coagulation precedes activation of platelets and aggregation during the formation of venous thrombi and that fibrin formation is a primary event in venous thrombosis (Poredos & Jezovnik, 2007). The presence of leucocytes in the red thrombus may suggest that the initiation of the coagulation cascade in the case of venous thrombi could be a consequence of inflammation (Sevitt, 1974).

Marked activation of the endothelium and also of platelets and leucocytes is present in the event of venous thrombosis, and is associated with the release of endothelial microparticles, which then interact with leucocytes (Chirinos *et al*., 2005; Poredos & Jezovnik, 2007). Tissue factor, which is the main trigger of thrombus formation, is expressed by activated endothelial cells (Poredos & Jezovnik, 2007). Also, activated endothelial cells express both P-selectin and phosphatidylserine where microparticles, which are also sources of tissue factor, can bind (Hamilton *et al*., 1990; Poredos & Jezovnik, 2007). It is thus possible that, in large veins where thrombi do not develop from damage to the vessel wall, thrombosis is initiated by

tissue factor-bearing particles interacting with the activated endothelium in the same way activated platelets would (Del Conde & Lopez, 2005).

With the differences shown between arterial and venous thrombi, there may be differences in the lysis of these clots. These differences can be likened to differences in methods of investigating lysis (as discussed in section 2.3). In some methods lytic agents (t-PA) are added after clot formation and in others these agents are added during clot preparation and in this way incorporated in the clot (as, for example, in the Plasma fibrinolytic potential clot lysis assay of Lisman *et al*., 2001) (Sakharov *et al*., 1996). The possible differences in lysis for arterial and venous thrombosis are summarised by Sakarov *et al.* (1996). It is possible that in some cases of arterial thrombosis, the sudden rise in blood pressure across the occluding thrombus will rapidly force lytic agents from fibrinolytic therapy to the inside of the clot, from where this clot can be quickly lysed (Sakarov *et al.,* 1996). When no pressure is applied, as in the case of large venous thrombi, lytic agents slowly diffuse into the clot and lysis proceeds from the outer surface of the clot towards the middle (Sakarov *et al.,* 1996).

Just as inflammation may increase the risk for thrombosis, as described in the above section, various other factors may also be associated with increased, or perhaps decreased, risk for hypofibrinolysis and, possibly, CVD. Only a small number of studies have investigated associations of fibrinolysis (using global assays) with CVD risk factors, and therefore data on this subject are scarce. The available data are presented in Table 2.2.

2.4.3. Associations between fibrinolysis and individual (traditional) CVD risk factors

In Table 2.1, studies reporting on the associations between fibrinolysis and traditional cardiovascular disease risk factors are summarised. Many studies report on global fibrinolytic activity, when in fact they measured only t-PA and PAI-1 activity; these studies are not included in the table below as this is not the focus of this thesis. Data for the association of increased PAI-1 activity with CVD are abundant and are reviewed in Kohler & Grant, 2000 and Gils & Declerck, 2004.

Table 2.2. CVD risk factors and their association with fibrinolysis

Risk factor	Reference	Participants	Method of measurement	Association with fibrinolysis
BMI	Colucci *et al*., 2008	176 VTE patients (sub-sample of 124 patients used for regression analyses; results reported here)	Modified turbidimetric plasma fibrinolytic potential clot lysis assay (Lisman *et al*., 2001)	Fibrinolysis time increased with increasing BMI.
	Meltzer *et al*., 2008	2 564 Caucasian control participants aged between 18 and 70 years from the Multiple Environmental and Genetic Assessment (MEGA) study	Plasma fibrinolytic potential (Lisman *et al*., 2001)	CLT increased substantially with increasing BMI (Increase in CLT per kg/m^2 was 2.0 minutes).
	Meltzer *et al*., 2009a	642 male Caucasian control participants (mean age 57.4 years) from the Study of Myocardial Infarctions Leiden (SMILE)	Plasma fibrinolytic potential (Lisman *et al*., 2001)	CLT increased with increasing BMI.
	Guimarães *et al*., 2009	330 Caucasian men and women (mean age 38 years) without a history of arterial thrombosis from the Arterial thrombosis at young age: the role of TAFI and other coagulation factors (ATTAC) study	Plasma fibrinolytic potential (Lisman *et al*., 2001)	Obese individuals had significantly longer CLTs than non-obese and a linear trend was seen between CLTs and BMI.

Lipid profile	Mussoni *et al*., 1992	30 Caucasian hypertriglyceridemic patients free of other recognised risk factors compared with healthy controls (n=27)	Euglobulin lysis area (ELA)	Basal fibrinolytic activity similar in hypertriglyceridemic patients and healthy controls.
	Undas *et al*., 2006	Control group (apparently healthy n=76), CAD group (patients with advanced CAD n=33), diabetes mellitus group (newly diagnosed DM type 2 n=16) and the hypercholesterolemia group (TC > 7.0 mmol/l n=17). All men, aged 35−65 years	Turbidimetric clot lysis assay (lysis time defined as the time required for gel turbidity of a plasma clot to decrease by 50% from the maximum optical density)	Clot lysis times were significantly longer in the hypercholesterolemia group than in the control group.
	Colucci *et al*., 2008	176 VTE patients (sub-sample of 124 patients used for regression analyses; results reported here)	Modified turbidimetric plasma fibrinolytic potential clot lysis assay (Lisman *et al*., 2001)	Fibrinolysis time increased with increasing levels of cholesterol and triglycerides.
	Meltzer *et al*., 2009a	642 male Caucasian control participants (mean age 57.4 years) from the SMILE	Plasma fibrinolytic potential (Lisman *et al*., 2001)	CLT increased with increasing total cholesterol and triglyceride concentration, and decreased with increasing HDL-cholesterol.
Glucose	Harmanci *et al*., 2006	30 Turkish type-1 diabetic and 27 healthy control group men and women	Global fibrinolytic capacity (GFC). A commercial kit was used to measure D-dimer generated and hypofibrinolysis was as D-dimer less than 1 µg/l.	GFC of diabetic men and women was significantly lower than that of the controls.

	Undas *et al*., 2006	Control group (apparently healthy n=76), CAD group (patients with advanced CAD n=33), diabetes mellitus group (newly diagnosed DM type 2 n=16) and the hypercholesterolemia group (TC > 7.0 mmol/l n=17). All men, aged 35−65 years	Turbidimetric clot lysis assay (lysis time defined as the time required for gel turbidity of a plasma clot to decrease by 50% from the maximum optical density)	Participants with DM type 2 had significantly longer clot lysis times than healthy controls.
	Colucci *et al*., 2008	176 VTE patients (sub-sample of 124 patients used for regression analyses; results reported here)	Modified turbidimetric plasma fibrinolytic potential clot lysis assay (Lisman *et al*., 2001)	Fibrinolysis time increased with increasing glucose levels.
	Meltzer *et al*., 2008	2 564 Caucasian control participants aged between 18 and 70 years from the MEGA study (n=71 diabetic, n=2389 not diabetic, diabetes data unavailable for n=95)	Plasma fibrinolytic potential (Lisman *et al*., 2001)	Diabetes was associated with a 17% increase in CLT compared with participants without diabetes.
	Meltzer *et al*., 2009a	642 male Caucasian control participants (mean age 57.4 years) from the SMILE (n=22 diabetic, n=620 not diabetic)	Plasma fibrinolytic potential (Lisman *et al*., 2001)	No significant differences were found in CLT between diabetic and non-diabetic participants.
	Guimarães *et al*., 2009	330 Caucasian men and women (mean age 38 years) without a history of arterial thrombosis from the ATTAC study (n=5 diabetics)	Plasma fibrinolytic potential (Lisman *et al*., 2001)	Participants with diabetes presented with significantly longer CLTs than those participants without diabetes.
Blood pressure	Guimarães *et al*., 2009	330 Caucasian men and women (mean age 38 years) without a history of arterial thrombosis from the ATTAC study	Plasma fibrinolytic potential (Lisman *et al*., 2001)	No association was found between blood pressure and CLT.

	Meltzer *et al*., 2009a	642 male Caucasian control participants (mean age 57.4 years) from the SMILE	Plasma fibrinolytic potential (Lisman *et al*., 2001)	CLT increased with increasing systolic and diastolic blood pressure.
Age	Meade *et al*., 1979	1 601 Caucasian men and 707 women from the Northwick Park Heart Study (NPHS) aged 18−64 and 18−59 respectively	Fibrinolytic activity (FA) expressed as dilute blood clot lysis time (100/ lysis time)	In men FA declined with age up to 58 years after which there was a small increase. FA increased in women between 18 and 40 years, and was followed by decreased FA between 40 and 59 years.
	MacCallum *et al*., 1998	150 healthy Caucasian men (n=73) and women (n=77) aged 23−80	Dilute clot lysis time (DCLT) (whole blood)	Lysis time decreased significantly with age in men, but increased non-significantly with age in women.
	Lisman *et al*., 2005	469 healthy Caucasian men and women (Leiden Thrombophilia Study (LETS) control group), aged 14−72 years	Plasma fibrinolytic potential (Lisman *et al*., 2001)	CLT seemed to progressively increase with age in men and women.
	Colucci *et al*., 2008	176 VTE patients aged 31−66 years (sub-sample of 124 patients used for regression analyses; results reported here)	Modified turbidimetric plasma fibrinolytic potential clot lysis assay (Lisman *et al*., 2001)	Fibrinolysis time increased with increasing age.
	Meltzer *et al*., 2008	2 564 Caucasian control participants aged between 18 and 70 years from the MEGA study	Plasma fibrinolytic potential (Lisman *et al*., 2001)	CLT increased with age in men and women, but reached a plateau in men at age 60 years while still increasing with age in women.
	Guimarães *et al*., 2009	330 Caucasian men and women (mean age 38 years) without a history of arterial thrombosis from the ATTAC study	Plasma fibrinolytic potential (Lisman *et al*., 2001)	CLT was not significantly associated with age in men or women; however, a trend of increasing CLTs with increasing age was

				observed.
Gender	Lisman *et al*., 2005	469 healthy Caucasian men (n=198) and women (n=271) (LETS control group), aged 14−72 years	Plasma fibrinolytic potential (Lisman *et al*., 2001)	Slightly longer CLTs were observed in men than women.
	Meltzer *et al*., 2008	2 564 Caucasian control participants (men n=1226, women n=1338) aged between 18 and 70 years from the MEGA study	Plasma fibrinolytic potential (Lisman *et al*., 2001)	Mean CLT in men was slightly higher than mean CLT in women.
	Guimarães *et al*., 2009	330 Caucasian men (n=122) and women (n=208), mean age 38 years, without a history of arterial thrombosis from the ATTAC study	Plasma fibrinolytic potential (Lisman *et al*., 2001)	No association was found between CLT and gender.
CRP levels	Singh *et al*., 2005 (population information from Devaraj *et al.,* 2005*)*	21 healthy men and women divided into a low CRP group (n=11, CRP levels <0.5 mg/l on three separate occasions in previous 12 months) and a high CRP group (n=10, CRP 2.1-9.9 mg/l on three separate occasions in previous 12 months)	Euglobulin clot lysis time (ECLT)	Fibrinolytic activity of plasma from volunteers with high CRP was significantly lower than in samples with low CRP levels.
	Meltzer *et al*., 2009a	642 male Caucasian control participants (mean age 57.4 years) from the SMILE	Plasma fibrinolytic potential (Lisman *et al*., 2001)	CLT increased with increasing CRP concentrations.
Homocysteine levels	Undas *et al*., 2006	Control group (apparently healthy n=76), CAD group (patients with advanced CAD n=33), diabetes mellitus group (newly diagnosed DM type 2 n=16) and the	Turbidimetric clot lysis assay (lysis time defined as the time required for gel turbidity of a plasma clot to decrease by	Clots from controls with tHcy > 14.5 µmol/L were less susceptible to lysis than those of controls with ≤ 14.5 µmol/L.

		hypercholesterolemia group (TC > 7.0 mmol/l n=17). All men, aged 35−65 years	50% from the maximum optical density)	
	Colucci *et al*., 2008	VTE patients (75 men, 101 women), 118 with normal homocysteinemia and 58 with mild hyperhomocysteinemia	Modified turbidimetric plasma fibrinolytic potential clot lysis assay (Lisman *et al*., 2001)	Plasma clots from patients with mild hyperhomocysteinemia had lower fibrinolytic potential than clots from patients with normal homocysteine levels.
Smoking	Meade *et al*., 1979	1 601 Caucasian men and 707 women from the NPHS aged 18−64 and 18−59 respectively (smokers n=950, non-smokers n=1014)	Fibrinolytic activity (FA) expressed as dilute blood clot lysis time (100/ lysis time)	Lower fibrinolytic activity was found in smokers compared with non-smokers.
	Guimarães *et al*., 2009	330 Caucasian men and women, mean age 38 years, without a history of arterial thrombosis from the ATTAC study (current smokers n=88, former smokers n=79, never smoked n=163)	Plasma fibrinolytic potential (Lisman *et al*., 2001)	CLT did not differ significantly between participants who were current smokers and those who did not smoke or smoked formerly.
	Meltzer *et al*., 2009a	642 male Caucasian control participants (mean age 57.4 years) from the SMILE (smokers n=213, non-smokers n=429)	Plasma fibrinolytic potential (Lisman *et al*., 2001)	No apparent differences were found between smokers and non-smokers.
	Stępień *et al.*, 2011	21 healthy participants from the CRABIS (mean age 52 years current smokers, 58 years former smokers, 55 years non-smokers)	Turbidimetric lysis assay	Lysis times of current smokers were significantly longer than lysis times of former smokers and non-smokers. Lysis times also differed significantly between former smokers and non-smokers.

Alcohol consumption	Meade *et al*., 1979	1 601 Caucasian men and 707 women from the NPHS aged 18−64 and 18−59 respectively (Men: drinkers n=950, non-drinkers n=651, frequencies for women not reported)	Fibrinolytic activity (FA) expressed as dilute blood clot lysis time (100/ lysis time)	A higher, but not significantly higher, FA was observed in male and female drinkers compared with non-drinkers.
	Guimarães *et al*., 2009	330 Caucasian men and women, mean age 38 years, without a history of arterial thrombosis from the ATTAC study (used alcohol n=109, did not use alcohol n=221)	Plasma fibrinolytic potential (Lisman *et al*., 2001)	No significant differences were found between participants who used alcohol and those who did not.
	Meltzer *et al*., 2009a	642 male Caucasian control participants (mean age 57.4 years) from the SMILE (regularly n=557, occasionally n=21, never n=64)	Plasma fibrinolytic potential (Lisman *et al*., 2001)	No significant differences were found between participants who regularly consumed alcohol and those who consumed it only occasionally or not at all.

ATTAC: The arterial thrombosis at young age: the role of TAFI and other coagulation factors study; CAD: Coronary artery disease; CLT: Clot lysis time; LETS: Leiden Thrombophilia Study; MEGA: Multiple Environmental and Genetic Assessment study; NPHS: Northwick Park Heart Study; BMI: Body mass index; CRP: C-reactive protein; tHcy: total homocysteine; MI: myocardial infarction; CRABIS: Prevention Program Krakow – Atherosclerosis Bio-imaging Study; ELA; euglobulin lysis area; SMILE: Study of Myocardial Infarctions Leiden.

The summary of the associations of various CVD risk factors with fibrinolysis presented in Table 2.2 makes it clear that BMI has a linear relationship with CLT (Colucci *et al*., 2008; Meltzer *et al*., 2008; Meltzer *et al*., 2009a; Guimarães *et al*., 2009). When looking at the relationships of lipid markers with CLT, Mussoni *et al*. (1992) did not find differences in ELA between hypertriglyceridemic patients and healthy controls, while other studies found that CLT increased with increasing levels of cholesterol and triglycerides (Undas *et al*., 2006; Colucci *et al*., 2008). One study found decreased CLTs with higher HDL-cholesterol concentrations (Meltzer *et al*., 2009a). However, different methods for the determination of fibrinolysis were used in these studies and this may be the reason for the differences in the results.

Most studies investigating the association of fibrinolysis and glucose found longer clot lysis times or decreased fibrinolytic capacity in diabetic participants than in controls (Undas *et al*., 2006; Colucci *et al*., 2008; Meltzer *et al*., 2008 & Guimarães *et al*., 2009). However, this association was not found in all studies (Harmanci *et al*., 2006; Meltzer *et al*., 2009a). In the study of Harmanci *et al.* (2006) participants were type 1 diabetics, while in the study of Meltzer *et al.* (2009a) the type of diabetes was not defined. Most of the studies indicating associations between prolonged lysis times of clots and diabetes or glucose defined diabetics as patients with newly diagnosed type 2 diabetes (Undas *et al*., 2006) or patients using anti-diabetic medication or insulin (Guimarães *et al*., 2009). The studies from Colucci *et al*. (2008) and Meltzer *et al*. (2008) did not indicate the type of diabetes. Although the majority of studies found CLT to be longer in diabetes, we cannot make definite conclusions on the association between CLT and diabetes, although it does seem to be related to the type and/or degree of diabetes.

Guimarães *et al*. (2009) found no association between blood pressure and clot lysis time while another study found increased clot lysis times with increased blood pressure (Meltzer *et al*., 2009a). In these two studies the same clot lysis time assay was used; however, the population sizes and the mean age of each population differed and this may be a reason for the difference in results. The mean age of participants in the study of Guimarães *et al*. (2009) was 38 years, while the mean age of the SMILE participants was 57.4 years. Age has been shown to be positively

associated with PAI-1 antigen in Caucasian populations, while increased PAI-1 has been associated with increased blood pressure (Cigolini *et al*., 1995; Mansfield *et al.,* 1995), possibly explaining the positive association found between CLT and blood pressure in the study of Meltzer *et al.*(2009a).

As regards the association of age and CLT, some researchers have found that lysis time increased with age (Lisman *et al*., 2005), whereas others found CLTs that appeared to be lower in men older than 70 years compared with younger men (Meltzer *et al*., 2009a), or that appeared to increase, but not significantly (Guimarães *et al*., 2009). A possible reason for the difference in results may be differences in the age ranges included in these studies (Guimarães *et al*., 2009). In the LETS study (Lisman *et al*., 2005) the age range of control participants was 14−72 years whereas the age range for the ATTAC study controls were 18−45 years for men and 18−55 for women (Guimarães *et al*., 2009). Others have found decreased fibrinolytic activity in men until the age of 58 years, after which a small increase was observed, and increased fibrinolytic activity in women up until age 40, after which a decrease was seen (Meade *et al*., 1979). The results of these studies suggest that the relationship between age and CLT is probably not linear.

MacCallum *et al*. (1998) observed decreased lysis times in men, but non-significant increases in lysis times with age in women. Slightly longer mean clot lysis times have been reported for men compared with women (Lisman *et al*., 2005; Meltzer *et al*., 2008), while others found no associations between clot lysis time and gender (Guimarães *et al*., 2009). In the study of MacCallum *et al.* (1998), the main determinants of dilute clot lysis time were found to be t-PA and PAI-1 activities, while PAI-1_{act} alone was a much stronger determinant of DCLT in women. Unfortunately, these factors were not measured in the LETS population (Lisman *et al.,* 2005). Results of the studies discussed are conflicting and a conclusion on the association of clot lysis times and sex cannot be drawn, although it does seem that CLT may be longer in Caucasian men that in women.

Meltzer *et al.* (2009a) and Singh *et al.* (2005) found longer clot lysis times and decreased fibrinolytic activity in healthy individuals with increased CRP

concentrations. The association between fibrinogen and CRP, both acute-phase proteins, may explain these observations because clots formed under conditions with increased fibrinogen concentrations are dense and may take longer to lyse than clots formed under conditions with normal fibrinogen concentrations (Koenig, 2003; Wolberg, 2010; Lord, 2011). It is also possible that increased CRP concentrations are a reflection of inflammation, and the presence of inflammation may influence clot lysis in various ways.

Raised homocysteine levels have been shown to be associated with an increased CVD risk (Wald *et al.,* 2002). Undas *et al.* (2006) and Colucci *et al.* (2008) found clots less susceptible to lysis and decreased fibrinolytic potential in healthy controls with increased homocysteine compared with those with normal homocysteine levels. It has been reported that possible ways through which homocysteine may impair fibrinolysis could be by influencing plasma levels of fibrinolytic factors PAI-1 and t-PA, or by altering the structure of fibrinogen (Colucci *et al.,* 2008). However, in the study of Colucci *et al.* (2008) it was found that the fibrinolytic abnormalities seen in individuals with high levels of homocysteine vary according to experimental conditions. The effect of high homocysteine levels on fibrinolysis seems to be mediated largely through TAFI in fibrinolysis assays using physiological or near-physiological concentrations of t-PA, whereas the effect is related mainly to fibrinogen at high t-PA concentrations (Colucci *et al.,* 2008). Increased levels of homocysteine seem to be associated with decreased clot lysis times, although the precise mechanism is not yet clear.

Meade *et al*. (1979) found decreased fibrinolytic activity in smokers compared with non-smokers, while more recent studies found no apparent differences in clot lysis times of smokers, former smokers or non-smokers (Guimarães *et al*., 2009; Meltzer *et al*., 2009a). Another study, however, showed significant structural differences and differences in lysis times of clots from current and former smokers and non-smokers, with the shortest lysis times in the non-smokers (Stępień *et al.,* 2011). Reasons for differences in the results of Meade *et al.* (1979), Guimarães *et al*. (2009), and Meltzer *et al*. (2009a) may lie in the difference in the numbers of cigarettes or the amount of tobacco smoked per day, as these data were reported only by Meade *et al.* (1979).

Meade *et al.* (1979) found non-significant increased fibrinolytic activity in drinkers compared with non-drinkers. The studies of Guimarães *et al*. (2009) and Meltzer *et al*. (2009a) also did not find significant differences between participants who regularly consumed alcohol and those who did not consume alcoholic beverages. Individual coagulation and fibrinolytic factors have been shown to be influenced by acute and habitual alcohol consumption (Salem & Laposatsa, 2005; Pieters *et al.,* 2010a; Pieters *et al.,* 2010b); however, from the studies summarised above there seems to be no strong association between alcohol consumption and clot lysis.

Thus it seems that increased BMI, triglyceride concentrations, blood pressure, CRP and homocysteine concentrations are associated with prolonged lysis times, while the association of CLT with diabetes, gender, smoking and alcohol consumption is less clear. In the following section, associations of clot lysis time and haemostatic factors which have been shown to contribute to the variance in CLT will be discussed.

2.5. CLOT LYSIS AND HAEMOSTATIC VARIABLES

Clot formation and lysis are the result of a complex interplay of various coagulation and lytic factors which can all potentially influence plasma fibrinolytic potential. There is relatively limited literature available, however, investigating the contribution of the respective haemostatic variables to CLT *per se*. In a study done by Meltzer *et al*. (2010a), which specifically investigated this question, it was found that the haemostatic variables investigated explained 77% of the variance in CLT, using the same assay that will be used in this thesis. PAI-1 antigen ($PAI\text{-}1_{ag}$) was the main determinant of CLT, followed by plasminogen, TAFI, prothrombin and α2-antiplasmin. CLT is not sensitive to endogenous t-PA because large amounts of exogenous t-PA are added to the plasma as part of the experimental design of the assay (Lisman *et al.,* 2001). Fibrinogen, FVII, FX and XI contributed only minimally (Meltzer *et al.,* 2010a). These factors and their roles in fibrinolysis will be described in the following sub-sections.

2.5.1. Plasminogen activator inhibitor type-1 and its influence on clot lysis

PAI-1 is a member of the serine protease inhibitors superfamily (SERPINS) and has a molecular weight of 52 kd (Pannekoek *et al*., 1986). This inhibitor is synthesised by adipocytes, hepatocytes, endothelial cells, and vascular and non-vascular smooth muscle cells, and inhibits clot lysis by forming complexes with t-PA and u-PA, which prevents these plasminogen activators from binding to plasminogen to form plasmin (Dellas & Loskutoff, 2005). Higher $PAI\text{-}1_{act}$ and antigen levels are not only associated with longer clot lysis times (Lisman *et al*., 2001; Colucci *et al.,* 2008; Meltzer *et al*., 2010a), but PAI-1 is also considered to be the strongest determinant of CLT (Meltzer *et al*., 2010a). A number of polymorphisms of the PAI-1 gene have been identified that could potentially influence PAI-1 levels. Of these polymorphisms, the 4G/5G polymorphism has shown the strongest link with PAI-1 levels (Ye *et al.,* 1995; Ossei-Gerning *et al.,* 1997; Festa *et al*., 2003). The effects of other PAI-1 polymorphisms on PAI-1 levels are less clear.

Because of the prominent role of PAI-1 in determining CLT, it is worthwhile investigating polymorphisms of the PAI-1 gene which might affect PAI-1 levels or activity. The following section will provide details on the 4G/5G polymorphism. To date, this is the most prominent PAI-1 gene polymorphism described in the literature, which can potentially influence PAI-1 levels.

2.5.2. PAI-1 genotypes and their influence on PAI-1 levels

PAI-1 4G/5G polymorphism

The human PAI-1 gene is located on chromosome 7, with eight introns and nine exons (Strandberg *et al*., 1988). To date, the PAI-1 4G/5G polymorphism, a single nucleotide insertion or deletion polymorphism situated 675 base pairs upstream of the transcriptional site in the promoter area, is the most researched polymorphism of the PAI-1 gene (Dawson *et al*., 1993; Nordt *et al*., 2001). In this polymorphism the insertion or deletion of a guanine nucleotide leads to a sequence of either four or five guanine nucleotides (Eriksson *et al*., 1995). Both the 4G and 5G alleles bind transcription-regulation proteins (Eriksson *et al*., 1995). When a 5G allele is present, a repressor protein, namely the 5G allele-specific transcriptional repressor, in addition to the transcription factor, may bind, leading to a reduction in transcription of

the PAI-1 gene and lower PAI-1 levels (Eriksson *et al*., 1995). When a 4G allele is present, the repressor protein cannot bind (Eriksson *et al*., 1995). High PAI-1 levels have been found in participants who are homozygous for the 4G allele, intermediate levels for heterozygous participants and low PAI-1 levels in 5G homozygotes (Ye *et al.,* 1995; Ossei-Gerning *et al.,* 1997; Festa *et al*., 2003). In fact, study participants who were 4G homozygotic have been shown to have 25% higher PAI-1 levels that those who were 5G homozygotic (Ye *et al.*, 1995). In diabetic subjects with the 4G allele even higher PAI-1 levels than in healthy controls with this same allele have been found (Eriksson *et al*., 1995; Mansfield *et al*., 1995). The 4G/5G polymorphism, and specifically, the 4G allele of this genotype, which is associated with higher transcription rates of PAI-1, has also been linked to CVD (Iacoviello *et al.*, 1998; Gardemann *et al*., 1999).

The 4G/5G polymorphism is considered to be a response polymorphism, meaning that the effect the polymorphism will have on PAI-1 levels is exacerbated under certain conditions. A condition that has been shown to influence PAI-1 levels through the 4G/5G polymorphism is hypertriglyceridemia. Hypertriglyceridemic patients homozygous for the 4G allele presented with much higher PAI-1 concentrations than patients who were homozygous for the 5G allele (Ossei-Gerning *et al*., 1997). A VLDL response region has been found in the promoter area of the PAI-1 gene adjacent to the 4G/5G polymorphic site (Eriksson *et al.,* 1998). A transcription factor which is induced by VLDL can bind to the VLDL response region adjacent to and partly overlapping the binding site of the 5G allele (Eriksson *et al.,* 1998). Competition between the VLDL-induced factor and the 5G transcriptional repressor could cause increased transcription of PAI-1 (Eriksson *et al*., 1998), thus causing even bigger differences in PAI-1 levels between the different genotypes.

Different frequencies of the 4G and 5G alleles have been found in different populations, along with different PAI-1 levels corresponding to the genotypes (Festa *et al.*, 2003). Naran *et al*. (2008) investigated the 4G/5G polymorphism frequencies in Indian, Caucasian and black South Africans. These researchers found 4G/4G genotype frequencies of 33.9%, 36.4% and 2.8% respectively in Indian, Caucasian and Black Africans (Naran *et al*., 2008). Black South Africans have been shown to present with significantly lower PAI-1 levels than Caucasians (Greyling *et al*., 2007;

Jerling *et al*., 1994; Pieters & Vorster, 2008). One of the research questions that will be addressed in this thesis is to determine the contribution of the 4G/5G polymorphism to the variance in PAI-1_{act} levels in the South African PURE population. We will additionally identify other polymorphisms in the promoter area of the PAI-1 gene and determine their influence on PAI-1_{act} and CLT.

2.5.3. Plasminogen and its influence on clot lysis

Plasminogen is a 92-kd, single-chain glycoprotein and is converted by plasminogen activators, such as t-PA and u-PA, to plasmin on the surface of fibrin, although u-PA can also be activated to plasminogen in the absence of fibrin (Forsgren *et al*., 1987). Plasminogen has been shown to increase with increasing triglycerides, total cholesterol levels, CRP and smoking and to be strongly related to α2-antiplasmin (Meltzer *et al*., 2010b). Plasma plasminogen circulates at high concentrations in healthy individuals, with little variation (Meltzer *et al.,* 2010a). This could be the reason why the contribution of plasminogen to the variance in CLT is much smaller than the contribution of PAI-1 (variation of which is much greater). Meltzer *et al*. (2010b) found that the risk of myocardial infarction increased with increasing plasminogen levels, which is not what one would expect, considering the role of plasminogen in fibrin lysis. Upon adjustment for lipids, CRP, smoking and alcohol consumption, however, the association disappeared (Meltzer *et al*., 2010b). Apart from its role in fibrinolysis, plasminogen is also considered an acute-phase protein which is increased during inflammation (Lackner & Javid, 1973; Haverkate *et al.,* 1995). Meltzer *et al*. (2010a) found decreasing levels of plasminogen to be associated with longer clot lysis times. Thus it seems that even though decreased levels of plasminogen are associated with prolonged clot lysis times, increased plasminogen levels may possibly also increase the risk for myocardial infarction through other roles plasminogen plays apart from its effect in fibrinolysis, such as, for instance, its role in inflammation.

2.5.4. Thrombin-activatable fibrinolysis inhibitor (TAFI) and its influence on clot lysis

TAFI, a 60-kd zymogen, is activated by thrombin or plasmin and in its activated form (TAFIa) inhibits the binding of t-PA and plasminogen to fibrin (Colucci & Semeraro,

2012). Thrombin is a relatively weak activator of TAFI; however, in the presence of thrombomodulin, the efficiency of thrombin as an activator increases more than 1000-fold (Leurs & Hendriks, 2005; Colucci & Semeraro, 2012). The cleavage of fibrin by plasmin generates C-terminal lysine residues, to which additional plasminogen and t-PA can bind, to form more plasmin (Colucci & Semeraro. 2012). However, proteolysis of these lysine residues by TAFI prevents the further generation of plasmin and fibrin lysis (Mosnier & Bouma, 2006). The half-life of TAFIa is approximately 8−9 minutes at 37°C and the effect TAFI exerts on clot lysis is through a threshold-dependent mechanism (Boffa *et al*., 1998; Walker & Bajzar, 2004). If the TAFI concentration falls below a threshold value, which is dependent on the tempo of TAFIa formation, the intrinsic instability of TAFIa, plasmin inhibitors present and t-PA concentration, the amount of lysine residues on fibrin to which t-PA and plasminogen can bind will increase exponentially (Mosnier & Bouma, 2006).
Similar to assays available for global fibrinolytic potential, assays used to measure thrombin generation are also available. Results from these thrombin generation assays may aid in the interpretation of global fibrinolytic assays as it provides information on the activation of the coagulation cascade. Since thrombin generation can vary significantly between individuals, this can ultimately influence lysis rates.

The function of TAFI is to stabilise the formed clot (Mosnier & Bouma, 2006). Increased TAFI levels have consequently been associated with longer CLTs (Meltzer *et al*., 2010a) and increased risk of venous thrombosis and recurrent venous thrombosis (van Tilburg *et al*., 2000; Eichinger *et al*., 2004; Meltzer *et al*., 2010b).

Colucci *et al.* (2008) investigated the importance of TAFI on lysis time by adding a specific TAFIa inhibitor (PTCI) to their method and found that lysis times shortened by approximately 30 minutes, thus indicating that TAFIa plays an important role in lysis time. These researchers also varied concentrations of t-PA used in their models and found that high concentrations of t-PA completely overcame the inhibitory effect of TAFI on fibrinolysis (Colucci *et al.,* 2008). In this study TAFI did not emerge as an independent predictor of lysis time even when low (physiological) t-PA concentrations were used, possibly because other variables entered into the multiple regression model took the prediction of TAFI (Colucci *et al.,* 2008).

2.5.5. Prothrombin and its influence on clot lysis

As mentioned in section 2.2.1, prothrombin is converted to thrombin through the binding of FXa to Va (Monroe & Hoffman, 2006). Meltzer *et al*. (2010a) found increased clot lysis times with increased prothrombin levels. This is probably due to increased TAFI activation (Meltzer *et al.,* 2010a). These results are in line with studies that showed longer clot lysis times in patients with the G20210A mutation, which is a mutation on the prothrombin gene associated with increased prothrombin levels (Poort *et al*., 1996; Colucci *et al*., 2004; Meltzer *et al*., 2008).

The higher levels of prothrombin can be converted to high levels of thrombin. It has been shown that high levels of thrombin lead to the formation of clots with thin fibres and a more dense structure, which may be one of the reasons why these clots are lysed more slowly in comparison with clots formed with lower thrombin concentrations (Ryan *et al.,* 1999). Similar to assays available for global fibrinolytic potential, assays used to measure thrombin generation are also available. Results from these thrombin generation assays may aid in the interpretation of global fibrinolytic assay as it provides information on the activation of the coagulation cascade and thrombin generation van vary significantly between individuals, ultimately influencing lysis rates.

2.5.6. Alpha 2-antiplasmin and its influence on clot lysis

Alpha 2-antiplasmin has a molecular weight of 70 kd and is the main inhibitor of plasmin (Holmes *et al*., 1987). This single-chain glycoprotein forms a complex with plasmin, inactivating it, which then prevents the plasmin-mediated lysis of fibrin (Collen & Wiman, 1978). It circulates in plasma at high concentrations and is a strong inhibitor of fibrinolysis (Meltzer *et al.,* 2009b; Rijken & Lijnen, 2009).

Alpha 2-antiplasmin has been found to be negatively associated with HDL-cholesterol levels, age, von Willebrand factor and systolic blood pressure, while it was positively associated with total cholesterol, triglyceride concentrations and plasminogen (Meltzer *et al*., 2010b). Longer CLTs have been found with increased α2-antiplasmin levels (Meltzer *et al*., 2010a). Meltzer *et al*. (2010b) also found

α2-antiplasmin to be associated with risk of myocardial infarction in a dose-dependent manner.

2.5.7. Fibrinogen and its influence on clot structure and lysis

The structure of fibrinogen is described in section 2.2.1. A normal fibrinogen plasma concentration is approximately 2.5 g/l (Weisel, 2005). A direct association between fibrinogen levels and severity of coronary artery disease has been found (Jackson *et al.,* 2000). Levels of fibrinogen have also been shown to be increased after acute thrombosis and in patients with unstable angina or myocardial infarction (Koenig *et al.,* 2003). High fibrinogen concentrations are seen as an independent risk factor for cardiovascular events (Ernst & Resch, 1993). The Fibrinogen Studies Collaboration review published in 2005 shows strong associations between fibrinogen concentrations and increased risk of various CVD events.

Although the reasons why elevated fibrinogen levels are involved in CVD and atherosclerosis are not completely understood, possible pathophysiological mechanisms may involve the role of fibrinogen in the coagulation cascade as the substrate for thrombin, the role fibrinogen plays in platelet aggregation, modulation of endothelial function, the proliferation and migration of smooth muscle cells, and the role of fibrinogen as an acute-phase protein (Koenig, 2003). Also, higher fibrinogen levels are associated with increased plasma viscosity, which further increases the risk of CVD (Lowe *et al.,* 1997; Lominadze *et al.,* 2010). Fibrinogen concentration has been found to significantly affect fibrin network structure, which in turn has been shown to influence lysis rates (Weisel, 2007; Wolberg, 2010; Lord, 2011).

Upon activation by thrombin, fibrinogen is converted to fibrin, which forms the scaffold of the clot. Leander *et al.* (2012) found a significant association between increased fibrin formation and risk of myocardial infarction. However, the structure of the fibrin fibres themselves can also greatly influence the lysis of a clot (Lord, 2011). Fibrin structure and clot architecture influence the accessibility of fibrinolytic agents in the clot (Collet *et al*., 2000). For instance, a clot with thin fibres which are tightly packed will be less susceptible to lysis, as the fibrinolytic agents facilitating

fibrinolysis will permeate at a slower rate through the clot (Lord, 2011), while a clot with thicker fibres will have larger pores and lyse more easily (Collet *et al*., 2000). Thus, changes in fibre diameter can influence plasmin generation and the resistance of fibres to lysis (Gabriel *et al*., 1992). However, even though increased levels of fibrinogen and increased fibrin formation are risk factors for CVD, fibrinogen was found to contribute to the variation in CLT only to a very small extent (Meltzer *et al.,* 2010a). It may be that the contribution of fibrinogen to CLT is less prominent in plasma models, possibly owing to the presence and interaction of other plasma components that also affect clot lysis.

2.6. CONCLUSION

The prevalence of CVD has increased significantly in the black population of South Africa in recent years. This increase may be attributed to the process of urbanisation which this population is undergoing. The role of fibrinolysis in CVD has been investigated in Caucasian populations but no data on this topic are available for black South Africans.

In this literature review, the processes of clot formation and lysis, methods by which lysis is measured, the role of clot lysis in CVD, the prevalence of CVD in black South Africans, various traditional CVD risk factors and their relation to clot lysis, as well as the main contributing haemostatic factors playing important roles in clot lysis have been described. While hypofibrinolysis has been linked to both venous and arterial thrombosis, the exact mechanisms behind these associations have not yet been fully elucidated. Data on the associations between CVD risk factors and fibrinolysis are scarce and deserve further investigation, especially in under-studied ethnic groups such as black Africans. The remainder of this thesis consists of two articles and a general conclusion chapter. The first article will focus on the global fibrinolytic potential in the South African PURE population and its relation to individual CVD risk factors. The second article will focus on the effect of polymorphisms in the promoter area of the PAI-1 gene on $PAI\text{-}1_{act}$ and CLT, and also investigate possible gene-environment interactions.

CHAPTER 3:

Plasma clot lysis time and its association with cardiovascular risk factors in black Africans

This chapter includes:

- the instructions given to authors by the journal *PLOS One*;
- the article titled “Plasma clot lysis time and its association with cardiovascular risk factors in black Africans” (e48881), published 8 November 2012 and presented in the technical style specified by the journal;
- the published article, which is provided as Addendum C (page 169).

INSTRUCTIONS TO AUTHORS: PLOS One

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•Figures (guidelines for preparing figures can be found at the Figure and Table Guidelines)

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You should supply an approximately one page cover letter that:

•Concisely summarizes why your paper is a valuable addition to the scientific literature

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•Specifies the type of article you are submitting (for example, research article, systematic review, meta-analysis, clinical trial)

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All manuscripts should include line numbers and page numbers.

Manuscripts should begin with the ordered sections:

•Title

•Authors

•Affiliations

•Abstract

•Introduction

and end with the sections of:

•Acknowledgments

•References

•Figure Legends

•Tables

Figures should not be included in the main manuscript file. Each figure must be prepared and submitted as an individual file.

The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file.

There are no explicit requirements for section organization between these beginning and ending sections. Articles may be organized in different ways and with different section titles, according to the authors' preference. In most cases, internal sections include:

•Materials and Methods

•Results

•Discussion

•Conclusions (optional)

PLOS ONE has no specific requirements for the order of these sections, and in some cases it may be appropriate to combine sections. Guidelines for individual sections can be found below.

Abbreviations should be kept to a minimum and defined upon first use in the text. Non-standard abbreviations should not be used unless they appear at least three times in the text.

Standardized nomenclature should be used as appropriate, including appropriate usage of species names and SI units.

Manuscript File Type Requirements

Authors may submit their manuscript files in Word (as .doc or .docx), LaTeX (as .pdf), or RTF format. Only RTF and .doc files can be used during the production process.

Submissions with equations. If your manuscript is or will be in .docx format and contains equations, you must follow the instructions below to make sure that your equations are editable when the file enters production.

If you have not yet composed your article, you can ensure that the equations in your .docx file remain editable in .doc by enabling "Compatibility Mode" before you begin. To do this, open a new document and save as Word 97-2003 (*.doc). Several features of Word 2007/10 will now be inactive, including the built-in equation editing tool. You can insert equations in one of the two ways listed below.

If you have already composed your article as .docx and used its built-in equation editing tool, your equations will become images when the file is saved down to .doc. To resolve this problem, re-key your equations in one of the two following ways.

1. Use MathType to create the equation (recommended)

2. Go to Insert > Object > Microsoft Equation 3.0 and create the equation

If, when saving your final document, you see a message saying "Equations will be converted to images," your equations are no longer editable and PLoS will not be able to accept your file.

2. Guidelines for Standard Sections

Title

Manuscripts must be submitted with both a full title and a short title, which will appear at the top of the PDF upon publication if accepted. Only the full title should be included in the manuscript file; the short title will be entered during the online submission process.

The full title must be 150 characters or fewer. It should be specific, descriptive, concise, and comprehensible to readers outside the subject field. Avoid abbreviations if possible. Where appropriate, authors should include the species or model system used (for biological papers) or type of study design (for clinical papers).

Examples:

•Impact of Cigarette Smoke Exposure on Innate Immunity: A Caenorhabditis elegans Model

•Solar Drinking Water Disinfection (SODIS) to Reduce Childhood Diarrhoea in Rural Bolivia: A Cluster-Randomized, Controlled Trial

The short title must be 50 characters or fewer and should state the topic of the paper.

Authors and Affiliations

All author names should be listed in the following order:

•First names (or initials, if used),

•Middle names (or initials, if used), and

•Last names (surname, family name)

Each author should list an associated department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. If the article has been submitted on behalf of a consortium, all author names and affiliations should be listed at the end of the article.

This information cannot be changed after initial submission, so please ensure that it is correct.

To qualify for authorship, a researcher should contribute to all of the following:

1. Conception and design of the work, acquisition of data, or analysis and interpretation of data
2. Drafting the article or revising it critically for important intellectual content
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All persons designated as authors should qualify for authorship, and all those who qualify should be listed. Each author must have participated sufficiently in the work to take public responsibility for appropriate portions of the content. Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments.

When a large group or center has conducted the work, the author list should include the individuals whose contributions meet the criteria defined above, as well as the group name.

One author should be designated as the corresponding author, and his or her email address or other contact information should be included on the manuscript cover page. This information will be published with the article if accepted.

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The abstract should:
•Describe the main objective(s) of the study
•Explain how the study was done, including any model organisms used, without methodological detail
•Summarize the most important results and their significance
•Not exceed 300 words

Abstracts should not include:
•Citations
•Abbreviations, if possible
The introduction should:
•Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
•Define the problem addressed and why it is important
•Include a brief review of the key literature
•Note any relevant controversies or disagreements in the field
•Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

Materials and Methods
This section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.

We encourage authors to submit detailed protocols for newer or less well-established methods as Supporting Information. These are published online only, but are linked to the article and are fully searchable. Further information about formatting Supporting Information files, can be found here.

Methods sections of papers on research using human or animal subjects and/or tissue or field sampling must include required ethics statements. See the Reporting Guidelines for human research, clinical trials, animal research, and observational and field studies for more information.
Methods sections of papers with data that should be deposited in a publicly available database should specify where the data have been deposited and provide the relevant accession numbers and version numbers, if appropriate. Accession numbers should be provided in parentheses after the entity on first use. If the accession numbers have not yet been obtained at the time of submission, please state that they will be provided during review. They must be provided prior to publication.
Methods sections of papers using cell lines must state the origin of the cell lines used. See the Reporting Guidelines for cell line research for more information.

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Results, Discussion, and Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled "Results and Discussion") or a mixed Discussion/Conclusions section (commonly labeled "Discussion"). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn. Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

PLOS ONE editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the PLOS ONE Publication Criteria for more information.

Acknowledgments

People who contributed to the work but do not fit the PLOS ONE authorship criteria should be listed in the acknowledgments, along with their contributions. You must ensure that anyone named in the acknowledgments agrees to being so named.

Funding sources should not be included in the acknowledgments, or anywhere in the manuscript file. You will provide this information during the manuscript submission process.

References

Only published or accepted manuscripts should be included in the reference list. Manuscripts that have been submitted but not yet accepted should not be cited. Limited citation of unpublished work should be included in the body of the text only as “unpublished data.”

References must be listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, citations should be indicated by the reference number in brackets. Journal name abbreviations should be those found in the NCBI databases. A number of reference software companies supply PLOS style files (e.g., Reference Manager, EndNote).

Proper formatting of the references is crucial; some examples are shown below.

•Published papers. Hou WR, Hou YL, Wu GF, Song Y, Su XL, et al. (2011) cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (Ailuropoda melanoleuca). Genet Mol Res 10: 1576-1588.

Note: Use of a DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers.

•Accepted, unpublished papers. Same as above, but “In press” appears instead of the page numbers.

•Electronic journal articles. Huynen MMTE, Martens P, Hilderlink HBM (2005) The health impacts of globalisation: a conceptual framework. Global Health 1: 14. Available: http://www.globalizationandhealth.com/content/1/1/14. Accessed 25 January 2012.
•Books. Bates B (1992) Bargaining for life: A social history of tuberculosis. Philadelphia: University of Pennsylvania Press. 435 p.
•Book chapters Hansen B (1991) New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health. pp. 21-28.

Tables

Tables should be included at the end of the manuscript. All tables should have a concise title. Footnotes can be used to explain abbreviations. Citations should be indicated using the same style as outlined above. Tables occupying more than one printed page should be avoided, if possible. Larger tables can be published as Supporting Information. Please ensure that table formatting conforms to our Guidelines for table preparation.

Table Guidelines

Tables submitted for publication should be included at the very end of the article file (.doc, .rtf, .tex). Supporting Information tables should be submitted as separate files in any of the following formats (although authors should aim to ensure that the file type is most appropriate to the information displayed): Word (.doc), Excel (.xls), PDF, PPT, JPG, EPS, or TIFF.

Title and Footnotes

Each table needs a concise title of no more than one sentence, placed above the table with the table number (e.g., Table 1). The legend and footnotes should be placed below the table. Footnotes may be used to explain abbreviations.

Specifications

Tables that do not conform to the following requirements may give unintended results when published. Problems may include the movement of data (rows or columns), loss of spacing, or disorganization of headings. Note: Multi-part tables with varying numbers of columns or multiple footnote sections should be divided and renumbered as separate tables.

In the published version, tables will be formatted in PLOS style. This includes alternate row shading, content left-aligned in cells, title above the table and legend/footnotes below the table.

Tables must:

◦Be cell-based (e.g., created in Word with Tables tool (preferred) or in Excel).
◦Be editable (i.e., not a graphic object).
◦Have heading/subheading levels in separate columns.
◦Be no larger than one printed page (7 in x 9.5 in). Larger tables can be published as online supporting information. Note: some wide tables may be printed sideways in the PDF.

Tables must not:

◦Use returns or tabs within a cell.

◦Have color or shading.

◦Use lines, rules, or borders.

◦Contain spaces within cells to align text.

◦Have vertically merged cells; horizontally merged cells are fine.

◦Have inserted text boxes or pictures.

◦Have tables within tables.

◦Include empty columns, rows, or cells to create spacing.

◦Include hyperlinked text.

Figure Legends

Figures should not be included in the manuscript file, but figure legends should be. Guidelines for preparing figures can be found here.

Figure legends should describe the key messages of a figure. Legends should have a short title of 15 words or less. The full legend should have a description of the figure and allow readers to understand the figure without referring to the text. The legend itself should be succinct, avoid lengthy descriptions of methods, and define all non-standard symbols and abbreviations.

Further information about figure legends can be found in the Figure Guidelines.

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Methods sections of papers on research using human subject or samples must include ethics statements that specify:

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•Whether informed consent was written or oral. If informed consent was oral, it must be stated in the manuscript:

◦Why written consent could not be obtained

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•Define categories in as much detail as the study protocol allows

•Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency

•Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: "Caucasian" should be changed to "white" or "of [Western] European descent" (as appropriate); "cancer victims" should be changed to "patients with cancer."

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal (PDF), which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual's case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

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ARTICLE: Plasma clot lysis time and its association with cardiovascular risk factors in black Africans

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Abstract

Studies in populations of European descent show longer plasma clot lysis times (CLT) in patients with cardiovascular disease (CVD) than in controls. No data are available on the association between CVD risk factors and fibrinolytic potential in black Africans, a group undergoing rapid urbanisation with increased CVD prevalence. We investigated associations between known CVD risk factors and CLT in black Africans and whether CLTs differ between rural and urban participants in light of differences in CVD risk.

Data from 1000 rural and 1000 urban apparently healthy black South Africans (35–60 years) were cross-sectionally analysed.

Increased PAI-1_{act}, BMI, HbA1c, triglycerides, the metabolic syndrome, fibrinogen concentration, CRP, female sex and positive HIV status were associated with increased CLTs, while habitual alcohol consumption was associated with decreased CLT. No differences in CLT were found between age and smoking categories, contraceptive use and non-use or hypertensive and normotensive participants. Urban women had longer CLTs than rural women while no differences were observed for men.

CLT was associated with many known CVD risk factors in black Africans. Differences were observed, however, when results were compared with data available in the literature relating to populations of European descent, suggesting possible ethnic differences. The effect of urbanisation on CLT is influenced by traditional CVD risk factors and their prevalence in urban and rural communities.

Introduction

Cardiovascular disease (CVD) is a global problem and CVD risk factors and disease rates continue to rise [1]. The development of CVD may start early in life but the immediate underlying cause of a CVD event is the occlusion of a critically situated blood vessel by a blood clot, which results in loss of blood flow to vital organs [2]. Therefore, the optimal breakdown of clots is an important protective mechanism in CVD [3].

Various proteins are involved in the lysis of blood clots. These proteins, such as plasminogen activator inhibitor type-1 (PAI-1), tissue-type plasminogen activator (tPA) and plasminogen, can be measured individually. They then serve as proxy markers for fibrinolysis. Alternatively, one can make use of global fibrinolytic assays to measure the global ability of blood or plasma to lyse clots. These assays give an indication of the speed at which the body can lyse clots, often reported as lysis time. In recent years, a plasma fibrinolytic potential assay has been developed that mimics the physiological initiation of coagulation by tissue factor and clot breakdown by tPA from the endothelium [4].

Various studies conducted in populations of European descent have shown that plasma clot lysis times (CLT) are associated with CVD. In general, CVD patients had longer CLTs than controls [5–9], although this was not the case in all studies [10]. CLT has been shown, furthermore, to be associated with individual CVD risk factors such as increased body mass index (BMI), diabetes, increased total cholesterol, triglycerides and CRP in populations of European descent [7, 8].

No information is available regarding the association of clot lysis times and CVD risk factors in Africans, a little-studied population in CVD epidemiology. CVD, which has often been thought of as a specific to developed countries is now a major problem in developing countries, including South Africa [11, 13]. This increase in CVD prevalence is considered to be attributable to the urbanisation of the black South African population, a transition characterised by decreased physical activity and a shift to a Westernised lifestyle and diet. Furthermore, it has previously been shown that CVD risk factors and their contribution to CVD risk may differ between blacks and populations of European descent [11, 12]. Two large epidemiological studies in black South Africans have shown that various CVD risk factors increased with urbanisation. Vorster [14] reviewed data from the Transition and Health during Urbanisation in South Africa (THUSA) study and reported that BMI, smoking prevalence in men, total serum cholesterol levels and blood pressure increased with urbanisation. In the Prospective Urban and Rural Epidemiological (PURE) population, Pieters et al. [15] found blood pressure, BMI, waist circumference, triglyceride concentrations, fasting plasma glucose, and PAI-1_{act}, all factors associated with CVD, to be significantly higher in the urban participants than in the rural group.

The purpose of this study was, therefore, to determine whether urbanisation, with its increased prevalence of CVD risk factors, is associated with hypofibrinolysis, and secondly, to determine the association between CLT and traditional CVD risk factors in black South Africans.

Materials and methods

Study population

Participants were recruited to take part in the South African arm of the international PURE study. This is a large-scale cohort study that tracks changing lifestyles, risk factors and chronic disease, using periodic standardised data collection in rural and urban areas of 17 countries in transition over 12 years [16, 17]. The data reported here are from the baseline data of just over 2000 randomly selected participants from well-established rural (living under tribal law) and urban (living in informal and formal settlements surrounding cities) communities in the North West Province of South Africa. The sample size decided upon was based on power calculations performed on the results of the THUSA study, a cross-sectional epidemiological study conducted in similar communities in the same province 10 years prior to PURE [14]. In order to obtain 2000 participants, it was decided to include randomly 6000 households (3000 from rural and urban respectively), based on previous experience from the THUSA study. From these 6000 households, 4000 subjects were identified who fitted the inclusion criteria. Inclusion criteria were that participants had to be apparently healthy black South African men and women between the ages of 35 and 60 years. Use of chronic medication for non-communicable diseases and/or any self-reported acute illness were bases for exclusion. Of these 4000 eligible subjects, 2792 (rural = 1444, urban = 1348) agreed to take part in the study, indicated their availability during the blood collection period and had no plans to relocate in the foreseeable future. During the 12-week blood collection period in 2005, blood was finally collected from 1006 rural and 1004 urban participants. The Ethics committee of the North-West University, South Africa, approved this study. The study procedure was explained to participants in their home language, after which

participants signed informed consent forms (Addendum A) and the study commenced. All data were treated confidentially and all analyses were performed with coded data.

Blood collection

Between 07:00 and 11:00 on days of data collection, qualified nursing sisters, using sterile winged infusion sets and syringes, collected fasting blood samples with minimum stasis from the antecubital veins of participants. For the analysis of lipids and C-reactive protein (CRP) in serum, blood was collected in tubes without anticoagulant. Blood was collected in EDTA tubes for the determination of glycosylated haemoglobin (HbA1c) and in fluoride tubes for glucose measurements. For the analysis of PAI-1_{act}, fibrinogen concentration and plasma fibrinolytic potential, blood was collected in citrate tubes and kept on ice until centrifugation. 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

Serum lipids and high-sensitivity CRP were measured with a Sequential Multiple Analyser Computer (SMAC), using the Konelab™ autoanalyser (Thermo Fischer Scientific, Vantaa, Finland). HbA1c was determined by means of the D-10 Haemoglobin testing system (Biorad, Hercules, CA, USA). A hexokinase method, using the Synchron®System(s) (Beckman Coulter Co., Fullerton, CA, USA) and reagents, was employed to measure plasma glucose. Participants' status with regard to the human immunodeficiency virus (HIV) was determined according to the

South African Department of Health protocol and the UNAIDS/WHO Policy Statement on HIV-testing with the Rapid HIV test, and if positive, a Pareeshak test was performed to confirm the results. Participants received pre-test counselling, and for those who chose to know the results of their HIV test, post-test counselling was given in privacy. $PAI\text{-}1_{act}$ was measured using an indirect enzymatic method (Spectrolyze PAI-1, Trinity Biotech, Bray, Ireland). A modified Clauss method (Multifibrin U-test, Dade Behring, Deerfield, IL, USA) on the Dade Behring BCS coagulation analyser was used to determine fibrinogen concentrations. Plasma fibrinolytic potential of tissue factor-induced clots, lysed by exogenous tPA, was measured over a period of four weeks, using the method of Lisman et al. [5] with slightly modified tissue factor and tPA concentrations in order to obtain comparable CLTs of about 60 min (intra-assay CV = 3.6%, between plate CV= 4.5%). Final concentrations were tissue factor (125 x diluted; 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). CLT was defined as the time from the midpoint of clear to maximum turbidity, which is representative of clot formation, to the midpoint of maximum turbidity to clear, which represents the lysis of the clot [5] (for detailed protocol refer to Addendum B).

Dietary intake analysis and anthropometrical measurements

Quantitative Food Frequency questionnaires, designed and validated for use in this population, were used to determine the habitual alcohol consumption of study participants.

Anthropometric measurements were taken according to the International Standards of Anthropometric assessment [International society for the advancement of Kinanthropometry] and included weight and height as well as waist circumference. The recommended waist circumference cut-off for central obesity in sub-Saharan Africans, which is ≥94cm for men and ≥80cm for women [18], was used to define central obesity and used as the cut-off for metabolic syndrome criteria. Blood pressure was measured using an automatic digital blood pressure monitor (Omron HEM-757), with subjects sitting relaxed but upright and the right arm supported at heart level.

Statistical analysis

Data were analysed with the computer software package Statistica (Statsoft Inc., Tulsa, Oklahoma, USA). A p-value of 0.05 or less was regarded as statistically significant. Normally distributed data are reported as mean (95% confidence interval or SD). Data that were not normally distributed were log transformed to improve normality and reported as median (25^{th} – 75^{th} percentile). T-tests for independent samples were used when comparing parametric data between two groups and analysis of variance (ANOVA) with Tukey's Honestly Significant Difference post-hoc test was used for comparisons between three or more groups. The Mann-Whitney U test was used for comparison of non-parametric data between two groups. Analysis of co-variance (ANCOVA) was used when comparisons between groups required adjustment. Mean differences with corresponding 95% confidence intervals are also reported. CLT was not significantly different between women who used contraceptives and those who did not, so we did not stratify or adjust for contraceptive use. Forward stepwise multiple regression analysis was used to

determine the main contributors to the variance in CLT in the PURE population, using parametric and log-transformed data.

Results

General characteristics of rural and urban participants

Table 1 provides population characteristics for the total study population as well as for the urban and rural groups separately. Sex differences are also indicated for variables with sex-specific cut-offs. CLT could be determined for only 1802 participants, owing to inadequate sample volume and/or haemolysis of some samples. Baseline characteristics of the 1802 participants did not differ from those of the total group. The mean CLT was 57.3 (± 11.2) minutes (Table 1). CLT was longer in urban than in rural women (mean difference 1.79 min, 95% CI 0.60–2.99), while no difference was observed for men before adjustments. Taking the standard deviation into consideration, the likelihood of this difference being clinically significant is small. Urban participants had significantly higher blood pressure, BMI (women only), waist circumference (women only), triglycerides, PAI-1_{act} and fasting blood glucose than the rural participants. While urban women consumed more alcohol than rural women, the opposite was seen for men. Fibrinogen concentration and CRP were, however, higher in the rural participants.

Associations between CLT and CVD risk factors

The associations between CLT and various non-biochemical cardiovascular risk factors are presented in Table 2. ANCOVAs were used to adjust for factors that

could potentially influence/obscure independent associations or for intermediate variables that could, at least in part, explain the associations. These include variables that were themselves associated with CLT and which differed between the respective sub-categories. There was no significant difference in CLT between different age categories, between contraceptive use and, non-use, or between hypertensive and normotensive participants. Women had significantly longer CLTs than men (mean difference 1.3 min 95%CI 0.27–2.38), even after adjustment for differences in BMI and PAI-1_{act} (which differed between men and women, and which were associated with CLT). CLTs were significantly increased with increasing BMI categories (mean difference between lowest and highest BMI categories: 13.8 min, 95%CI 12.2–15.4) and in participants with abdominal obesity (mean difference 9.3 min, 95%CI 8.31–10.3). These differences were also likely to be clinically significant, taking the standard deviations into consideration. Although CLT correlated with both waist circumference ($r = 0.42$, $p<0.0001$) and BMI ($r = 0.47$, $p<0.0001$), the correlation with BMI was stronger and BMI was used in further analyses. Since sex differences were present amongst the BMI and waist circumference categories, we adjusted for sex, but significance remained. Participants who were diagnosed with the metabolic syndrome, using the criteria recommended by Alberti et al. [18], also had significantly longer CLTs than those without metabolic syndrome (mean difference 4.1 min 95% CI 2.84–5.36). Current smokers had significantly shorter CLTs than non-smokers (mean difference 4.5 min 95% CI 3.48–5.52). This significance remained after adjustment for BMI, but disappeared after adjustment for habitual alcohol consumption (which differed between smoking categories). Participants who were non-drinkers had significantly longer CLTs than participants who were reported to be moderate (mean difference 5.2 min 95% CI 4.03–6.37) or

heavy drinkers (mean difference 6.9 min 95% CI 5.41–8.39). Because non-drinkers had significantly higher waist circumference measures (also associated with longer CLT) and significantly lower PAI-1_{act} (associated with shorter CLTs) than drinkers, we adjusted for both, but significance remained ($p<0.0001$). Men drank significantly more than women did, and since sex differences for CLT were found, we also adjusted for sex, but again significance remained ($p<0.0001$).

In Table 3, associations between CLT and biochemical CVD risk factors are presented. CLT increased significantly over HbA1c quartiles (mean difference between lowest and highest quartiles: 8.7 min 95% CI 7.38–10.0) and fasting glucose categories (mean difference 4.5 min 95% CI 2.97–6.03). Since a positive association was found between both HbA1c and fasting plasma glucose with BMI, we adjusted for BMI, but the significance between the HbA1c quartiles and fasting glucose quartiles remained. Furthermore, significantly longer CLTs were observed in participants who had increased serum triglycerides compared with normal triglyceride levels (mean difference 7.6 min 95% CI 6.29–8.91), increased total cholesterol levels compared with normal cholesterol (mean difference 3.5 min 95% CI 2.44–4.56) and decreased HDL-cholesterol levels compared with the recommended values (mean difference 5.8 min 95% CI 4.70–6.90). CLT also increased significantly over the PAI-1_{act} (mean difference between lowest and highest quartiles 15.4 min 95% CI 14.0–16.8), fibrinogen (mean difference between lowest and highest quartiles 4.9 min 95% CI 3.43–6.37) and CRP quartiles (mean difference between lowest and highest quartiles 6.9 min 95% CI 5.42–8.38). Taking the standard deviation into consideration, these differences were all likely to be clinically significant. Significance remained for fibrinogen after adjustment for CRP

and also for CRP after adjustment for PAI-1_{act} and fibrinogen. CLTs tended to be longer in HIV+ compared with HIV- participants (mean difference 1.3 min 95% CI 0.11–2.49). Because there is a difference in BMI between HIV+ and HIV- participants (data not shown), and CLT correlated with BMI, we adjusted for BMI. After the adjustment, HIV+ participants now had significantly longer CLTs than the HIV- participants (59.7 vs 56.9 minutes).

In order to determine the main contributors to the variance of CLT, the variables in Table 2 and 3 were included in a forward stepwise regression model. The model explained 45% of the variance in CLT. PAI-1_{act} explained 27% of the variance, while BMI, alcohol consumption and HbA1c explained 8%, 3% and 2% respectively. Triglycerides, CRP, HDL-cholesterol and HIV status each explained only 1% of the variance. Blood pressure, total cholesterol, fibrinogen, smoking and age each explained less than 0.5%. BMI, but not waist circumference, and HbA1c, but not fasting glucose, were included in order to prevent inter-correlation between the variables. Metabolic syndrome per se was also not included as the individual components were entered into the model separately.

Discussion

This study investigated for the first time whether urbanisation, with its resultant increased CVD risk, is associated with hypofibrinolysis. This is also the first paper to investigate the association between known CVD risk factors and global fibrinolytic potential in blacks.

Rural and urban differences for CLT

Clot lysis time was not found to be significantly longer in the urban than in the rural participants, despite an increase in most CVD risk factors and positive associations of these risk factors with CLT (which will be discussed below). This can probably be explained through the association of CLT with individual CVD risk factors. CVD risk factors that were increased in the urban group and that are associated with increased clot lysis, e.g. triglycerides, PAI-1 and BMI, could potentially increase CLT in the urban group. On the other hand, some CVD risk factors, for example, fibrinogen and CRP, were higher in the rural group and were also found to have positive associations with CLT, therefore contributing to increased CLT in the rural group. Separate analysis for men and women indicated that urban women had a mean CLT that was only slightly longer than that of rural women, but still statistically significant, while no differences were observed for men. A possible reason for the rural–urban differences in CLT observed in women but not in men is that the rural–urban differences in the CVD risk factors were more pronounced in the women than in the men. This was indeed the case for BMI, the rural–urban difference in women being seven times that of the difference in men. The relative importance of BMI in CLT was additionally established with the multiple regression analysis.

Association of CLT with CVD risk factors

Of the known CVD risk factors investigated, the main contributors to the variance in CLT in this population, as determined by a forward stepwise multiple regression model which included the variables in Tables 2 and 3, were PAI-1_{act} (27%), BMI (8%), alcohol consumption (3%) and HbA1c (2%). A previous study conducted in a population of European descent found triglycerides, BMI, diastolic blood pressure,

systolic blood pressure and CRP to be the main contributors to CLT. PAI-1, however, was not measured [8].

In a separate study investigating the main coagulation and fibrinolytic factors associated with CLT, PAI-1_{ag}, in agreement with our PAI-1_{act} results, was found to have the strongest association, explaining 24% of CLT variation in a multiple regression model [19]. The differences in CLT, especially across the PAI-1 and BMI quartiles, may be of clinical relevance. The difference between the lowest and highest quartiles was about 15 minutes while differences between arterial thrombosis patients and controls in previous studies ranged from 1.9 to 10.8 minutes [7, 8, 19].

Interestingly, in the PURE population, CLT correlated better with BMI than with waist circumference even though PAI-1_{act} had a significantly stronger correlation with waist circumference [20]. This stronger correlation of PAI-1 with waist circumference is probably due to the fact that visceral adipose tissue is a major source of PAI-1 and that its levels are further increased by hepatic production in response to adipocyte-derived cytokines [21–24]. Adjustment for PAI-1_{act} did not significantly affect the results and CLT remained longer with increased BMI categories and in individuals with abdominal obesity. While PAI-1_{act} seems to be more related to central obesity, CLT seems to be related to general obesity. Although increased PAI-1_{act} levels probably play a major role in this relationship, there seem to be additional mechanisms, unrelated to the link between PAI-1 and visceral fat, involved in the association between body fat and CLT.

CLT was significantly associated with both glucose and HbA1c. In agreement with these results, Guimarães et al. [7] and Meltzer et al. [6] found that white diabetic

subjects had longer CLT than non-diabetic subjects. Another study, however, found no association between CLT and diabetes in control subjects [8]. CLT was also longer in participants diagnosed with the metabolic syndrome, than in those without. These results are to be expected since most of the criteria for the metabolic syndrome were found to be associated with longer CLTs. These results are in agreement with the results of Carter et al. [25], who found that CLT was longer in white patients with the metabolic syndrome, although a different classification system was used to diagnose metabolic syndrome and their clot lysis assay differs from the assay we used.

Although CLT was increased across fibrinogen quartiles, fibrinogen concentration explained less than 0.5% of the variance in CLT and correlated only weakly with CLT ($r = 0.18$, $p<0.0001$), indicating that fibrinogen concentration was not one of the main contributing factors to CLT variance in this population. Theoretically, fibrinogen could influence fibrin lysis rates through its effect on clot structure, as has been demonstrated in purified models [26-28]. This effect, however, is less prominent in plasma models, probably because of the presence and interaction of other plasma components that also affect clot lysis. It is also possible that other factors included in the model influenced the prediction value of fibrinogen. We also saw a significant increase in CLT across CRP quartiles, even after adjustment for PAI-1 and fibrinogen, indicating an independent positive association between inflammation and CLT. These results are in agreement with associations found between CLT, fibrinogen and CRP data in the literature [5, 8].

We found no association between CLT and age and between CLT and blood pressure whereas studies in populations of European descent found trends of increased CLT with increased age [5, 7, 8] and with increased systolic and diastolic blood pressure [8]. In agreement with this, no association was found between PAI-1_{act} and age in the PURE study population [20].

Our results also show women to have significantly longer CLTs than men, while other studies found either longer CLTs in white men than in women, although the difference was not significant [5, 6], or no differences between men and women [7]. Possible factors that might have explained the longer CLT in women in our study are PAI-1_{act} and BMI, which were both found to be higher in the women than in the men [20]. Adjustment for PAI-1_{act} and BMI did not significantly affect the results, however, and CLTs remained longer in women than in men, indicating a possible real sex difference in this study population.

We found significantly shorter CLTs for moderate and heavy drinkers than for non-drinkers, while studies in populations of European descent reported no apparent differences between regular users of alcohol and participants who do not use alcohol or do so only occasionally [7, 8]. Three factors that differed significantly between drinkers and non-drinkers [20] and that were found to be associated with CLT were considered in order to explain the association between CLT and alcohol consumption. These factors were sex, waist circumference and PAI-1_{act} [20]. However, after separate adjustment for these three factors, significant differences in CLT between drinkers and non-drinkers remained. The fact that CLT is shorter in drinkers in this population, while PAI-1_{act}, a main factor determining CLT, was found

to be increased, suggests that alcohol affects CLT, at least in part, in a manner that is unrelated to PAI-1_{act}.

HIV+ participants had longer CLTs than HIV- participants after adjustments for BMI differences. One would expect the HIV- group which had the higher BMI values to have longer CLTs because of the link between PAI-1_{act} and adipose tissue or overall body fat. Positive HIV status has, on the other hand, been shown to be associated with increased PAI-1 antigen and probably, therefore, impaired fibrinolysis. This increase may be attributed to fat redistribution in patients infected with the HIV virus [29]. However, in the PURE population, PAI-1_{act} did not differ between the HIV+ and HIV- participants [30].

Because many haemostatic factors play a role in and/or influence CLT, the fact that only PAI-1_{act} and fibrinogen concentrations were measured in this study population may be a limitation to the interpretation of the results of this study and could potentially lead to residual confounding. Additionally, since this was a cross-sectional study, causality could not be determined for CLT. 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, CLT in black Africans associated significantly with many known CVD risk factors. However, differences were observed in these associations compared with available data on white populations, suggesting possible ethnic differences in the association of CLT with CVD risk. Of the variables measured, CLT was most strongly related to PAI-1_{act} and BMI. CLT seems to be strongly affected by total

body fat, but only partly, it seems, through the link between PAI-1 and visceral fat. Additional research is required to determine which factors associated with obesity influence CLT. Alcohol consumption in this population was significantly associated with shorter CLT, despite increased PAI-1_{act}. This also deserves further attention. Urbanisation per se is not associated with hypofibrinolysis despite an increase in presence of many CVD risk factors. The effect of urbanisation on CLT is dependent on the relationship of the individual CVD risk factors with CLT and on the extent to which urbanisation affects these risk factors.

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Legends to Tables

Table 1 Characteristics of the South African PURE study population

Parametric data reported as mean ± SD and non-parametric data as median (25^{th}–75^{th} percentile); SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index; HDL: high-density lipoprotein; LDL: low-density lipoprotein; PAI-1: plasminogen activator inhibitor type-1; HbA1c: glycosylated haemoglobin; CLT: clot lysis time; * Means differed significantly between sexes; rural vs urban p-value adjusted for age and sex, and only for age where values are reported for men and women separately.

Table 2 Non-biochemical cardiovascular disease risk factors and their association with CLT

* Moderate drinking: >0 < 15 g/day for women; > 0 < 30 g/day for men; ** Heavy drinking: ≥15 g/day for women; ≥ 30 g/day for men; †§: Means with the same symbol differed significantly; ¶ Mean differed significantly from other means in subgroup. WC: waist circumference; BMI: Body mass index; PAI-1: Plasminogen activator inhibitor type-1. For groups with more than two subgroups, the difference between the highest and lowest value is reported. N varies among variables owing to lack of sample availability, which occurred randomly during sample collection. • Model 1: adjusted for age; $^{\Delta}$ Model 2: adjusted for age as well as variables indicated in table.

Table 3 Biochemical cardiovascular disease risk factors and their association with CLT

†‡§‖ Means with the same symbol differed significantly. HbA1c: Glycosylated haemoglobin; HDL-cholesterol: high density lipoprotein cholesterol; PAI-1: Plasminogen activator inhibitor type-1; CRP: C-reactive protein; HIV: Human

Immunodeficiency Virus. For groups with more than two subgroups, the difference between the highest and lowest value is reported. N varies among variables owing to lack of sample availability which occurred randomly during sample collection. • Model 1: adjusted for age; Δ Model 2: adjusted for age as well as variables indicated in table.

Table 1. Characteristics of the South African PURE study population

Variable	Total population (n=2010)	Urban (n=1004)	Rural (n=1006)	Rural vs urban p-value
Age (years)	48 (41 – 56)	48 (42 – 57)	47 (41 – 55)	0.0002
Men/women n (%)	749 (37.3)/1260 (62.7)	401 (39.9)/602 (60.0)	348 (34.6)/658 (65.4)	0.01
CLT (minutes)	57.3 ± 11.2	57.6 ± 12.0	57.0 ± 10.5	0.09
Men	52.9 ± 11.6*	52.6 ± 12.4*	53.3 ± 10.6*	0.55
Women	59.9 ± 10.2*	60.8 ± 10.5*	59.0 ± 10.0*	0.015
SBP (mm/Hg)	133.5 ± 24.5	137.3 ± 25.1	129.7 ± 23.3	<0.0001
DBP (mm/Hg)	87.7 ± 14.5	89.3 ± 14.5	86.2 ± 14.5	<0.0001
BMI (kg/m^2)	22.9 (19.3 – 28.6)	23.4 (19.5 – 29.4)	22.4 (19.1 – 28.1)	<0.0001
Men	19.8 (18.1 – 22.4)	20.0 (18.3 – 22.8)	19.7 (18.0 – 22.2)	0.60
Women	25.8 (21.4 – 31.7)	27.1 (22.3 – 32.5)	24.9 (20.8 – 30.7)	<0.0001
Waist circumference (cm)	77.5 (70.2 – 87.7)	78.5 (70.9 – 89.0)	76.0 (69.7 – 86.9)	0.001
Men	74.4 (69.9 – 81.3)*	74.3 (69.7 – 81.8)*	74.5 (70.2 – 80.5)*	0.49
Women	81.1 (70.6 – 91.3)*	82.8 (73.1 – 92.8)*	78.8 (69.5 – 89.5)*	<0.0001
HDL-cholesterol (mmol/l)	1.52 ± 0.63	1.52 ± 0.65	1.52 ± 0.62	0.83
Men	1.58 ± 0.66*	1.61 ± 0.66*	1.55 ± 0.66	0.22
Women	1.48 ± 0.62*	1.46 ± 0.63*	1.50 ± 0.61	0.17
Triglycerides (mmol/l)	1.08 (0.82 – 1.55)	1.11 (0.84 – 1.65)	1.05 (0.80 – 1.43)	<0.001
Total cholesterol (mmol/l)	4.82 (4.01 – 5.87)	4.89 (4.00 – 5.97)	4.75 (4.02 – 5.80)	0.32
LDL-cholesterol (mmol/l)	2.79 (2.08 – 3.65)	2.81 (2.07 – 3.66)	2.77 (2.09 – 3.63)	0.95

PAI-1 (U/ml)	4.26 (1.27 – 7.92)	5.01 (1.76 – 9.11)	3.58 (0.81 6.85)	<0.0001
Fibrinogen (g/l)	2.90 (2.30 – 5.00)	2.70 (2.20 – 4.30)	3.00 (2.40 – 5.40)	<0.0001
Men	2.60 (2.10 – 3.70)*	2.50 (2.00 – 3.30)*	2.80 (2.20 – 4.30)*	0.04
Women	3.10 (2.30 – 5.50)*	2.90 (2.30 – 5.40)*	3.20 (2.50 – 5.70)*	<0.001
HbA1c	5.50 (5.30 – 5.80)	5.50 (5.20 – 5.80)	5.60 (5.30 – 5.80)	0.89
Fasting plasma glucose (mM)	4.80 (4.30 – 5.30)	4.90 (4.30 – 5.40)	4.70 (4.40 – 5.20)	0.06
CRP (mg/l)	3.29 (0.96 – 9.34)	3.25 (1.12 – 9.85)	3.33 (0.85 – 9.02)	0.07
Alcohol consumption (g/day)				
(Alcohol consumers only)				
Total group (n = 872)	15.4 (6.43 – 34.7)	15.9 (7.71 – 30.9)	15.0 (5.14 – 44.8)	0.96
Men (U n = 281, R n = 186)	19.7 (7.71 – 40.0)*	19.3 (11.4 – 34.7)*	21.9 (5.86 – 56.6)*	0.88
Women (U n = 252, R n = 153)	13.4 (4.29 – 30.9)*	14.8 (4.86 – 26.8)*	11.4 (3.21 – 38.6)*	0.92

Parametric data reported as mean ± SD and non-parametric data as median (25^{th}–75^{th} percentile); SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index, HDL, high density lipoprotein; LDL, low-density lipoprotein, PAI-1, plasminogen activator inhibitor type-1; HbA1c, glycosylated haemoglobin; CLT, clot lysis time; R, rural; U, urban; * Means differed significantly between sexes; rural vs urban p-value adjusted for age and sex, and only for age where values are reported for men and women separately.

Table 2. Non-biochemical cardiovascular disease risk factors and their association with CLT

Variable	N	Mean CLT (95% CI) minutes	ANCOVA p-value: Model 1• (Model 2) Δ variables adjusted for	Mean difference (95% CI)
Age groups				
30-39 years	332	56.9 (55.9 – 57.9)	0.71	0.8 (-0.95 – 2.55)
40-49 years	700	57.5 (56.7 – 58.3)	(0.56) Smoking, alcohol	
50-59 years	482	57.0 (55.9 – 58.0)	consumption and BMI	
≥60 years	288	57.7 (56.3 – 59.2)		
Sex			<0.0001	
Men	670	52.9 (52.1 – 53.9)	(<0.0001) BMI	1.3 (0.27 – 2.38)
Women	1132	59.9 (59.3 – 60.5)	(0.003) PAI-1act, BMI	
Contraceptive use				
Yes	403	60.5 (59.5 – 61.6)	0.10	1.1 (-0.18 – 2.38)
No	667	59.4 (58.7 – 60.2)		
Blood pressure				
Normal	946	57.3 (56.6 – 58.0)	0.93	0 (-1.05 – 1.05)
Hypertensive	842	57.3 (56.5 – 58.1)		
BMI				
<18.5 kg/m^2	314	50.8 (49.6 – 52.0)†	<0.0001	13.8 (12.2 – 15.4)
18.5 – 24.9 kg/m^2	739	55.3 (54.5 – 56.0)†	(0.0001) PAI-1act	
25 – 29.9 kg/m^2	297	60.8 (59.6 – 61.9)†	(<0.001) PAI-1act, sex	
≥30 kg/m^2	354	64.6 (63.6 – 65.6)†		
Waist circumference			<0.0001	
Normal	1168	54.1 (53.5 – 54.7)†	(<0.0001) PAI-1act	9.3 (8.31 – 10.3)
Abdominal obesity	617	63.4 (62.6 – 64.2)†	(<0.0001) PAI-1act, sex	
Metabolic syndrome				
Yes	434	60.4 (59.2 – 61.5)†	<0.0001	4.1 (2.84 – 5.36)
No	1358	56.3 (55.7 – 56.9)†		
Metabolic Syndrome risk score				
0	278	56.4 (55.1 – 57.6)	<0.0001	5.5 (3.81 – 7.19)
1	604	54.9 (54.0 – 55.8)		
2	476	58.0 (57.1 – 59.0)		
≥3	434	60.4 (59.2 – 61.5)¶		
Smoking status				
Yes	943	55.3 (54.6 – 56.1)†	<0.0001	4.5 (3.48 – 5.52)
No	785	59.8 (59.0 – 60.5)†	(0.016) BMI	
Former	66	56.0 (52.9 – 59.0)	(0.78) BMI, alcohol	
Alcohol consumption			<0.001	
Non-drinkers	964	60.0 (59.4 – 60.7)†§	<0.0001) Sex, WC	6.9 (5.41 – 8.39)
Moderate drinkers *	467	54.8 (53.9 – 55.8)†	(<0.0001) Sex, WC,	
Heavy drinkers **	320	53.1 (51.7 – 54.4)§	PAI-1act	

* Moderate drinking: >0 < 15 g/day for women; > 0 < 30 g/day for men; ** Heavy drinking: ≥15 g/day for women; ≥ 30 g/day for men; †‡§ǁ Means with the same symbol differed significantly; ¶ Mean differed significantly from other means in subgroup. WC: waist circumference; BMI: Body mass index; PAI-1: Plasminogen activator inhibitor type-1. For groups with more than two subgroups, the difference between the highest and lowest value is reported. N varies

among variables owing to lack of sample availability, which occurred randomly during sample collection. ·Model 1: adjusted for age; △Model 2: adjusted for age as well as variables indicated in table.

Table 3. Biochemical cardiovascular disease risk factors and their association with CLT

Variable	N	Mean CLT (95% CI) minutes	ANCOVA p-value: Model 1• (Model 2)Δ variables adjusted for	Mean difference (95% CI)
HbA1c				
<5.3	443	53.1 (52.2 – 54.0)†	<0.0001	8.7 (7.38 – 10.0)
≥5.3 - <5.5	289	54.1 (52.9 – 55.3)†	(<0.0001) BMI	
≥5.5 - <5.8	490	57.8 (56.8 – 58.8)		
≥5.8	564	61.8 (60.9 – 62.8)		
Fasting glucose				
≤5.5 mmol/l	1435	56.5 (55.9 – 57.0)	<0.0001	4.5 (2.97 – 6.03)
>5.5 mmol/l	297	61.0 (59.6 – 62.5)	(0.003) BMI	
Triglycerides				
<1.7mmol/l	1417	55.8 (55.2 – 56.3)†	<0.0001	7.6 (6.29 – 8.91)
≥1.7mmol/l	342	63.4 (62.2 – 64.6)†		
Total cholesterol				
<5.2 mmol/l	1059	55.8 (55.2 – 56.5)†	<0.0001	3.5 (2.44 – 4.56)
≥5.2 mmol/l	710	59.3 (58.5 – 60.1)†		
HDL-cholesterol				
Men >1, women>1.2mmol/l	1233	55.5 (54.9 – 56.1)†	<0.0001	5.8 (4.70 – 6.90)
Men<1, women<1.2mmol/l	536	61.3 (60.4 – 62.2)†		
PAI-1act				
<1.27 U/ml	456	50.2 (49.3 – 51.2)†	<0.0001	15.4 (14.0 – 16.8)
≥1.27 - <4.26 U/ml	460	54.9 (54.1 – 55.7)†	(<0.0001) Sex	
≥4.26 -<7.92 U/ml	446	58.7 (57.9 – 59.5)†		
≥7.92 U/ml	440	65.6 (64.5 – 66.7)†		
Fibrinogen				
<2.3 g/L	471	55.1 (54.0 – 56.1)†ǁ	<0.0001	4.9 (3.43 – 6.37)
≥2.3 - <2.9 g/L	380	56.3 (55.2 – 57.4) ‡§	(<0.001) CRP	
≥2.9 - <5 g/L	406	58.7 (57.6 – 59.8) †§		
≥5 g/L	421	60.0 (59.0 – 61.0) ‡ǁ		
CRP				
<0.964 mg/L	431	53.8 (52.8 – 54.7)†	<0.0001	6.9 (5.42 – 8.38)
≥0.964 - <3.286 mg/L	443	56.2 (55.2 – 57.1)†	(<0.0001) PAI-1act	
≥3.286 - <9.340 mg/L	442	58.3 (57.3 – 59.4)†	(<0.0001) Fibrinogen	
≥9.340 mg/L	445	60.7 (59.5 – 61.8)†	(<0.0001) PAI-1act, fibrinogen	
HIV status				
Positive	306	58.4 (57.4 – 59.4)†	0.052	1.3 (0.11 – 2.49)
Negative	1486	57.1 (56.5 – 57.7)†	(<0.0001) BMI	

†‡§ǁ Means with the same symbol differed significantly. HDL-cholesterol: high density lipoprotein cholesterol; PAI-1: Plasminogen activator inhibitor type-1; CRP: C-reactive protein; HbA1c: Glycosylated haemoglobin; HIV: Human Immunodeficiency Virus. For groups with more than two subgroups, the difference between the highest and lowest value is reported. N varies among variables owing to lack of sample availability, which occurred randomly during sample collection. • Model 1: adjusted for age; Δ Model 2: adjusted for age as well as variables indicated in table.

CHAPTER 4:

The effect of polymorphisms of the PAI-1 gene on PAI-1 activity levels and plasma clot lysis time in black South Africans: the influence of urbanisation

This chapter includes:

- the instructions given to authors by the journal *Thrombosis and haemostasis*;
- proof of submission for publication;
- the article titled “The effect of genetic polymorphisms of the PAI-1 gene on PAI-1 activity levels and plasma clot lysis time in black South Africans: the influence of urbanisation”, submitted for publication in April 2013 and presented in the technical style specified by the journal; and
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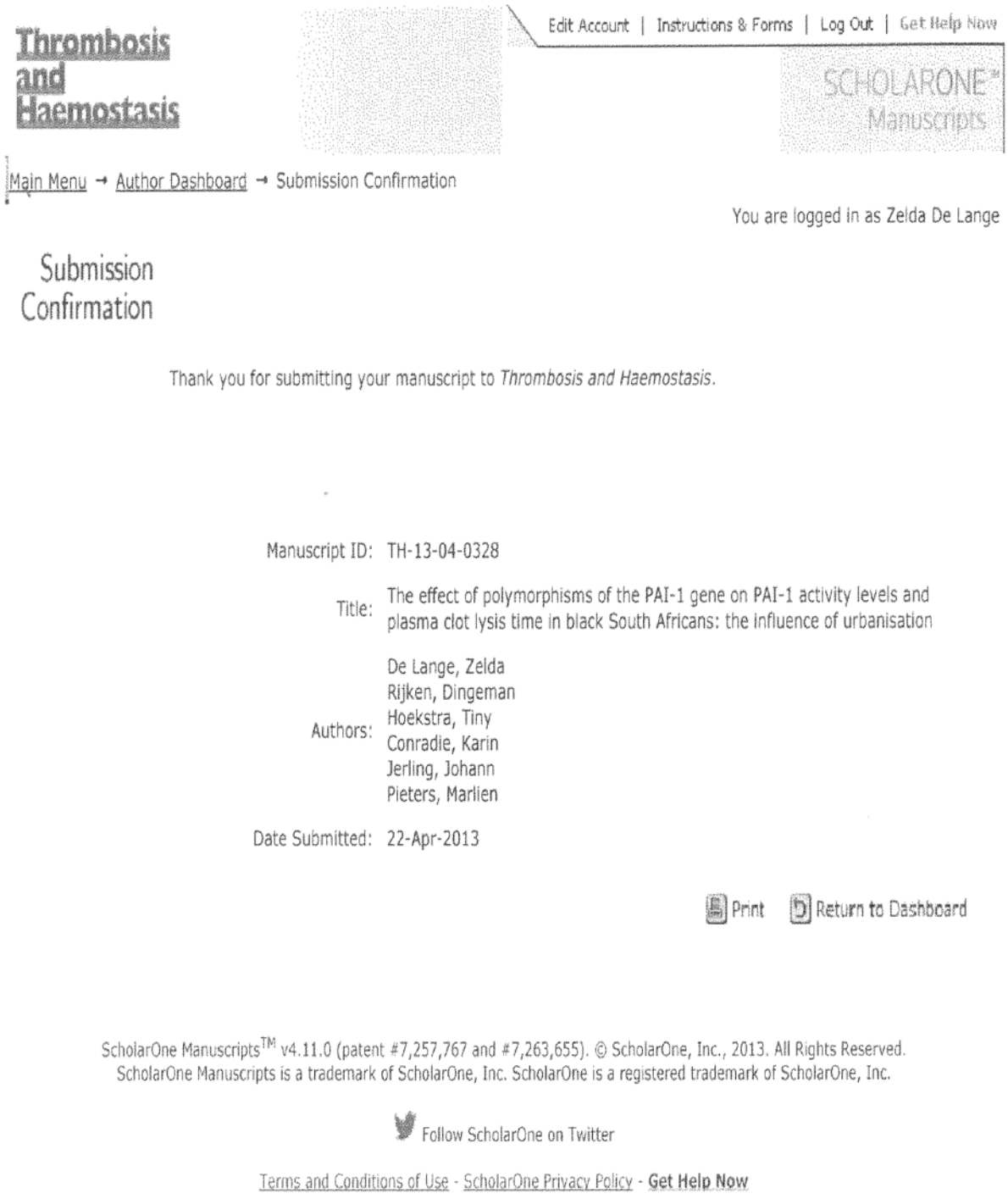

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MANUSCRIPT: The effect of polymorphisms of the PAI-1 gene on PAI-1 activity levels and plasma clot lysis time in black South Africans: the influence of urbanisation

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Abstract

Data on genetic and environmental factors influencing PAI-1 levels and their consequent effect on clot lysis in black African populations are limited. We identified polymorphisms in the promoter area of the PAI-1 gene and determined their influence on PAI-1_{act} levels and plasma clot lysis time (CLT). We also describe gene-environment interactions and the effect of urbanisation. Data from 2010 apparently healthy urban and rural black participants from the South African arm of the PURE study were cross-sectionally analysed. The 5G allele frequency of the 4G/5G polymorphism was 0.85. PAI-1_{act} increased across genotypes in the urban subgroup (p=0.009) but not significantly in the rural subgroup, while CLT did not differ across genotypes. Significant interaction terms were found between the 4G/5G polymorphism and BMI, waist circumference and triglycerides in determining PAI-1_{act}, and between the 4G/5G polymorphism and fibrinogen and fibrinogen gamma prime in determining CLT. The C428T and G429A polymorphisms did not show direct relationships with PAI-1_{act} or CLT but they did influence the association of other environmental factors with PAI-1_{act} and CLT. Several of these interactions differed significantly between rural and urban subgroups, particularly in individuals harbouring the mutant alleles. In conclusion, although the 4G/5G polymorphism significantly affected PAI-1_{act}, it contributed less than 1% to the PAI-1_{act} variance. (Central) obesity was the biggest contributor to PAI-1_{act} variance (12.5%). Urbanisation significantly influenced the effect of the 4G/5G polymorphism on PAI-1_{act} as well as gene-environment interactions for the C428T and G429A genotypes in determining PAI-1_{act} and CLT.

Introduction

The prevalence of cardiovascular disease (CVD) has increased significantly in developing countries, and will continue to do so in the future (1). The increase in morbidity and mortality from CVD is also seen in the black population of South Africa (2, 3), particularly as a result of urbanisation, which is the change in lifestyle patterns brought about by the on-going migration from rural to urban settings. One of the factors that have repeatedly been shown to be associated with an increased risk of CVD is increased levels of plasminogen activator inhibitor type-1 (PAI-1) (4, 5). PAI-1 can contribute to the development of CVD through several mechanisms, including influencing plaque formation as well as being a major inhibitor of blood clot lysis (6, 7). The speed with which the body can lyse clots, often reported as lysis time, has been linked to CVD (8). This reflects an individual's fibrinolytic potential and can be measured with the use of global fibrinolytic assays. PAI-1 antigen (PAI-1_{ag}) levels have been found to explain a large portion (24%) of the variance in clot lysis time (CLT) in a Caucasian population (9), while PAI-1 activity (PAI-1_{act}) explained 27% of CLT variance in the black African PURE population (10).

PAI-1 levels have previously been shown to be consistently lower in black African and African American populations than in Caucasian populations (11-16). However, as a result of urbanisation and the increased prevalence of various CVD risk factors associated with it, PAI-1 levels in urbanised black South Africans are increasing (reviewed by 16, 17). Environmental factors known to influence PAI-1 differ significantly between rural and urban settings (17).

PAI-1 levels are influenced by both environmental and genetic factors. There is, however, not much information available in the literature regarding the genetic determinants of PAI-1 levels, especially in black Africans. One of the most extensively studied genetic polymorphisms known to influence PAI-1 levels is the 4G/5G polymorphism (18, 19). This is a single base-pair insertion/deletion polymorphism in the promoter area of the PAI-1 gene (18, 20). The results of a recent large genome-wide association study (GWAS) meta-analysis showed that a SNP (rs2227631), considered a proxy SNP for the 4G/5G polymorphism, was strongly associated with PAI-1 levels (21). High PAI-1 levels have been found in participants who are homozygous for the 4G allele, intermediate levels in heterozygous participants and low PAI-1 levels in 5G homozygotes (12, 22, 23). The 4G/5G polymorphism is, furthermore, considered to be a response polymorphism. This implies that the difference in PAI-1 levels between 4G and 5G becomes more obvious in the presence of disease and/or environmental factors which stimulate PAI-1 expression (24). A limited number of studies in African Americans showed a higher prevalence of the 5G allele than in Caucasians (12, 25). Much less data is available for continental African populations. To date, only three small-scale studies have been performed in black African individuals, showing an even higher prevalence of the 5G allele than in African Americans (15, 26, 27).

The aim of this study was therefore to identify genetic polymorphisms in the promoter area of the PAI-1 gene and to determine their effect on PAI-$1_{act.}$ In addition we investigated whether these possible differences in PAI-1_{act} levels translated into altered global fibrinolytic potential (plasma clot lysis time). Owing to large differences in environmental factors between the rural and urban setting, we also

aimed to investigate the effect of gene-environment interactions (e.g. BMI, blood lipids) on PAI-1_{act} and CLT and whether these interactions were influenced by urbanisation.

Methods

Study population

Participants were recruited to take part in the South African arm of the international PURE study. This is a large-scale cohort study that tracks changing lifestyles, risk factors and chronic disease, using periodic standardised data collection in rural and urban areas of 17 countries in transition over 12 years (28, 29). The data reported here are from the baseline data of 2010 randomly selected participants from well-established rural and urban 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 are reported elsewhere (10, 17). In short, apparently healthy black South African men and women between the ages of 35 and 65 years were eligible to participate. Use of chronic medication for non-communicable diseases and/or any self-reported acute illness were bases for exclusion.

Equal numbers of rural and urban participants were included. The Ethics Committee of the North-West University, South Africa, approved this study. The study procedure was explained to participants in their home language, after which participants signed informed consent forms (Addendum A) and the study commenced. All data were treated confidentially and all analyses were performed with coded data.

Blood collection and laboratory analysis

Fasting blood samples were collected between 08:00 and 11:00. For the analysis of the lipid profile, blood was collected in tubes without anticoagulant - in EDTA tubes for the determination of glycosylated haemoglobin (HbA1c) and total homocysteine, and in fluoride tubes for glucose determination. For the determination of PAI-1_{act}, fibrinogen concentration, fibrinogen gamma prime, plasma fibrinolytic potential and for DNA isolation, blood was collected in citrate tubes. 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.

Methods for the measurement of fibrinogen, lipids, HbA1c, total homocysteine and plasma glucose have been described previously (17). PAI-1_{act} was measured using an indirect enzymatic method (Spectrolyze PAI-1, Trinity Biotech, Bray, Ireland). A modified Clauss method (Multifibrin U-test, Dade Behring, Deerfield, IL, USA) on the Dade Behring BCS coagulation analyser was used to determine fibrinogen concentrations. Fibrinogen gamma prime was determined with an enzyme-linked immunosorbent assay (ELISA) method using a 2.G2.H9 mouse monoclonal coating antibody against the human gamma prime 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 (Antibody 7539, Cambridge, USA) (30). Plasma fibrinolytic potential using tissue-factor-induced plasma clots, lysed by exogenous tPA, was measured using the method of Lisman *et al*. (8), validated by Talens *et al.* (31) with slightly modified tissue factor and tPA concentrations in order to obtain comparable CLTs of about 60 min (intra-assay CV

= 3.6%, between plate CV= 4.5%). Final concentrations were tissue factor (125x diluted – an estimated final concentration of 59 pM (32); 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). CLT was defined as the time from the midpoint of the transition of clear to maximum turbidity, which is representative of clot formation, to the midpoint in transition from maximum turbidity to clear, which represents the lysis of the clot (8). For more detail on this assay, please refer to Addendum B.

PAI-1 promoter area genotyping

The promoter region of the PAI-1 gene was sequenced in a subgroup of 25 randomly chosen participants and used as a representative group of the study population. PCR products were purified and bi-directionally sequenced using the BigDye®Terminatorv3.1Cycle Sequencing kit (AppliedBiosystems, CA, USA). After this, capillary electrophoresis was performed by the Central Analytical Facility of the Stellenbosch University on a 3130*xl* GeneticAnalyser (AppliedBiosystems) (33) (Supplemental Table 1).

Sequences were aligned to a reference sequence (NT_007933.15) using BioEdit v7 and were manually checked for known and novel polymorphisms. Seventeen polymorphisms were observed (Supplemental table 2). After linkage disequilibrium analysis and exclusion of two SNPs in linkage with the 4G/5G polymorphism, the polymorphisms with the highest frequencies, the 4G/5G polymorphism (rs1799889), located at chromosome position 100769710, and two SNPs located next to each other, at chromosome position 100768428 and 100768429 (rs36228614) were selected for analysis in the PURE population. The novel polymorphism at position

100768428 will be referred to as C428T, and the previously reported polymorphism next to it as G429A in the remainder of this article. The participants from the subgroup were used as controls in further analysis.

The 4G/5G-polymorphism was genotyped using TaqMan-based assays, as previously described (34), using the Biorad IQ5 real-time polymerase chain reaction (PCR) machine (Bio-Rad, Hercules, USA). A final concentration of 1.2 µM of each primer was used and 0.4 µM of each probe at an annealing temperature of 63°C.

The double nucleotide polymorphism C428T-G429A was genotyped by using a TaqMan-based assay using the BioRad IQ5 real-time PCR machine. A multiplex real-time PCR reaction was then performed, using four different probes (Table 3 of Supplement). A final concentration of 0.4 µM of each primer and 0.2 µM of each probe was used. The annealing temperature for the reaction was optimised at 62.4°C. Sequences are available in the supplement.

Statistical analysis

Data were analysed with the computer software package Statistica (Statsoft Inc., Tulsa Oklahoma, USA). A p-value of 0.05 or less was regarded as statistically significant. Normally distributed data are reported as means (95% confidence interval or standard deviation). Data that were not normally distributed were log-transformed to improve normality and are reported as median (25^{th}–75^{th} percentile). Owing to the large number of participants who had PAI-1_{act} values of 0 as calculated

from the standard curve of the assay, a value of 1 was added to all PAI-1_{act} values before log transformation. This was subtracted again when reporting the data. Pearson correlation coefficients were used to determine the relationships of PAI-1 and CLT with various CVD risk factors. T-tests were used 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. To investigate possible gene-environment interactions between PAI-1 genotypes and other factors and whether these interactions differed for rural and urban groups, interaction terms were entered into an analysis of co-variance (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, i.e. 5G/5G compared with the 4G/X allele group, CC compared with the T/X group, and GG compared with the A/X group. Analyses of the C428T and G429A SNPs combined did not yield different results from when analysed separately (data not reported).

Results

PAI-1_{act} and CLT across PAI-1 genotypes

General population characteristics of the South African PURE population have been described previously (10, 17) and are included in the supplement (Supplemental table 4) for ease of reference. The distribution of all three genotype classes of each

of the polymorphisms investigated adhered to the assumptions of Hardy Weinberg equilibrium. For the three polymorphisms we investigated, the linkage disequilibrium in the total study population between polymorphisms 4G/5G and C428T was: D' 1.0, r^2=0.007; between 4G/5G and G429A D' 0.72, r^2=0.011; and between C428T and G429A D' 0.72, r^2=0.003). Because of the low r^2, we report results for the three SNPs separately. The allele frequencies of the three polymorphisms are presented in Table 1.

The median PAI-1_{act} level of the South African PURE population was 4.26 (1.27-7.92) U/ml. Data for PAI-1_{act} levels and CLT across genotypes are presented for the total population as well as for the urban and rural participants separately in Table 1. PAI-1_{act} levels for the total population and for the urban subgroup differed significantly across the 4G/5G genotypes, where 5G homozygous participants presented with the lowest PAI-1_{act} levels and 4G homozygous participants with the highest PAI-1_{act} levels, while heterozygous participants had intermediate PAI-1_{act} levels. In the rural group, PAI-1_{act} was higher in the 4G/4G group only. BMI, however, differed across genotypes in the rural group (5G/5G: 22.6, 4G/5G: 21.3 and 4G/4G: 25.1 kg/m^2 p=0.008), but not in the urban group (5G/5G: 23.4, 4G/5G: 23.3 and 4G/4G: 25.5 kg/m^2 p=0.83). Because of the known relationship between BMI and PAI-1 levels, we adjusted for BMI. After this adjustment, differences between PAI-1_{act} levels across the genotypes remained in the urban subgroup but had only borderline significance in the rural group (p=0.06). No significant differences in clot lysis times between the genotype subclasses of the 4G/5G polymorphism were detected in the total population (p=0.12) or in the urban group (p=0.40). However, in the rural participants, those who had the homozygous 5G and

heterozygous genotypes had significantly shorter clot lysis times than participants with the 4G/4G genotype (57.0 and 56.6 minutes vs 63.1 minutes, p=0.04). Again after the adjustment for BMI, this significance disappeared.

Because the homozygous mutant genotype group (TT) of C428T was too small to include in further analysis as a separate group, it was combined with the CT heterozygous group. Neither PAI-1_{act} levels nor clot lysis times differed significantly between the homozygous C or T-allele group of C428T for the total population or for the rural and urban subgroups.

For the G429A SNP, the homozygous mutant (AA genotype) was also combined with the GA genotype for further analysis. No significant differences in PAI-1_{act} levels or clot lysis times were observed between the GG and A-allele genotypes in the total, rural or urban groups, except for PAI-1_{act}, which was higher in the GG-genotype than in the group carrying the A-allele in the urban subgroup.

Associations of cardiovascular disease risk factors with PAI-1_{act}

The contributions of various traditional CVD risk factors to PAI-1_{act} variance in this population were determined using forward stepwise multiple regression. Risk factors included in the regression model were: age, gender, waist circumference, triglycerides, HDL-cholesterol, LDL-cholesterol, HbA1c, total homocysteine, fibrinogen, fibrinogen gamma prime, blood pressure, smoking and 4G/5G genotype. These factors explained 24% of the variance in PAI-1_{act} in this population. Waist circumference explained most of the variance with 12.5%, gender, triglyceride and fibrinogen concentration explained 5%, 4% and 1% respectively, while the 4G/5G

polymorphism, fibrinogen gamma prime, blood pressure, smoking and HDL-cholesterol each explained less than 1%. Age and LDL-cholesterol did not enter the model. Associations of CLT and CVD risk factors have been reported in de Lange *et al.* (10).

Gene-environment interactions

Next we investigated the possibility of gene-environment interactions on PAI-1_{act} levels and CLT using the CVD risk factors that were significantly associated with PAI-1 and CLT. Results are presented for the interactions found for the 4G/5G polymorphism with PAI-1_{act} and CLT. Results reporting interactions of the C428T and G429A polymorphisms with PAI-1_{act} and CLT are available in the supplement.

When the participants were divided according to BMI, PAI-1_{act} differed across the genotypes of the 4G/5G polymorphism in individuals in the normal, overweight and obese BMI range, but not in underweight participants (BMI <18.5kg.m^2), and in participants with central adiposity but not in participants with normal waist circumferences (Table 2). CLT did not differ across the genotypes in any of the BMI categories, but was borderline significantly longer (p=0.05) in the 4G allele carriers in participants with central obesity. Distribution of the 4G/5G genotypes were similar in individuals with a BMI of <25kg/m^2 and individuals with a BMI of >25kg/m^2 (BMI <25kg/m^2: 5G/5G 73%; 4G/5G 25%; 4G/4G/ 2%: BMI >25kg/m^2: 5G/5G 73%; 4G/5G 23%; 4G/4G/ 3.7%). Furthermore, there was no difference in genotype distribution between individuals with abdominal obesity and those with normal waist circumference (abdominal obesity: 5G/5G 70%; 4G/5G 26%; 4G/4G/ 4%: normal waist circumference: 5G/5G 73%; 4G/5G 24%; 4G/4G/ 2.3%). There was also no

difference in BMI and waist circumference across the 4G/5G genotypes (data not shown) in the total study population.

The 4G/5G polymorphism had significant interactions with WC ($p<0.001$), BMI ($p=0.006$) and triglyceride concentration ($p=0.04$) in determining PAI-1_{act} (Table 3). In participants with the 4G allele, there was a greater increase in PAI-1_{act} with an increase in WC, BMI and triglycerides than in 5G/5G homozygotes. The 4G/5G polymorphism had significant interactions with fibrinogen ($p=0.005$) and fibrinogen gamma prime ($p=0.04$) concentration in determining CLT (Table 3). In participants with the 4G allele, there was a greater increase in CLT with an increase in fibrinogen and fibrinogen gamma prime concentration than in 5G/5G homozygotes. Urbanisation did not influence these gene-environment interactions.

The genotypes of the C428T and G429A polymorphisms also had significant interactions with various CVD risk factors in determining PAI-1_{act} and CLT (see supplement for details). In general it seems that, for both polymorphisms, differences in gene-environment interactions were observed between rural and urban participants in individuals harbouring the respective mutant alleles, while no rural-urban differences were present in participants homozygous for the respective wild-type alleles.

Discussion

This study provides the first population-based data for genotype frequencies of the 4G/5G polymorphism in a large black African population. To date there have been

only three small-scale studies and our data indicate an even higher prevalence of the 5G allele than previously reported for African Americans. In addition we aimed to identify other factors contributing to PAI-1_{act} variance in Africans by identifying other polymorphisms in the PAI-1 promoter area and determining their relation to PAI-1_{act} levels and CLT. Lastly we demonstrated that gene-environment interactions between the three investigated polymorphisms and several traditional CVD risk factors significantly affected PAI-1_{act} and CLT, and that level of urbanisation (rural or urban), representing two different sets of specific combinations of environmental factors, significantly influenced phenotypic expression of these polymorphisms.

PAI-1_{act} and CLT across PAI-1 genotypes

In this large black African population we found a high prevalence of the 5G/5G genotype (72.5%) of the 4G/5G polymorphism. These results agree with the results of two small-scale studies conducted in black South African participants, in which the 5G homozygous genotype was present in 76% and 77.6% of the populations in comparison with 36% and 19.3% in South African Caucasians (15, 26). The 5G allele frequency (0.85) was higher than that reported for African American, (0.74 and 0.72), and Caucasian populations (0.38 and 0.28) (12, 25).

In agreement with results from other studies, we also found PAI-1_{act} levels of the 4G homozygous genotype to be higher than those of the 4G/5G heterozygous and 5G homozygous genotypes, with the 4G/5G genotype group presenting with intermediate PAI-1_{act} levels in the overall population (12, 22, 23). Interestingly, this difference in PAI-1_{act} levels between genotypes of the 4G/5G polymorphism was much more pronounced in the urban than in the rural subgroup, suggesting that

urbanisation-dependent stimuli might modulate gene expression through an interaction with the 4G/5G polymorphism. Future studies identifying these stimuli are warranted to confirm this hypothesis.

Despite increased PAI-1_{act} levels in both the rural and urban subgroups in the 4G/4G genotype, this change did not translate to longer CLTs. The longer CLTs in the 4G/4G genotype in the rural subgroup could probably be attributed to higher BMI values in this group, since CLTs were no longer different after adjustment for BMI.

Neither PAI-1_{act} nor CLT differed across the novel C428T or the G429A genotype except in the urban subgroup, where homozygous wild-type participants had significantly higher PAI-1_{act} levels than the A-allele group. Although the G429A polymorphism is not novel, no information is available in the literature regarding its effect on PAI-1 levels. A possible reason for the apparent lack of effect of these two SNPs may be that the SNPs were not present within a known transcription factor binding site (as determined with the UCSC genome browser using the TFBS conserved and ENCODE regulation tracks).

Associations of cardiovascular disease risk factors with PAI-1_{act}

The 4G/5G polymorphism explained less than one percent of the variance of PAI-1_{act} in this population. The main contributor to PAI-1_{act} variance in this population was waist circumference, followed by gender and triglyceride concentration. This is in agreement with Verschuur *et al*. (35), who found obesity to be a more important determinant of PAI-1 levels than genetic variation in the PAI-1 promoter area. It is

also possible, however, that PAI-1 levels in Africans can be influenced by polymorphisms outside the PAI-1 gene. Huang *et al*. (21), for example recently found SNPs in the ARNTL and the PPARG genes to have genome-wide significant association with circulating PAI-1 levels.

Gene-environment interactions

4G/5G polymorphism

The relationship between PAI-1 levels, the 4G/5G genotype and BMI, although extensively studied, remains controversial. Genotype-specific differences in PAI-1_{act} levels were present in all BMI ranges in our study population, except for BMI <18.8kg/m^2, whereas in Caucasian populations, they have sometimes been observed in obese individuals only (36-38), while in other studies (35) they have appeared in lean individuals only. In agreement with results from Sartori *et al*. (38), these differences were also present in individuals with central obesity but not in those with a normal waist circumference. We additionally found significant interactions of the 4G/5G genotypes with waist circumference and BMI in determining PAI-1_{act} levels in this apparently healthy population, with PAI-1_{act} increasing more rapidly with increasing BMI and WC in the 4G allele group. These results are in agreement with McCormack *et al*. (39), who investigated these associations in Pima Indians. Ossei-Gerning *et al*. (23), however, found the association between BMI and PAI-1 to be stronger in the 5G/5G genotype in a group of 453 Caucasian patients with a history of myocardial infarction. *In vitro* studies aiming at elucidating possible mechanisms for this interaction found no effect of genotype on promoter activity in HepG2 or BAEC cells (35), nor did it influence the

adipose secretion rate of PAI-1 (40). With the 4G/5G polymorphism being a response polymorphism, ethnic differences as well as differences in study population characteristics (healthy vs diseased), probably contribute to these discrepancies in the literature.

The relationship between PAI-1_{act} and triglyceride levels was also found to be influenced by the 4G/5G genotype in our study population, with PAI-1_{act} increasing more rapidly with increasing triglycerides in the 4G allele group. Similar associations were found in studies investigating CVD patients (23, 41, 42), although in studies investigating healthy Caucasian individuals, genotype was not found to influence this relationship (22, 43). A possible explanation for the observed 4G/5G–triglyceride interaction is that the activity of a VLDL-response element in the PAI-1 promoter area was found to be influenced by the 4G/5G polymorphism located adjacent to and upstream of the binding site of the VLDL-inducible transcription factor (44). This gene-environment interaction between the 4G/5G polymorphism and triglycerides may partly explain the interaction found with BMI and abdominal obesity, as individuals with a BMI >25kg/m^2 and/or with central obesity were found to have increased triglyceride levels (data not shown).

As yet, it is still unclear whether PAI-1 plays a role in the evolution of obesity and whether the 4G/5G polymorphism could possibly contribute to this. While some case-control studies found differences in genotype distribution between obese and non-obese participants (45 46), others did not (38). Our data, providing the first population-based evidence, indicated that there were no differences in genotype distribution between individuals of normal weight and those who were

overweight/obese. Additionally, BMI and WC did not differ across the genotypes. This is in agreement with Gardemann *et al*. (47) but in contradiction to Hoffsted *et al*. (45), who found the prevalence of obesity to be twice as high in the 4G allele as in the 5G allele group. In an *in vitro* experiment, Demiralp *et al.* (48) suggest that overexpression of PAI-1 in 3T3-L1 cells can increase adipocyte differentiation and that the 4G allele was significantly more active than the 5G allele in driving PAI-1 gene transcription, in this way contributing to adipogenesis. More evidence from population-based studies, comparing different ethnicities is required, however, to determine the role of PAI-1 and its gene regulation in obesity. Genome wide association studies (GWAS) have shown that various genes influence obesity (49, 50), but, to our knowledge no GWAS have been published investigating the influence of the polymorphisms we investigated and their associations with obesity.

We found significant interactions of the 4G/5G genotypes with fibrinogen and fibrinogen gamma prime in determining CLT. Fibrinogen gamma prime was included in this investigation as it has been shown to affect clot structure, which may influence lysis (51). In participants with the 4G allele, there was a greater increase in clot lysis time with an increase in fibrinogen and fibrinogen gamma prime than in 5G/5G homozygotes. Olman *et al.* (52) identified an AP-1-like DNA element as important in transcriptional control in the PAI-1 gene. According to Olman *et al.* (52), a negative regulatory feedback loop is initiated, wherein D-dimers, formed after fibrin degradation, generate a signal that results in PAI-1 transcription through the AP-1 expression element, inhibiting fibrinolysis. The influence of increasing fibrinogen and fibrinogen gamma prime concentrations on clot lysis in the present study may,

therefore, amongst other reasons, be through the increased generation of D-dimer. If the AP-1 expression element enhances PAI-1 mRNA in the 4G but not the 5G allele, as has been demonstrated for interleukin-1 (18) and VLDL, it could at least in part explain the observed gene-environment interactions. The enhanced transcription of PAI-1 could consequently result in prolonged CLT. Additional research is required, however, to test this hypothesis.

C428T and G429A polymorphisms

Despite there being no direct relationship between these polymorphisms and PAI-1_{act} and CLT, we did find evidence that the association of other environmental factors with PAI-1_{act} and CLT was influenced by these genotypes. Additionally, many, but not all, of the gene-environment interactions differed consistently between rural and urban participants in subjects harbouring the mutant alleles, but not in subjects harbouring the most common genotype. These data suggest that significant differences in environmental factors in urban and rural living conditions could potentially influence phenotypic expression of the investigated SNPs in subjects harbouring the mutant variants and that these individuals may be more sensitive towards environmental factors than individuals harbouring the respective common genotypes.

In conclusion, this black African population had a high prevalence of the 5G allele (0.85). The 4G/5G polymorphism significantly affected PAI-1_{act} but not CLT. The polymorphism contributed, however, to less than 1% in the PAI-1_{act} variance. Obesity had a much more pronounced effect on PAI-1_{act} than the measured polymorphisms. The C428T and G429A SNPs had no direct association with either

PAI-1_{act} or CLT. There were significant interactions between the 4G/5G polymorphism and BMI, central obesity and triglycerides in determining PAI-1_{act} and between the polymorphism and fibrinogen and fibrinogen gamma prime in determining CLT. Both the C428T and G429A polymorphisms also showed significant gene-environment interactions in determining PAI-1_{act} and CLT. Urbanisation significantly affected the phenotypic expression of the 4G/5G polymorphism, with PAI-1_{act} showing larger differences across the 4G/5G genotypes in the urban community than in the rural community. Urbanisation additionally influenced gene-environment interactions, with differences between rural and urban participants observed particularly in participants harbouring the mutant alleles of the C428T and G429 polymorphisms. From these results it seems clear that environmental factors and combinations thereof, such as specific combinations present in a rural or urban environment, significantly influence phenotypic expression of genes. This should be taken into consideration when developing treatment modalities addressing CVD risk factors such as PAI-1 and global fibrinolytic potential.

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Legends to tables

Table 1. Log-transformed data (PAI-1) reported as geometric means (95% CI) after adjustment for BMI (4G/5G polymorphism), normally distributed data (CLT) reported as mean (95% CI) after adjustment for BMI (4G/5G polymorphism). *† Values with the same symbol differed significantly. # p-values after adjustment for BMI; •p-value reported for CC vs CT and TT groups combined for C428T, and GG vs GA and AA groups combined for G429A because of small sample size in homozygous mutant groups.

Table 2. PAI-1_{act}: Plasminogen activator inhibitor type-1 activity; CLT: clot lysis time; BMI: Body mass index; WC: Waist circumference; Normal WC: <80cm for women and <94cm for men; Central obesity, ≥80cm for women and ≥94cm for men.

Table 3. PAI-1_{act}: plasminogen activator inhibitor-1 activity; BMI: body mass index; CLT: clot lysis time. • Slope: regression line obtained by plotting PAI-1_{act} or CLT (y-axis) against environmental factors (x-axis). * p-values indicate significance of the interaction term of the 4G/5G genotype and environmental factor in an ANCOVA.

Table 1. PAI-1_{act} levels and clot lysis time of PAI-1 promoter area polymorphisms

	Allele Freq.	N (%)	PAI-1_{act} (U/ml)			CLT (min)		
			Total group	Urban	Rural	Total group	Urban	Rural
4G/5G:								
5G/5G	5G: 0.85	1304 (72.5)	3.27 (3.06-3.48)* (n=1241)	3.84 (3.48–4.22)*† (n=543)	2.87 (2.62-3.13)* (n=698)	57.0 (56.5-57.6) (n=1223)	57.2 (56.3-58.2) (n=557)	56.9 (56.1-57.6)* (n=666)
4G/5G		448 (24.9)	4.54 (1.72-8.77)* (n=415)	4.85 (4.18-5.62)* (n=213)	3.16 (2.69-3.69)† (n=202)	57.9 (56.9-58.9) (n=426)	58.5 (57.0-59.9) (n=229)	57.3 (56.0-58.7)† (n=197)
4G/4G	4G: 0.15	47 (2.61)	6.39 (2.96-9.70)* (n=45)	5.96 (3.82-9.04)† (n=24)	4.96 (3.10-7.66)*† (n=21)	59.3 (56.2-62.4) (n=43)	57.7 (53.2-62.2) (n=24)	61.3 (57.0-65.6)*† (n=19)
ANOVA p-value			p<0.001 (p=0.0003)#	p=0.02 (p=0.009)#	p=0.03 (p=0.06)#	p=0.12 (p=0.15)#	p=0.40 (p=0.39)#	p=0.04 (p=0.12)#
C428T:								
CC	C: 0.96	1743 (92.3)	3.50 (3.31-3.70) (n=1646)	4.12 (3.81-4.46) (n=771)	3.02 (2.79-3.26) (n=875)	57.3 (56.7-57.8) (n=1640)	57.5 (56.7-58.3) (n=801)	57.1 (56.3-57.8) (n=839)
CT		143 (7.57)	3.18 (2.59-3.87) (n=134)	4.34 (3.17-5.83) (n=52)	2.55 (1.94-3.30) (n=82)	57.3 (55.5-59.1) (n=134)	57.9 (54.6-61.3) (n=55)	56.9 (54.8-59.0) (n=79)
TT	T: 0.04	2 (0.11)	12.5 (2.88-45.7) (n=2)	12.9 (2.96-47.8) (n=2)	- (n=0)	61.4 (-71.8-195) (n=2)	61.4 (-71.8-195) (n=2)	- (n=0)
ANOVA p-value			p=0.14•	p=0.75•	p=0.15•	p=0.92•	p=0.74•	p=0.90•

G429A:								
GG	G: 0.90	1528 (80.9)	3.57 (3.36-3.79) (n=1437)	4.39 (4.03-4.77) (n=676)	2.95 (2.71-3.21) (n=761)	57.3 (56.7-57.9) (n=1443)	57.6 (56.7-58.4) (n=713)	57.0 (56.2-57.8) (n=730)
GA		333 (17.6)	3.10 (2.72-3.53) (n=320)	3.08 (2.51-3.74) (n=138)	3.12 (2.62-3.68) (n=182)	57.5 (56.2-58.7) (n=306)	57.3 (55.3-59.4) (n=132)	57.6 (56.1-59.1) (n=174)
AA	A: 0.10	27 (1.43)	3.38 (2.08-5.22) (n=25)	4.92 (2.48-9.09) (n=11)	2.41 (1.15-4.42) (n=14)	54.6 (50.9-58.4) (n=27)	57.7 (51.8-63.6) (n=13)	51.8 (46.7-56.8) (n=14)
ANOVA p-value			p=0.18•	p=0.02•	p=0.45•	p=0.97•	p=0.86•	p=0.85•

Log-transformed data (PAI-1) reported as geometric means (95% CI) after adjustment for BMI (4G/5G polymorphism), normally distributed data (CLT) reported as mean (95% CI) after adjustment for BMI (4G/5G polymorphism). *† Values with the same symbol differed significantly. # p-values after adjustment for BMI; •p-value reported for CC vs CT and TT groups combined for C428T, and GG vs GA and AA groups combined for G429A because of small sample size in homozygous mutant groups.

Table 2. PAI-1_{act} and CLT across BMI and waist circumference categories for 5G/5G participants and those harbouring the 4G genotype

		PAI-1_{act} (U/ml)			CLT (minutes)		
	N	5G/5G Median (25-75th percentile)	4G-allele Median (25-75th percentile)	p-value	5G/5G Mean (95% CI)	4G-allele Mean (95% CI)	p-value
BMI (kg/m^2)							
<18.5	319	1.91(0.00-4.75)	1.72 (0.00-4.51)	0.44	50.7 (49.2-52.2)	50.9 (48.6-53.2)	0.86
18.5-24.9	731	2.98 (0.62-6.11)	4.41 (1.72-7.03)	0.004	54.9 (54.0-55.8)	56.1 (54.5-57.7)	0.18
25-29.9	298	5.41 (2.80-8.71)	6.20 (3.64-11.0)	0.05	60.7 (59.4-61.9)	61.6 (58.8-64.4)	0.49
≥30	353	7.01 (3.38-10.3)	9.08 (3.58-16.8)	0.009	64.2 (63.1-65.3)	65.1 (63.0-67.3)	0.43
Normal WC	1422	3.46 (0.70-6.63)	3.82 (1.14-7.03)	0.27	55.3 (54.6-56.0)	55.3 (54.2-56.5)	0.93
Centrally obese	356	7.17 (3.64-10.5)	10.2 (4.89-18.5)	<0.0001	64.4 (63.3-65.5)	66.7 (64.3-69.2)	0.05

PAI-1_{act}: Plasminogen activator inhibitor type-1 activity; CLT: clot lysis time; BMI: Body mass index; WC: Waist circumference; Normal WC: <80cm for women and <94cm for men; Central obesity, ≥80cm for women and ≥94cm for men.

Table 3. 4G/5G genotype gene-environment interactions for PAI-1_{act} and CLT

Interaction	Interaction p-value*	N	5G/5G Slope (95% CI)	N	4G allele Slope (95% CI)
PAI-1_{act}:					
Waist circumference (cm)	0.001	1289	1.89 (1.57; 2.21)	489	2.92 (2.42; 3.43)
BMI (kg/m^2)	0.006	1241	1.15 (0.96; 1.34)	460	1.66 (1.35; 1.97)
Triglycerides (mmol/l)	0.04	1259	0.56 (0.46; 0.66)	481	0.78 (0.60; 0.95)
CLT:					
Fibrinogen (g/L)	0.005	1149	2.96 (1.55; 3.82)	425	5.94 (3.83; 8.06)
Fibrinogen gamma prime (g/L)	0.04	1173	6.78 (5.69; 7.88)	444	9.04 (7.06; 11.0)

PAI-1_{act}: plasminogen activator inhibitor-1 activity; BMI: body mass index; CLT: clot lysis time. • Slope: regression line obtained by plotting PAI-1_{act} or CLT (y-axis) against environmental factors (x-axis). * p-values indicate significance of the interaction term of the 4G/5G genotype and environmental factor in an ANCOVA.

Supplement:

The effect of polymorphisms of the PAI-1 gene on PAI-1 activity levels and plasma clot lysis time in black South Africans: the influence of urbanisation

PAI-1 promoter area genotyping

Supplemental table 1. Sequences used for PAI-1 promoter area

Primer name	Sequence 5' – 3'	Design
PAI_1_F	TTCCACCCACTGAAACTTCC	Own design
PAI_1_R	GATGGGAGACCGTGACAGAT	Own design
PAI_2_F	GGTTGCAAGCTCCCTATGAG	Own design
PAI_2_R	CAGCCACGTGATTGTCTAGG	Own design
PAI_3_F	GGGAGTCAGCCGTGTATCAT	Own design
PAI_3_R	AGTTCTCAGAGGTGCCTTGC	Own design

Supplemental table 2. PAI-1 promoter area polymorphisms

Number	Polymorphism	Frequency of polymorphism in n=25 participants	Measured in PURE population
1	C/T	n=2	Excluded
2	T/C	n=2	C428T included because of position next to G429A
3	A/G	n=8	G429A included
4	G/A	n=4	Excluded
5	T/C	n=1	Excluded
6	Insertion A	n=3	Excluded
7	C/T	Sequencing unclear	Excluded
8	A/T	Sequencing unclear	Excluded
9	C/T	n=0	Excluded
10	A/G	n=7	In linkage with 11, therefore excluded
11	4G/5G	n=6	4G/5G included
12	A/G	n=4	In linkage with 11, therefore excluded
13	G/A	n=3	Excluded
14	A/T	n=1	Excluded
15	CA Insertion/deletion	n=5	Still under investigation
16	Insertion T	n=1	Excluded
17	T/C	n=0	Excluded

Supplemental table 3.

Primers and probes used for genotyping of PAI-1 polymorphisms

Polymorphism	Name	Sequence 5' – 3'	Design
4G/5G	Primer 4G5GF:	TCTTTCCCTCATCCCTGCC	Tjarland et
	Primer 4G5GR:	CCAACCTCGCCAGACAAGG	al., 2003
	Probe: PAI 4C:	5HEX/ACACGGCTGACTCCCCACGT/3BHQ_1	
	Probe: PAI 5C:	56-FAM/ACGGCTGACTCCCCCACGT/3BHQ_1	
C428T	Primer PAI_F:	TCCCACCCACTGAAACTTCC	Own design
G429A	Primer PAI_R:	GGTGAGCATGTAGGGCTAGACT	
	Probe PAI_TG:	5CY5/ATCCAGACCACA**TG**GCCAAG/3lAbRQSp	
	Probe PAI_TA:	5TexRd-XN/ACCACA**TA**GCCAAGGGCACC/3lAbRQSp	
	Probe PAI_CA:	5HEX/TCCAGACCACA**CA**GCCAAGG/3BHQ_1	
	Probe PAI_CG:	56-FAM/CAGACCACA**CG**GGCCAAGG/3BHQ_1	

South African PURE population

Supplemental table 4. Characteristics of total study population, urban and rural participants

Variable	Total population N=2010	Urban N=1004	Rural N=1006	Rural vs Urban p-value
Age (yr)	48 (41-56)	48 (42-57)	47 (41-55)	0.0002
Gender M/F (%)	37.3 / 62.7	39.9 / 60.1	34.6 / 65.4	0.01
HIV + (%)	16.2	15.7	16.8	0.5
Smoking status (%)				
Never	43.8	42.7	44.9	0.32
Past	3.80	3.90	3.8	0.65
Current	51.8	52.6	51.1	0.5
Blood pressure (mmHg)				
Systolic	133.5 ± 24.5	137 ± 25.1	129.7 ± 23.3	<0.0001
Diastolic	87.7 ± 14.5	89.3 ± 14.5	86.2 ± 14.5	<0.0001
Body mass index (kg/m^2)	22.9 (19.3-28.6)	23.4 (19.5-29.4)	22.4 (19.1-28.1)	0.003
Waist circumference (cm)	77.5 (70.2-87.7)	78.5 (70.9-89.0)	76.0 (69.7-86.9)	0.002
Men	74.4 (69.9-81.3) ‡	74.3 (69.7-81.8) ‡	74.5 (70.2-80.5) ‡	0.61
Women	81.0 (70.6-91.3) ‡	82.8 (73.1-92.8) ‡	78.8 (69.5-89.5) ‡	<0.0001
Serum total cholesterol (mM)	5.01 ± 1.38	5.05 ± 1.4	4.96 ± 1.36	0.17

Serum LDL-cholesterol (mM)	2.92 ± 1.17	2.93 ± 1.18	2.92 ± 1.17	0.86
Serum HDL-cholesterol (mM)	1.52 ± 0.63	1.52 ± 0.65	1.52 ± 0.62	0.91
Men	1.58 ± 0.66 ‡	1.61 ± 0.66 ‡	1.55 ± 0.66	0.22
Women	1.48 ± 0.62 ‡	1.46 ± 0.63 ‡	1.50 ± 0.61	0.26
Serum triglycerides (mM)	1.07 (0.82-1.55)	1.11 (0.84-1.65)	1.05 (0.80-1.43)	<0.0001
Fasting plasma glucose (mM)	5.02 ± 2.73	5.17 ± 3.7	4.87 ± 1.23	0.02
Serum CRP (mg/L)	3.29 (0.96-9.34)	3.25 (1.12-9.85)	3.33 (0.85-9.02)	0.07
Plasma fibrinogen (g/L)	2.90 (2.30-5.00)	2.70 (2.20-4.30)	3.00 (2.40-5.40)	0.0001 *†◦
Men	2.60 (2.10-3.70) ‡	2.50 (2.00-3.30) ‡	2.80 (2.20-4.30) ‡	0.048 *◦
Women	3.10 (2.30-5.50) ‡	2.90 (2.30-5.40) ‡	3.20 (2.50-5.70) ‡	0.003 *†◦
Plasma PAI-1 activity (U/mL)	4.26 (1.27-7.92)	5.01 (1.76-9.11)	3.58 (0.81-6.85)	<0.0001
Plasma homocysteine (μM)	9.18 (7.45-12.1)	8.90 (7.23-11.4)	9.48 (7.67-12.6)	<0.0001
Men	10.2 (8.30-13.16) ‡	9.58 (8.01-12.06) ‡	11.3 (8.67-14.4) ‡	<0.0001
Women	8.76 (7.09-11.15) ‡	8.39 (6.90-10.7) ‡	8.76 (7.09-11.15) ‡	0.004

Normally distributed data reported as: mean ± std and non-parametric data as median (25^{th}-75^{th} percentile); * Significant difference between rural and urban groups after adjustment for CRP; † Significant difference between rural and urban groups after adjustment for homocysteine; ◦ Significant difference between rural and urban groups after adjustment for HIV status; ‡ Significant difference between men and women; M: male; F: female; HIV + human immunodeficiency virus-infected; LDL: low density lipoprotein; HDL: High density lipoprotein; CRP: c-reactive protein

(Pieters *et al.,* 2011)

Gene-environment interactions of the C428T and G429A polymorphisms

The genotypes of the C428T polymorphism had significant interactions with triglyceride concentration (p=0.04), HDL-cholesterol (p=0.03), total homocysteine (p=0.01) and fibrinogen gamma prime concentration (p=0.05) in determining PAI-1_{act} levels. Differences between these gene-environment interactions were found, however, between the rural and urban subgroups. The significant interactions are presented in Table 3 of this supplement. PAI-1_{act} increased significantly with an increase in triglyceride concentration. In the homozygous wild-type participants, this association did not differ between rural and urban subgroups, but in participants harbouring the T-allele, the association between PAI-1_{act} and triglyceride concentration differed significantly between the rural and urban subgroups. This same pattern can be seen in participants harbouring the T-allele for the association between PAI-1_{act} and HDL-C and tHcy, where the association between PAI-1_{act} and these factors did not differ between the rural and urban subgroups for the homozygous wild type-genotype but differed significantly between rural and urban subgroups in participants harbouring the T-allele (Table 3). The genotypes of the C428T polymorphism showed a significant interaction with BMI (p=0.01) in determining CLT. The association between CLT and BMI was less pronounced in the participants harbouring the T-allele than in the homozygous wild-type genotype (Table 3).

The G429A polymorphism had significant interactions with LDL-cholesterol (p=0.02) and fibrinogen (p=0.002) in determining PAI-1_{act} levels. These interactions differed significantly between the rural and urban subgroups. As was the case for the C428T polymorphism, there was no difference in the association of PAI-1_{act} with LDL-

cholesterol and fibrinogen between the rural and urban subgroups in the homozygous wild-type participants. In the participants harbouring the A-allele, however, the association between PAI-1_{act} and LDL-cholesterol and fibrinogen differed significantly between the rural and urban subgroups. The genotypes of the G429A polymorphism showed a significant interaction with systolic blood pressure in determining PAI-1_{act} ($p=0.03$); this interaction was not influenced by urbanisation (Table 3).

The G429A polymorphism had significant interactions with fibrinogen ($p=0.06$) and systolic blood pressure ($p=0.04$) in determining CLT. In the homozygous wild-type group, CLT increased with an increase in fibrinogen concentration, while no association was found in the group harbouring the A-allele. On the other hand, no association was found between CLT and systolic blood pressure in the homozygous wild-type group, while CLT decreased with an increase in systolic blood pressure in the group harbouring the A-allele (Table 3).

Supplemental table 5. Significant gene-environment interactions for the C428T and G429A genotypes in determining PAI-1_{act} and CLT - the effect of urbanisation

Interaction	Interaction p-value*	Wild type Slope (95% CI)• (N)		Mutant allele Slope (95% CI)• (N)	
C428T					
PAI-1_{act}:					
Triglycerides (mmol/l)	0.04	R: 0.62 (0.49; 0.76) (851)	U: 0.55 (0.43; 0.67) (845)	R: 0.98 (0.55; 1.41)▪ (78)	U: 0.14 (-0.34; 0.66)▪ (61)
HDL-chol (mmol/l)	0.03	R: -0.20 (-0.30; -0.04) (861)	U: -0.30 (-0.40; -0.20) (847)	R:0.08 (-0.27; 0.44)▪ (78)	U:-0.59 (-0.87; -0.31)▪ (61)
tHcy (µmol/l)	0.01	R:-0.17 (-0.33; -0.01) (863)	U:-0.13 (-0.31; 0.06) (826)	R: 0.30 (-0.24; 0.85)▪ (82)	U:-0.72 (-1.23; -0.20)▪ (59)
Fibrinogen gamma prime (g/L)	0.05	R: 0.40 (0.30; 0.51)▪ (838)	U:0.15 (0.02; 0.29)▪ (775)	R:0.07 (-0.27; 0.40) (77)	U: 0.58 (-0.04; 1.20) (55)
CLT:					
BMI (kg/m^2)	0.01	19.5 (17.6; 21.4) (1552)		10.9 (4.45; 17.4) (128)	

G429A					
PAI-1_{act}:					
LDL-chol (mmol/l)	0.02	R: 0.38 (0.23; 0.52) (742)	U: 0.23 (0.08; 0.37) (735)	R: -0.15 (-0.47; 0.17)▪ (185)	U: 0.34 (-0.01; 0.70)▪ (160)
Fibrinogen (g/L)	0.002	R: 0.003 (-0.12; 0.13) (731)	U:0.04 (-0.08; 0.17) (693)	R: 0.28 (0.05; 0.52)▪ (189)	U: -0.33 (-0.60; -0.05)▪ (143)
SBP (mm/Hg)	0.03	0.004 (0.002; 0.006) (1514)		-0.001 (-0.0005; 0.003) (355)	
CLT:					
Fibrinogen (g/L)	0.06	3.86 (2.77; 4.95) (1349)		1.44 (-0.74; 3.63) (306)	
SBP (mm/Hg)	0.04	-0.001 (-0.02; 0.02) (1433)		-0.06 (-0.11; -0.01) (328)	

PAI-1_{act}: plasminogen activator inhibitor -1 activity; HDL-chol: high density lipoprotein cholesterol; tHcy: total homocysteine; CLT: clot lysis time; BMI: body mass index; LDL-chol: low density lipoprotein cholesterol; SBP: systolic blood pressure; R: rural; U: urban. • Slope: regression line obtained by plotting PAI-1_{act} or CLT (y-axis) against environmental factors (x-axis). * p-values indicate significance of interaction term between SNP and environmental factor in an ANCOVA. Where data are reported for rural and urban groups separately, urbanisation influenced the gene-environment interaction. ▪ Rural and urban slopes differed significantly.

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CHAPTER 5:

Conclusion and recommendations

5.1. INTRODUCTION

No information regarding global fibrinolytic potential and its relation to CVD risk factors, or the relationship of PAI-1 promoter area polymorphisms with PAI-1_{act} and CLT is available for black Africans. In this study we sought to investigate these associations in a large black South African population.

This final chapter provides a summary of the main findings in the two articles that form part of this thesis and draws conclusions from the findings of these articles. In Chapters 3 and 4 the results were discussed and compared with the relevant literature and in this chapter the relevance of this thesis regarding the greater scientific knowledge base will be highlighted. To enable easier reference, the main aim and objectives of this thesis are repeated below, followed by a discussion of each objective and, at the end of the chapter, a section providing recommendations for future research.

Aim and objectives

The main aim of this project was to investigate global fibrinolytic potential and its relation to CVD risk factors in apparently healthy black South Africans.

Objectives of this project were:

- To analyse global fibrinolytic potential using a turbidimetric plasma clot lysis assay (Lisman *et al.,* 2005) in the South African PURE study population and to determine the association between global fibrinolytic potential and CVD risk factors.
- To determine the effect of urbanisation on global fibrinolytic potential.
- To identify genetic polymorphisms in the promoter area of the PAI-1 gene in this study population and to measure the prevalence of selected polymorphisms.
- To determine both the independent effect of these polymorphisms and gene–environment interactions on PAI-1_{act} and global fibrinolytic potential in this study population and whether urbanisation influenced this.

5.2. THE ASSOCIATION BETWEEN GLOBAL FIBRINOLYTIC POTENTIAL AND CVD RISK FACTORS IN A BLACK SOUTH AFRICAN POPULATION

Global fibrinolytic potential, expressed as plasma clot lysis time (CLT), is a measure of the body's ability to lyse clots. The association of global fibrinolytic potential with CVD risk factors has been investigated in Caucasian populations and decreased fibrinolytic potential (prolonged CLT) was shown to be significantly associated with cardiovascular disease (CVD) risk factors and arterial and venous CVD (Lisman *et al.,* 2005*;* Meltzer *et al*., 2008; Guimarães *et al.,* 2009; Meltzer *et al.,* 2009; Siegerink *et al*., 2011; Leander *et al.,* 2012).

The prevalence of CVD and CVD risk factors in black South Africans has increased significantly in recent years. Only limited information is available, however, regarding haemostatic risk factors of black South Africans. It is postulated that their respective roles in CVD development may be different from those of Caucasian populations, as the levels of some of these risk factors, such as fibrinogen and PAI-1, have repeatedly been shown to be increased or decreased compared to non-black counterparts (Jerling *et al.,* 1994; Vorster *et al.,* 2002; Festa *et al.,* 2003; Pieters *et al.,* 2006; Greyling *et al.,* 2007; Nienaber *et al.,* 2008). Additionally, global fibrinolytic potential, as a proxy marker for the ability of the body to lyse existing clots, and its relationship with CVD risk factors has until now not been investigated in the black African population. The relationship between CVD risk factors and CLT in the black African population is not known. We determined the global fibrinolytic potential (expressed in minutes as CLT) of 1 802 black Africans, using the method of Lisman *et al.* (2005), which is considered to be a reliable reflection of global plasma fibrinolytic potential, since it includes all components of the coagulation and fibrinolytic pathways in plasma. Clot lysis time was then related to various traditional and novel CVD risk factors. Various environmental and haemostatic factors seem to influence CLT. In our study population there were no differences in CLT between age categories, women who used contraceptives compared with those who did not, or between hypertensive and normotensive participants. CVD risk factors in the PURE population that associated significantly with CLT were gender, smoking, alcohol, increased BMI, HbA1c, fasting glucose, triglyceride concentration, total cholesterol concentration, $PAI\text{-}1_{act}$, fibrinogen, CRP concentration, the metabolic

syndrome and HIV. Women had longer CLTs than men, while increased BMI, HbA1c, fasting glucose, triglycerides, total cholesterol, PAI-1_{act}, fibrinogen, CRP concentration, and the metabolic syndrome were all associated with prolonged CLTs. In this African population the habitual consumption of alcohol was associated with shorter CLTs than in non-drinkers, while an HIV-positive person would also have longer CLTs than someone who was HIV-negative.

In a Caucasian population, the main contributing factor to CLT was PAI-1 antigen (PAI-1_{ag}) (explaining 24% of the variance) (Meltzer *et al.,* 2010). We found that PAI-1 activity (PAI-1_{act}) explained 27% of the variance in CLT, with BMI, alcohol consumption and HbA1c explaining 8%, 3% and 2% respectively. Triglyceride concentration, CRP, HDL-cholesterol and HIV status each explained 1%, while blood pressure, total cholesterol, fibrinogen, smoking and age each explained less than 0.5% of the variance, all adding up to a total of 45% of the variance in CLT being explained by these haemostatic and CVD risk factors. Although CLT associates with many CVD risk factors, it is not clear whether it contributes to CVD only through its association with CVD risk factors or whether it plays a causal role in CVD development. Owing to the prospective design of the overall PURE study, however, follow-up and CVD event data and mortality which will be available in the future may enable us to answer this question of whether or not CLT can be regarded as an independent CVD risk factor.

The present study is the first to report on associations between CLT and both traditional and less well established CVD risk factors in a large, black African population. Most of the associations we found are similar to associations found in Caucasian populations; however, the results of this study do highlight possible ethnic differences in the association between CLT and CVD risk and the importance of determining healthy cut-offs and ranges for CVD risk factors for specific populations and ethnic groups.

5.3. THE EFFECT OF URBANISATION ON GLOBAL FIBRINOLYTIC POTENTIAL IN BLACK SOUTH AFRICANS

The prevalence of CVD risk factors as well as CVD events has previously been low in the black South African population but is increasing as this population is becoming more urbanised (Vorster, 2002; Amira *et al*., 2006; Stewart *et al*., 2011). Many CVD risk factors, namely BMI, waist circumference, triglyceride concentration, fasting glucose and PAI-1_{act} levels, have been shown to increase in this population with urbanisation (Pieters *et al.,* 2011b). Additionally, most of the CVD risk factors investigated in this population were associated with increased clot lysis time, reflecting decreased fibrinolytic potential. Owing to the important role fibrinolysis is thought to play in the development of CVD and its positive association with many traditional CVD risk factors, we anticipated that global fibrinolytic potential might be decreased in urban compared with rural participants and thus further increase the CVD risk in urban dwellers. In the South African PURE population CLT did not, however, differ significantly between rural and urban participants, despite the higher prevalence of CVD risk factors present in the urban population. This can probably be explained by the association of global fibrinolytic potential with individual CVD risk factors (as discussed in the previous section). Some CVD risk factors that were increased in the urban group were found to have a positive association with CLT, while others, such as heavy alcohol consumption, were associated with shorter CLTs. Additionally, while most CVD risk factors were increased in the urban group, some, such as fibrinogen and CRP, were higher in the rural than in the urban group and were also associated with longer CLTs. While no rural-urban differences were observed for CLT in the total population, the CLT of urban women was found to be longer than that of rural women. This can, in all likelihood, be explained by the larger rural-urban differences observed for CVD risk factors between rural and urban women compared with differences observed for men. For example, the rural-urban difference in BMI for women was 7 times that of the difference in men. It seems, therefore, that the specific combinations of CVD risk factors and their individual relationships with CLT determine the final CLT in the rural and urban subgroups and that urbanisation *per se* does not have a strong independent effect on CLT.

Because CVD is a relatively recent disease in black Africans, its pathophysiology in blacks has not been studied as extensively as it has been in Caucasian populations. Although preliminary evidence suggests that many of the same factors that were identified in Caucasian populations were also present in CVD in blacks (Steyn *et al.,* 2005, Pieters *et al.,* 2011a), differences in pathophysiology can also be expected because of differences in genetic make-up as well as differences in environmental factors, of which urbanisation is a prime example. The majority of Caucasian participants from European or American descent follow a Westernised lifestyle compared with rural blacks in Africa, with traditional lifestyles and eating patterns. In support of this theory, differences occur, for instance, in the prevalence of individual risk factors in black Africans compared with Caucasians, such as the lipid profile and hypertension, with the African population known to be especially vulnerable to hypertension (Steyn *et al.,* 1996) in comparison with Caucasians. On the other hand, black South Africans in general seem to have a favourable lipid profile compared with other ethnic groups in South Africa (Vorster, 2002), with even CAD patients presenting with normal lipid levels (Nethononda *et al.,* 2004; Stewart *et al.,* 2011).

It is therefore of the utmost importance to determine the individual contribution of possible CVD risk factors, such as global fibrinolytic potential, in the black African population, and not to rely on data collected in European/Caucasian populations only, in order to plan and implement successful prevention and treatment strategies tailor-made for the black African population.

5.4. GENETIC POLYMORPHISMS IN THE PROMOTER AREA OF THE PAI-1 GENE IN THIS STUDY POPULATION

PAI-1 plays an important role in fibrinolysis and has been shown to explain a large portion of the variance in CLT (Meltzer *et al.,* 2010; de Lange *et al.,* 2012). This inhibitor of fibrinolysis also plays a role in plaque formation and thus seems to be involved in the aetiology of CVD (Loskutoff *et al.,* 1993; Sobel *et al.,* 2003). PAI-1 levels have been shown to be low in African populations (Jerling *et al.,* 1994; Festa

et al., 2003; Lutsey *et al.,* 2006; Greyling *et al.,* 2007). The reasons for these low levels are, however, not clear. PAI-1 levels can be influenced genetically and even though some literature exists regarding the genetic factors influencing PAI-1 in Caucasian populations, not much is known about the genetic composition of Africans and its contribution to variance in PAI-1 levels in these populations. We therefore sequenced the promoter area of the PAI-1 gene in a subgroup of 30 randomly chosen PURE South Africa participants as a representative group of the study population. The sequence was manually searched for deviations from the reference sequence and ten polymorphisms were identified. Of these, three polymorphisms were chosen to be analysed in the total study population, based on their high frequencies.

One of the polymorphisms identified was the 4G/5G polymorphism (rs1799889), which is the PAI-1 polymorphism most extensively investigated in the literature and considered to influence PAI-1 levels. The influence of this polymorphism on $PAI\text{-}1_{act}$ and CLT in the PURE population will be discussed in section 5.5. From the literature, the most common genotype in Caucasian populations seems to be the 4G/5G genotype, at frequencies of 46% and 44%, followed by the 4G homozygous genotype, present in frequencies of 39% and 36%, with the 5G homozygous genotype being the least frequent (15% and 19%) in two different studies (Lanfear *et al.,* 2004; Naran *et al.,* 2008). In the study of Pegoraro *et al.* (2003), the 4G/5G genotype was also the most common genotype in a Caucasian population at 45%, while the 4G and 5G homozygous genotypes were in this case present at frequencies of 19% and 36%, with a lower 4G allele frequency than reported in the two previously mentioned studies. However, in African American populations, the frequency of the 5G homozygous genotype has been shown to be considerably higher than the other genotypes, with frequencies of 55% for the 5G/5G genotype and 37% and 8% for the 4G/5G and 4G homozygous genotypes respectively (Lanfear *et al.,* 2004). Results of three small-scale studies conducted among continental African individuals show an even higher prevalence (70%, 78% and 63%) of the 5G homozygous genotype than in African American populations (Pegoraro *et al.,* 2003; Naran *et al.,* 2008; Schoenhard *et al.,* 2008). We found a frequency of 72% for the 5G homozygous genotype in the South African PURE population and

frequencies of 25% and 2.6% respectively for the 4G/5G and 4G/4G genotypes. The frequency of the 5G allele in our population was 0.85, even higher than frequencies reported for African American populations (Festa *et al.,* 2003; Lanfear *et al.,* 2004). It is clear that the 5G homozygous genotype is the most common genotype in this black African population.

We also identified two additional single nucleotide polymorphisms (SNPs) in the promoter area of the gene. One SNP, C428T, has not been reported previously and is located at chromosome position 100768428. The CC genotype of this SNP was present at a frequency of 92%, while the CT and TT genotypes presented only at frequencies of 7.6% and 0.11%, respectively. The other SNP, located next to C428T in position 100768429, namely G429A, has been reported (rs36228614), but not described in the literature. For this SNP the GG genotype was the most common in this population at a frequency of 81%, followed by the GA and AA genotypes (17.6% and 1.43% respectively). The presence of these SNPs, one of which is novel and one identified previously only once, suggests the presence of ethnic-specific genetic variance and the need for population-based genetic studies.

Our data provide the first population-based evidence for a high prevalence of the 5G/5G genotype in black Africans. We have, in addition, identified two novel polymorphisms in the promoter area of the PAI-1 gene. The latest trend in genetic research, when identifying possible genetic polymorphisms involved in altering protein level and/or function, is to make use of the genome-wide association approach that assays hundreds of thousands of the most common SNPs across the entire human genome (Christensen & Murray, 2007) instead of identifying single nucleotide polymorphisms in a candidate gene (Pearson & Manolio, 2008). We could, not, however, use a GWAS approach in this study as information obtained from other populations cannot be extrapolated to black African populations owing to the larger genetic variability in African populations (Chen *et al*., 1995; Schuster *et al.,* 2010). GWAS data for black South Africans still needs to be determined.

5.5. THE EFFECT OF THE PAI-1 PROMOTER AREA POLYMORPHISMS ON PAI-1_{ACT} AND GLOBAL FIBRINOLYTIC POTENTIAL IN THIS BLACK SOUTH AFRICAN POPULATION

To date, there is not a large body of evidence describing the effect of genetic polymorphisms of the PAI-1 gene on PAI-1 levels, with most of the available literature focusing on the 4G/5G polymorphism. Owing to the prominent role PAI-1 plays in CLT it is worthwhile investigating also possible associations of PAI-1 genotypes with CLT. We therefore sought to investigate the contribution of these three polymorphisms in the promoter area of the PAI-1 gene to the variance in PAI-1_{act} levels and whether this will translate into altered CLT. We additionally investigated whether gene-environment interactions existed in determining PAI-1_{act} and CLT and whether urbanisation influenced the phenotypic expression of these genotypes.

In agreement with results from other studies, we found differences in PAI-1_{act} between the 4G/5G genotypes (Ye *et al.,* 1995; Ossei-Gerning *et al.,* 1997; Festa *et al*., 2003). Participants who were 4G homozygous had the highest PAI-1_{act} levels, followed by the 4G/5G heterozygous group and then the 5G homozygous group, with the lowest PAI-1_{act} levels. However, after performing a multiple regression analysis we found the contribution of the 4G/5G polymorphism to the variance in PAI-1_{act} to be less than 1%. This result concurs with the results of Festa *et al.* (2003), who also found that this polymorphism explained approximately 1% of the variance in PAI-1. These researchers concluded that it is unlikely that the 4G/5G polymorphism explains the differences in PAI-1 levels they found in different ethnic groups, owing to the low variability explained. The results additionally highlight the importance of potentially modifiable environmental factors like body weight and insulin resistance in determining PAI-1 levels (Festa *et al.,* 2003). In our study we found the largest contributor to PAI-1_{act} to be waist circumference, followed by gender and triglycerides. Waist circumference and triglyceride concentrations are known modifiable environmental factors that are strongly associated with PAI-1 levels (Panahloo *et al.,* 1995; Verschuur *et al.,* 2005). In the present study it is clear that obesity has a much larger effect on PAI-1_{act} than the 4G/5G polymorphism. Variations in PAI-1_{act} levels were also more pronounced in the urban than the rural

group, indicating that the urban environment enhanced 4G/5G polymorphism-related gene-expression. Because of the important role PAI-1 plays in fibrinolysis, we were also interested in determining whether the differences in PAI-1_{act} levels across the 4G/5G genotypes would translate into different CLTs in the genotypes. The different PAI-1_{act} levels across the 4G/5G genotypes did not, however, result in prolonged CLT. We did not find independent associations for the C428T or G429A SNPs with PAI-1_{act} or CLT, except within the G429A polymorphism. The participants with the A-allele presented with significantly lower PAI-1_{act} in the urban subgroup than the homozygous common genotype; however, this did not translate into shorter CLT for this specific group.

Apart from investigating possible independent relationships between the genetic polymorphisms and PAI-1_{act} and CLT, we also investigated possible gene–environment interactions, since environmental factors are known to influence phenotypic expression of genes. We found various significant gene–environment interactions between the investigated 4G/5G polymorphism and two SNPs and several CVD risk factors in determining PAI-1_{act} and CLT. The 4G/5G genotype had significant interactions with BMI, waist circumference and triglycerides in determining PAI-1_{act}. While our results provide the first population-based evidence for black Africans, this relationship has also been found consistently in Caucasian populations. The relationship between the 4G/5G genotype, PAI-1 levels and body composition is complex, however, and it is not yet clear whether the genetic regulation of PAI-1 levels is influenced by obesity or whether it contributes to the aetiology of obesity. Again, the prospective design of the PURE study may help to elucidate possible causal pathways in this interesting relationship when follow-up data become available. The 4G/5G genotype was also found to have significant interactions with fibrinogen and fibrinogen gamma prime concentration in determining CLT. A clear mechanism for this association still needs to be determined but it may be through D-dimer production and its effect on PAI-1 gene expression.

While the C428T and G429A polymorphisms did not have independent associations with PAI-1_{act} and CLT, we did find that the association of other environmental factors

with PAI-1_{act} and CLT was influenced by these genotypes. Additionally, many, but not all, of the gene–environment interactions differed consistently between rural and urban participants in subjects harbouring the mutant alleles, but not in subjects harbouring the most common genotype. These data suggest that significant differences in environmental factors in urban and rural living conditions could potentially influence phenotypic expression of the investigated SNPs in subjects harbouring the mutant variants and that these individuals may be more sensitive towards environmental factors than individuals harbouring the respective most common types

While gene–environment interactions investigate the association between a single SNP and a single environmental factor, the investigation into whether gene expression is influenced by urbanisation aims to determine whether a specific set/combination of environmental factors, such as those found in urban or rural areas, can influence phenotypic expression of genes – a systems biology approach. Although such an approach does not provide information on individual mechanisms, it does serve to answer questions on a public health level. In a developing country such as South Africa, where basic medical care is minimal and more than 50% of the population cannot afford expensive medical aid, directing government policies and guidelines for prevention and treatment strategies is of extreme importance, especially in the face of the rise in CVD incidence and mortality.

5.6. RECOMMENDATIONS FOR FUTURE RESEACH

Based on the results from the papers that form part of this thesis, the following recommendations for future research can be made:

1. The associations found in the cross-sectional analysis of the baseline data of the prospective PURE follow-up study should be further investigated. Five-year follow-up data were collected in 2010 and ten-year follow-up data will be collected in 2015. Total mortality of the PURE participants in 2010 was 323 and CVD mortality 66. Use of these data will aid in determining relationships of

many of the associations found in this thesis over the course of ten years. These results may be used specifically to aid in elucidating mechanisms and the time sequence of events. For instance, is CLT altered before the onset of disease?

2. It would be useful to develop a GWAS database for black Africans. However, determining the role of genetics in population-specific differences in black South African populations using rare genetic variance and epigenetic approaches may give us more information than the GWAS approach. An epigenetic approach, in which modifications in gene expression that are not due to changes in nucleotide sequence but mostly DNA methylation or histone modification, can also be followed. Determining influences of environmental factors on epigenetic systems may be important in understanding mechanisms and biological variations in populations (Cooney, 2006).

3. Research should be continued into the effect of urbanisation on the health status of black Africans and particularly into how it contributes to the double burden of disease. It is also important to determine how results of studies such as this can be used in directing government policies and prevention and treatment strategies. Strategies focusing on how to decrease the prevalence of CVD risk factors in rural and urban areas need to be implemented and further research is needed to identify the individual contribution of each CVD risk factor and the influence of each risk factor in rural and urban areas.

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ADDENDA

ADDENDUM A

PURE informed consent form

POTCHEFSTROOM CAMPUS

PURE-SA Project (Prospective Urban and Rural Epidemiology)

INFORMED CONSENT FORM (including the PRIMER-study)

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 industrialization and urbanization 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/her 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: 082 771 5778 / 018 299 4037(Office)

ADDENDUM B

Plasma fibrinolytic potential protocol

Plasma fibrinolytic potential protocol (as performed on the PURE 2005 samples at the NWU, Potchefstroom)
(modified set-up for Erasmus MC, Rotterdam - D.C. Rijken / J. Malfliet)

1. Start the microplate reader (Multiskan Ascent) and set the temperature at 37°C.
2. Make an Assay Mixture at room temperature. Prepare 1400 µl for a full microtiter plate (we did not use columns 1 and 12, only measured 80 samples per plate).

Stock solutions:

Tissue factor (Innovin): make aliquots of 120 µl of a 2-fold diluted stock in **assay buffer** and store frozen (-20°C, use only once).
$CaCl_2$: make a stock of 425 mM in **MQ-H_2O** and store at room temperature.
tPA (Actilyse): make a stock of 0.01 mg/ml in **buffer (in 50 mM HEPES, 0.1% (w/v) BSA pH 7.4)**, aliquot in 50 µl and store frozen.
phospholipids (phospholipids-TGT of Rossix): use the original stock solution of 0.5 mM, stored at 4 °C (use until done).
Assay buffer: 25 mM HEPES; 137 mM NaCl; 3.5 mM KCl; 1% BSA; pH 7.4

Stock solution	Volume for 1400 µl Assay Mixture (µl)	Concentration in Assay Mixture	Final concentration in clot
Tissue Factor	149.3	18.75 x diluted	125 x diluted (use 2 x diluted stock)
$CaCl_2$	373.4	113 mM	17 mM
tPA	93.4	167 ng/ml	100 ng/ml
Phospholipids	186.6	67 µM	10 µM
Assay buffer	597.3		

3. Dilution of plasma samples at room temperature: to each well of a separate microtiter plate (untreated plates) add 71.4 µl of citrated plasma sample followed by 50.0 µl of assay buffer using a multi-channel pipette and shake on a plateshaker 10 seconds at 1400 rpm. (Use 120 µl citrated plasma and 84 µl assay buffer for a measurement in duplicate).

4. Make Assay Mixture. Add 15 µl of the Assay Mixture per well of a microtiter plate (NUNC-Maxisorp)

5. Check if the platereader is at the right temperature. (**note!!!** The next steps have to be performed quickly because the reaction starts immediately when the plasma is pipetted into the assay mix)

6. Then, pipette with a multi-channel 85 µl of the diluted plasma samples over to the plate containing the Assay Mixture (because we also wanted to determine lagtime, clotting time and max

absorbance, a timer was set to determine the time from pipetting the plasma dilution to the assay mix to the start of the measurement).

7. Shake on a plateshaker 10 seconds at 1400 rpm

8. Cover each well with 50 µl liquid paraffin oil (Merck) (**note!!!** the tips of the multi-channel have to be cut to facilitate pipetting the viscous liquid OR use wide-bore tips from Lasec)

9. Place the plate into the platereader and start measuring the A405nm every 9 second for the first two minutes, every 15 seconds up to 30 minutes and every minute until the clots had broken down or up to 270 minutes (protocol “clot lysis Ton Lisman”)

10. Determine the clot lysis time (CLT), which is defined as the time from the midpoint of clear to maximum turbidity transition (clotting time) to the midpoint of maximum turbidity to clear transition (lysis time). Clotting and lysis times are measured by sigmoidal curve fitting using the computer program Origin.

Comments

1. Using a more diluted Assay Mixture and undiluted plasma migth also be possible. The final plasma concentration should be **50%**

2. A final tPA concentration of 100 ng/ml was selected to induce a clot lysis time of between 60 - 100 min using normal plasma. When samples with a low fibrinolytic potential are measured, one could consider to use a higher tPA concentration.

References

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ADDENDUM C

Plasma clot lysis time and its association with cardiovascular risk factors in black Africans – published article

OPEN ACCESS Freely available online

PLOS ONE

Plasma Clot Lysis Time and Its Association with Cardiovascular Risk Factors in Black Africans

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Abstract

Studies in populations of European descent show longer plasma clot lysis times (CLT) in patients with cardiovascular disease (CVD) than in controls. No data are available on the association between CVD risk factors and fibrinolytic potential in black Africans, a group undergoing rapid urbanisation with increased CVD prevalence. We investigated associations between known CVD risk factors and CLT in black Africans and whether CLTs differ between rural and urban participants in light of differences in CVD risk. Data from 1000 rural and 1000 urban apparently healthy black South Africans (35–60 years) were cross-sectionally analysed. Increased PAI-1_{act}, BMI, HbA1c, triglycerides, the metabolic syndrome, fibrinogen concentration, CRP, female sex and positive HIV status were associated with increased CLTs, while habitual alcohol consumption associated with decreased CLT. No differences in CLT were found between age and smoking categories, contraceptive use or hyper- and normotensive participants. Urban women had longer CLT than rural women while no differences were observed for men. CLT was associated with many known CVD risk factors in black Africans. Differences were however observed, compared to data from populations of European descent available in the literature, suggesting possible ethnic differences. The effect of urbanisation on CLT is influenced by traditional CVD risk factors and their prevalence in urban and rural communities.

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☯ These authors contributed equally to this work.

Introduction

Cardiovascular disease (CVD) is a global problem and CVD risk factors and disease rates continue to rise [1]. The development of CVD may start early in life but the immediate underlying cause of a CVD event is the occlusion of a critically situated blood vessel by a blood clot which results in loss of blood flow to vital organs [2]. Therefore, the optimal breakdown of clots is an important protective mechanism in CVD [3].

Various proteins are involved in the lysis of blood clots. These proteins, such as plasminogen activator inhibitor type-1 (PAI-1), tissue-type plasminogen activator (tPA) and plasminogen can be measured individually. They then serve as proxy markers for fibrinolysis. Alternatively one can measure the global ability of blood or plasma to lyse clots, with the use of global fibrinolytic assays. These assays give an indication of the speed with which the body can lyse clots, often reported as lysis time. In recent years a plasma fibrinolytic potential assay that mimics the physiological initiation of coagulation by tissue factor and clot breakdown by tPA from the endothelium was developed [4].

Various studies conducted in populations of European descent have shown plasma clot lysis times (CLT) to be associated with CVD. In general, CVD patients had longer CLTs than controls [5–9], although it was not the case in all studies [10]. CLT has furthermore been shown to be associated with individual CVD risk factors such as increased body mass index (BMI), diabetes, increased total cholesterol, triglycerides and CRP in European descendant populations [7,8].

No information is available regarding the association of clot lysis times and CVD risk factors in Africans, an under-studied population in CVD epidemiology. CVD, which has often been thought of as a problem of developed countries is now a major problem of developing countries including South Africa [11,13]. This increase in CVD prevalence is considered to be attributed to urbanisation of the black South African population due to decreased physical activity and changes to Westernised lifestyle and diet. It has furthermore previously been shown that CVD risk factors and their contribution to CVD risk may differ between blacks and European descendant populations [11,12]. Two large epidemiological studies in black South Africans have shown various CVD risk factors to increase with urbanisation. Vorster [14] reviewed data from the Transition and Health during Urbanisation in South Africa (THUSA) study and reported BMI, smoking prevalence in men, total serum cholesterol levels and blood pressure to increase with urbanisation. In the Prospective Urban and Rural Epidemiological (PURE) population Pieters et al. [15] found blood pressure, BMI, waist circumference, triglyceride concentrations, fasting plasma glucose, and PAI-1_{act}, all factors associated with CVD, to be

significantly higher in the urban participants than in the rural group.

The purpose of this study was therefore to determine whether urbanisation, with its increased prevalence of CVD risk factors, is associated with hypofibrinolysis, and secondly to determine the association between CLT and traditional CVD risk factors in black South Africans.

Materials and Methods

Study Population

Participants were recruited to take part in the South African arm of the international PURE study. This is a large-scale cohort study that tracks changing lifestyles, risk factors and chronic disease using periodic standardised data collection in rural and urban areas of 17 countries in transition over 12 years [16,17]. The data reported here are from the baseline data of just over 2000 randomly selected participants from well-established rural (living under tribal law) and urban (living in informal and formal settlements surrounding cities) communities in the North West Province of South Africa. The sample size was decided upon, based on power calculations performed on the THUSA results, which is a cross-sectional epidemiological study, performed on similar communities in the same province 10 years prior to PURE [14]. In order to obtain 2000 participants it was decided to randomly include 6000 households (3000 from rural and urban respectively) based on previous experience from the THUSA study. From these 6000 households, 4000 subjects were identified who fitted the inclusion criteria. Of these 4000, 2792 (rural = 1444, urban = 1348) agreed to take part in the study, indicated their availability during the blood collection period and had no plans to relocate in the foreseeable future. During the 12 week blood collection period in 2005 blood was finally collected from 1006 rural and 1004 urban participants. Apparently healthy black South African men and women between the ages of 35 and 60 years were eligible to participate. Use of chronic medication for non-communicable diseases and/or any self-reported acute illness were bases for exclusion. The Ethics committee of the North-West University, South Africa approved this study. The study procedure was explained to participants in their home language, after which participants signed informed consent forms and the study commenced. All data were treated confidentially and all analyses were performed with coded data.

Blood Collection

Qualified nursing sisters collected fasting blood samples with minimum stasis from the antecubital veins of participants using sterile winged infusion sets and syringes between 07:00 and 11:00 on days of data collection. For the analysis of lipids and C-reactive protein (CRP) in serum, blood was collected in tubes without anticoagulant. Blood was collected in EDTA tubes for the determination of glycosylated haemoglobin (HbA1c) and in fluoride tubes for glucose measurements. For the analysis of $PAI\text{-}1_{act}$, fibrinogen concentration and plasma fibrinolytic potential, blood was collected into citrate tubes and kept on ice until centrifugation. 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 2–4 days at −82°C until analysis.

Laboratory Analysis

Serum lipids and high-sensitivity CRP were measured using a Sequential Multiple Analyser Computer (SMAC), using the Konelab™ autoanalyser (Thermo Fischer Scientific, Vantaa, Finland). HbA1c was determined with the D-10 Haemoglobin testing system (Biorad, Hercules, CA, USA). A hexokinase method using the Synchron®Sytem(s) (Beckman Coulter Co., Fullerton, CA, USA) and reagents was used to measure plasma glucose. Human Immunodeficiency Virus (HIV) status of participants was determined according to the South African Department of Health protocol and UNAIDS/WHO Policy statement on HIV-testing with the Rapid HIV test, and if positive a Pareeshak test was performed to confirm the results. Participants received pre-test counselling and for those who chose to know the results of their HIV test, post-test counselling was done in privacy. $PAI\text{-}1_{act}$ was measured using an indirect enzymatic method (Spectrolyze PAI-1, Trinity Biotech, Bray, Ireland). A modified Clauss method (Multifibrin U-test, Dade Behring, Deerfield, IL, USA) on the Dade Behring BCS coagulation analyser was used to determine fibrinogen concentrations. Plasma fibrinolytic potential of tissue factor induced clots, lysed by exogenous tPA was measured over a period of 4 weeks with the method of Lisman et al. [5] with slightly modified tissue factor and tPA concentrations in order to obtain comparable CLTs of about 60 min (intra-assay CV = 3.6%, between plate CV = 4.5%). Final concentrations were tissue factor (125×diluted; 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). CLT was defined as the time from the midpoint of clear to maximum turbidity, which is representative of clot formation, to the midpoint of maximum turbidity to clear, which represents the lysis of the clot [5].

Dietary Intake Analysis and Anthropometrical Measurements

Quantitative Food Frequency questionnaires, designed and validated for use in this population, were used to determine habitual alcohol consumption of study participants.

Anthropometrical measurements were taken according to the International Standards of Anthropometric assessment [International society for the advancement of Kinanthropometry] and included weight and height as well as waist circumference. The recommended waist circumference cut-off for central obesity in Sub-Saharan Africans, which is ≥94 cm for men and ≥80 cm for women [18] were used to define central obesity and used as the cut-off for Metabolic syndrome criteria. Blood pressure was measured with subjects sitting relaxed but upright and the right arm supported at heart level using an automatic digital blood pressure monitor (Omron HEM-757).

Statistical Analysis

Data were analysed with the computer software package Statistica (Statsoft Inc., Tulsa, Oklahoma, USA). A p-value of 0.05 or less was regarded as statistically significant. Normally distributed data are reported as mean (95% confidence interval or SD). Data that were not normally distributed were log transformed to improve normality and reported as median (25^{th} –75^{th} percentile). T-tests for independent samples were used when comparing parametric data between two groups and analysis of variance (ANOVA) with Tukey's Honest Significant Difference post hoc test were used for comparisons between three or more groups. The Mann-Whitney U test was used for comparison of non-parametric data between 2 groups. Analysis of co-variance (ANCOVA) was used when comparisons between groups required adjustment. Mean differences with corresponding 95% confidence intervals are also reported. CLT was not significantly different between women who used contraceptives and those who did not,

so we did not stratify or adjust for contraceptive use. Forward Stepwise Multiple Regression analysis was used to determine the main contributors to the variance in CLT in the PURE population using parametric and log transformed data.

Results

General Characteristics of Rural and Urban Participants

Table 1 provides population characteristics for the total study population as well as for the urban and rural groups separately. Sex differences are also indicated for variables with sex specific cut-offs. CLT could be determined for 1802 participants only due to inadequate sample volume and/or haemolysis of some samples. Baseline characteristics of the 1802 participants did not differ from that of the total group. The mean CLT was 57.3 (±11.2) minutes (Table 1). CLT was longer in urban than in rural women (mean difference 1.79 min, 95% CI 0.60–2.99), while no difference was observed for men before adjustments. Taking the standard deviation into consideration, the likelihood of this difference being clinically significant is small. Urban participants had significantly higher blood pressure, BMI (women only), waist circumference (women only), triglycerides, PAI-1_{act} and fasting blood glucose than the rural participants. While urban women consumed more alcohol than rural women, the opposite was seen for men. Fibrinogen concentration and CRP were, however higher in the rural participants.

Associations between CLT and CVD Risk Factors

The associations between CLT and various non-biochemical cardiovascular risk factors are presented in Table 2. ANCOVA's were used to adjust for factors that could potentially influence/obscure independent associations or for intermediate variables that could, at least in part, explain the associations. These include variables that themselves were associated with CLT and which differed between the respective sub-categories. There was no significant difference in CLT between different age categories, contraceptive use or between hyper- and normotensive participants. Women had significantly longer CLTs than men (mean difference 1.3 min 95%CI 0.27–2.38), also after adjustment for differences in BMI and PAI-1_{act} (which differed between men and women, and which were associated with CLT). CLTs were significantly increased with increasing BMI categories (mean difference between lowest and highest BMI categories: 13.8 min, 95%CI 12.2–15.4) and in participants with abdominal obesity (mean difference 9.3 min, 95%CI 8.31–10.3). These differences were also likely to be clinically significant, taking the standard deviations into consideration. Although CLT correlated with both waist circumference ($r = 0.42$, $p<0.0001$) and BMI ($r = 0.47$, $p<0.0001$), the correlation with BMI was stronger and BMI was used in further analyses. Since sex differences were present amongst the BMI and waist circumference categories, we adjusted for sex, but significance remained. Participants who were diagnosed with the metabolic syndrome, using the criteria recommended by Alberti et al. [18] also had significantly longer CLTs than those without metabolic syndrome (mean difference 4.1 min 95% CI 2.84–5.36). Current smokers had significantly shorter CLTs than non-smokers (mean difference 4.5 min 95% CI 3.48–5.52). This significance remained after adjustment for BMI, but disappeared after adjustment for habitual alcohol consumption (which differed between smoking categories). Participants who were non-drinkers had significantly longer CLTs than participants who reported to be moderate (mean difference 5.2 min 95% CI 4.03–6.37) or heavy drinkers (mean difference 6.9 min 95% CI 5.41–8.39). Non-drinkers had significantly higher waist circumference measures (also associated with longer CLT) and significantly lower PAI-1_{act} (associated with shorter CLTs) than drinkers, therefore we adjusted for both, but significance remained ($p<0.0001$). Men drank significantly more than women, and since sex differences for CLT were found, we also adjusted for sex, but again significance remained ($p<0.0001$).

In Table 3 associations between CLT and biochemical CVD risk factors are presented. CLT increased significantly over HbA1c quartiles (mean difference between lowest and highest quartiles: 8.7 min 95% CI 7.38–10.0) and fasting glucose categories (mean difference 4.5 min 95% CI 2.97–6.03). Since a positive association between both HbA1c and fasting plasma glucose with BMI was found, we adjusted for BMI, but the significance between the HbA1c quartiles and fasting glucose quartiles remained. Furthermore significantly longer CLTs were observed in participants with increased serum triglycerides compared to normal triglyceride levels (mean difference 7.6 min 95% CI 6.29–8.91), increased total cholesterol levels compared to normal cholesterol (mean difference 3.5 min 95% CI 2.44–4.56) and decreased HDL-cholesterol levels compared to the recommended values (mean difference 5.8 min 95% CI 4.70–6.90). CLT also increased significantly over the PAI-1_{act} (mean difference between lowest and highest quartiles 15.4 min 95% CI 14.0–16.8), fibrinogen (mean difference between lowest and highest quartiles 4.9 min 95% CI 3.43–6.37) and CRP quartiles (mean difference between lowest and highest quartiles 6.9 min 95% CI 5.42–8.38). Taking the standard deviation into consideration, these differences were all likely to be clinically significant. Significance remained for fibrinogen after adjustment for CRP and significance also remained for CRP after adjustment for PAI-1_{act} and fibrinogen. CLTs tended to be longer in HIV+ compared to HIV- participants (mean difference 1.3 min 95% CI 0.11–2.49). Because there is a difference in BMI between HIV+ and HIV- participants (data not shown) and CLT correlated with BMI we adjusted for BMI. After the adjustment, HIV+ participants now had significantly longer CLTs than the HIV- participants (59.7 vs. 56.9 minutes).

In order to determine the main contributors to the variance of CLT the variables in Table 2 and 3 were included in a forward stepwise regression model. The model explained 45% of the variance in CLT. PAI-1_{act} explained 27% of the variance, while BMI, alcohol consumption and HbA1c explained 8%, 3% and 2% respectively. Triglycerides, CRP, HDL-cholesterol and HIV status each explained only 1% of the variance. Blood pressure, total cholesterol, fibrinogen, smoking and age each explained less than 0.5%. In order to prevent inter-correlation between the variables BMI but not waist circumference and HbA1c but not fasting glucose were included. Metabolic syndrome per se was also not included as the individual components were entered into the model separately.

Discussion

This study investigated for the first time whether urbanisation with its resultant increased CVD risk is associated with hypofibrinolysis. This is also the first paper to investigate the association between known CVD risk factors and global fibrinolytic potential in blacks.

Rural and Urban Differences for CLT

Clot lysis time was not found to be significantly longer in the urban than the rural participants, despite an increase in most CVD risk factors and positive associations of these risk factors with CLT (which will be discussed below). This can likely be explained through the association of CLT with individual CVD risk factors.

Table 1. Characteristics of the South African PURE study population.

Variable	Total population (n = 2010)	Urban (n = 1004)	Rural (n = 1006)	Rural vs. urban p-value
Age (years)	48 (41–56)	48 (42–57)	47 (41–55)	0.0002
Men/women n (%)	749 (37.3)/1260 (62.7)	401 (39.9)/602 (60.0)	348 (34.6)/658 (65.4)	0.01
CLT (minutes)	57.3±11.2	57.6±12.0	57.0±10.5	0.09
Men	52.9±11.6*	52.6±12.4*	53.3±10.6*	0.55
Women	59.9±10.2*	60.8±10.5*	59.0±10.0*	0.015
SBP (mm/Hg)	133.5±24.5	137.3±25.1	129.7±23.3	<0.0001
DBP (mm/Hg)	87.7±14.5	89.3±14.5	86.2±14.5	<0.0001
BMI (kg/m^2)	22.9 (19.3–28.6)	23.4 (19.5–29.4)	22.4 (19.1–28.1)	<0.0001
Men	19.8 (18.1–22.4)	20.0 (18.3–22.8)	19.7 (18.0–22.2)	0.60
Women	25.8 (21.4–31.7)	27.1 (22.3–32.5)	24.9 (20.8–30.7)	<0.0001
Waist circumference (cm)	77.5 (70.2–87.7)	78.5 (70.9–89.0)	76.0 (69.7–86.9)	0.001
Men	74.4 (69.9–81.3)*	74.3 (69.7–81.8)*	74.5 (70.2–80.5)*	0.49
Women	81.1 (70.6–91.3)*	82.8 (73.1–92.8)*	78.8 (69.5–89.5)*	<0.0001
HDL-cholesterol (mmol/l)	1.52±0.63	1.52±0.65	1.52±0.62	0.83
Men	1.58±0.66*	1.61±0.66*	1.55±0.66	0.22
Women	1.48±0.62*	1.46±0.63*	1.50±0.61	0.17
Triglycerides (mmol/l)	1.08 (0.82–1.55)	1.11 (0.84–1.65)	1.05 (0.80–1.43)	<0.001
Total cholesterol (mmol/l)	4.82 (4.01–5.87)	4.89 (4.00–5.97)	4.75 (4.02–5.80)	0.32
LDL-cholesterol (mmol/l)	2.79 (2.08–3.65)	2.81 (2.07–3.66)	2.77 (2.09–3.63)	0.95
PAI-1 (U/ml)	4.26 (1.27–7.92)	5.01 (1.76–9.11)	3.58 (0.81 6.85)	<0.0001
Fibrinogen (g/l)	2.90 (2.30–5.00)	2.70 (2.20–4.30)	3.00 (2.40–5.40)	<0.0001
Men	2.60 (2.10–3.70)*	2.50 (2.00–3.30)*	2.80 (2.20–4.30)*	0.04
Women	3.10 (2.30–5.50)*	2.90 (2.30–5.40)*	3.20 (2.50–5.70)*	<0.001
HbA1c	5.50 (5.30–5.80)	5.50 (5.20–5.80)	5.60 (5.30–5.80)	0.89
Fasting plasma glucose (mM)	4.80 (4.30–5.30)	4.90 (4.30–5.40)	4.70 (4.40–5.20)	0.06
CRP (mg/l)	3.29 (0.96–9.34)	3.25 (1.12–9.85)	3.33 (0.85–9.02)	0.07
Alcohol consumption (g/day)				
(Alcohol consumers only)				
Total group (n = 872)	15.4 (6.43–34.7)	15.9 (7.71–30.9)	15.0 (5.14–44.8)	0.96
Men (U n = 281, R n = 186)	19.7 (7.71–40.0)*	19.3 (11.4–34.7)*	21.9 (5.86–56.6)*	0.88
Women (U n = 252, R n = 153)	13.4 (4.29–30.9)*	14.8 (4.86–26.8)*	11.4 (3.21–38.6)*	0.92

Parametric data reported as mean ± SD and non-parametric data as median (25th-75th percentile); SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index, HDL, high density lipoprotein; LDL, low-density lipoprotein, PAI-1, plasminogen activator inhibitor type-1; HbA1c, glycosylated haemoglobin; CLT, clot-lysis time; R rural; U, urban; *Means differed significantly between sexes; rural vs. urban p-value adjusted for age and sex, and only for age where values are reported for men and women separately.

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CVD risk factors that were increased in the urban group and that are associated with increased clot lysis e.g. triglycerides, PAI-1 and BMI could potentially increase CLT in the urban group. On the other hand some CVD risk factors i.e. fibrinogen and CRP were higher in the rural group and was also found to have positive associations with CLT, therefore contributing to increased CLT in the rural group. Separate analysis for men and women indicated urban women to have a small but statistically significant longer mean CLT than rural women, while no differences were observed for men. A possible reason for rural-urban differences in CLT observed in women and not in men is that the rural-urban differences in the CVD risk factors were more pronounced in the women than the men. This was indeed the case for BMI, for which the rural-urban difference in women was 7 times that of the difference in men. The relative importance of BMI in CLT was additionally established with the multiple regression analysis.

Association of CLT with CVD Risk Factors

Of the known CVD risk factors investigated, the main contributors to the variance in CLT in this population, as determined with a forward stepwise multiple regression model which included the variables in Tables 2 and 3, were PAI-1_{act} (27%), BMI (8%), alcohol consumption (3%) and HbA1c (2%). A previous study conducted in a population of European descent found triglycerides, BMI, diastolic blood pressure, systolic blood pressure and CRP to be the main contributors to CLT, PAI-1 was, however, not measured [8].

In a separate study investigating the main coagulation and fibrinolytic factors associated with CLT, PAI-1_{ag}, in agreement with our PAI-1_{act} results, was found to have the strongest association, explaining 24% of CLT variation in a multiple regression model [19]. The differences in CLT across especially the PAI-1 and BMI quartiles may be of clinical relevance. The

Table 2. Non-biochemical cardiovascular disease risk factors and their association with CLT.

Variable	N	Mean CLT (95% CI) minutes	ANCOVA p-value: Model 1* (Model 2)^Δ variables adjusted for	Mean difference (95% CI)
Age groups				
30–39 years	332	56.9 (55.9–57.9)	0.71	0.8 (−0.95–2.55)
40–49 years	700	57.5 (56.7–58.3)	(0.56) Smoking, alcohol	
50–59 years	482	57.0 (55.9–58.0)	consumption and BMI	
≥60 years	288	57.7 (56.3–59.2)		
Sex			<0.0001	
Men	670	52.9 (52.1–53.9)	(<0.0001) BMI	1.3 (0.27–2.38)
Women	1132	59.9 (59.3–60.5)	(0.003) PAI-1act, BMI	
Contraceptive use				
Yes	403	60.5 (59.5–61.6)	0.10	1.1 (−0.18–2.38)
No	667	59.4 (58.7–60.2)		
Blood pressure				
Normal	946	57.3 (56.6–58.0)	0.93	0 (−1.05–1.05)
Hypertensive	842	57.3 (56.5–58.1)		
BMI				
<18.5 kg/m^2	314	50.8 (49.6–52.0)†	<0.0001	13.8 (12.2–15.4)
18.5–24.9 kg/m^2	739	55.3 (54.5–56.0)†	(0.0001) PAI-1act	
25–29.9 kg/m^2	297	60.8 (59.6–61.9)†	(<0.001) PAI-1act, sex	
≥30 kg/m^2	354	64.6 (63.6–65.6)†		
Waist circumference			<0.0001	
Normal	1168	54.1 (53.5–54.7)†	(<0.0001) PAI-1act	9.3 (8.31–10.3)
Abdominal obesity	617	63.4 (62.6–64.2)†	(<0.0001) PAI-1act, sex	
Metabolic syndrome				
Yes	434	60.4 (59.2–61.5)†	<0.0001	4.1 (2.84–5.36)
No	1358	56.3 (55.7–56.9)†		
Metabolic Syndrome risk score				
0	278	56.4 (55.1–57.6)	<0.0001	5.5 (3.81–7.19)
1	604	54.9 (54.0–55.8)		
2	476	58.0 (57.1–59.0)		
≥3	434	60.4 (59.2–61.5)		
Smoking status				
Yes	943	55.3 (54.6–56.1)†	<0.0001	4.5 (3.48–5.52)
No	785	59.8 (59.0–60.5)†	(0.016) BMI	
Former	66	56.0 (52.9–59.0)	(0.78) BMI, alcohol	
Alcohol consumption			<0.001	
Non-drinkers	964	60.0 (59.4–60.7)†§	<0.0001) Sex, WC	6.9 (5.41–8.39)
Moderate drinkers*	467	54.8 (53.9–55.8)†	(<0.0001) Sex, WC,	
Heavy drinkers**	320	53.1 (51.7–54.4)§	PAI-1act,	

*Moderate drinking: >0<15 g/day for women; >0<30 g/day for men; **Heavy drinking: ≥15 g/day for women; ≥30 g/day for men; †‡§Means with the same symbol differed significantly; ¶Mean differed significantly from other means in subgroup. WC: waist circumference; BMI: Body mass index; PAI-1: Plasminogen activator inhibitor type-1. For groups with more than two subgroups the difference between the highest and lowest value are reported. N varies among variables due to lack of sample availability that occurred randomly during sample collection. *Model 1: adjusted for age; ^ΔModel 2: adjusted for age as well as variables indicated in table.
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difference between the lowest and highest quartiles were around 15 minutes while differences between arterial thrombosis patients and controls in previous studies ranged from 1.9 to 10.8 minutes [7,8,19].

Interestingly, in the PURE population CLT correlated better with BMI than with waist circumference even though PAI-1_{act} had a significantly stronger correlation with waist circumference [20]. This stronger correlation of PAI-1 with waist circumference is likely due to the fact that visceral adipose tissue is a major source of PAI-1 and that its levels are further increased by hepatic production in response to adipocyte-derived cytokines [21–24]. Adjustment for PAI-1_{act} did not significantly affect the results and CLT remained longer with increased BMI categories and in individuals with abdominal obesity. While PAI-1_{act} seems to be

Table 3. Biochemical cardiovascular disease risk factors and their association with CLT.

Variable	N	Mean CLT (95% CI) minutes	ANCOVA p-value: Model 1* (Model 2)Δ variables adjusted for	Mean difference (95% CI)
HbA1c				
<5.3	443	53.1 (52.2–54.0)†	<0.0001	8.7 (7.38–10.0)
≥5.3–<5.5	289	54.1 (52.9–55.3)†	(<0.0001) BMI	
≥5.5–<5.8	490	57.8 (56.8–58.8)		
≥5.8	564	61.8 (60.9–62.8)		
Fasting glucose				
≤5.5 mmol/l	1435	56.5 (55.9–57.0)	<0.0001	4.5 (2.97–6.03)
>5.5 mmol/l	297	61.0 (59.6–62.5)	(0.003) BMI	
Triglycerides				
<1.7 mmol/l	1417	55.8 (55.2–56.3)†	<0.0001	7.6 (6.29–8.91)
≥1.7 mmol/l	342	63.4 (62.2–64.6)†		
Total cholesterol				
<5.2 mmol/l	1059	55.8 (55.2–56.5)†	<0.0001	3.5 (2.44–4.56)
≥5.2 mmol/l	710	59.3 (58.5–60.1)†		
HDL-cholesterol				
Men >1, women>1.2 mmol/l	1233	55.5 (54.9–56.1)†	<0.0001	5.8 (4.70–6.90)
Men<1, women<1.2 mmol/l	536	61.3 (60.4–62.2)†		
PAI-1act				
<1.27 U/ml	456	50.2 (49.3–51.2)†	<0.0001	15.4 (14.0–16.8)
≥1.27–<4.26 U/ml	460	54.9 (54.1–55.7)†	(<0.0001) Sex	
≥4.26–<7.92 U/ml	446	58.7 (57.9–59.5)†		
≥7.92 U/ml	440	65.6 (64.5–66.7)†		
Fibrinogen				
<2.3 g/L	471	55.1 (54.0–56.1)†‡	<0.0001	4.9 (3.43–6.37)
≥2.3–<2.9 g/L	380	56.3 (55.2–57.4)‡§	(<0.001) CRP	
≥2.9–<5 g/L	406	58.7 (57.6–59.8)†§		
≥5 g/L	421	60.0 (59.0–61.0)‡‖		
CRP				
<0.964 mg/L	431	53.8 (52.8–54.7)†	<0.0001	6.9 (5.42–8.38)
≥0.964–<3.286 mg/L	443	56.2 (55.2–57.1)†	(<0.0001) PAI-1act	
≥3.286–<9.340 mg/L	442	58.3 (57.3–59.4)†	(<0.0001) Fibrinogen	
≥9.340 mg/L	445	60.7 (59.5–61.8)†	(<0.0001) PAI-1act, fibrinogen	
HIV status				
Positive	306	58.4 (57.4–59.4)†	0.052	1.3 (0.11–2.49)
Negative	1486	57.1 (56.5–57.7)†	(<0.0001) BMI	

†‡§‖Means with the same symbol differed significantly. HDL-cholesterol: high density lipoprotein cholesterol; PAI-1: Plasminogen activator inhibitor type-1; CRP: C-reactive protein; HbA1c: Glycosylated haemoglobin; HIV: Human Immunodeficiency Virus. For groups with more than two subgroups the difference between the highest and lowest value are reported. N varies among variables due to lack of sample availability that occurred randomly during sample collection. *Model 1: adjusted for age; ΔModel 2: adjusted for age as well as variables indicated in table.

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more related to central obesity, CLT seems to be related to general obesity. Although increased PAI-1_{act} levels likely play a major role in this relationship, there seem to be additional mechanisms involved in the association between body fat and CLT, unrelated to the PAI-1 and visceral fat link.

CLT was significantly associated with both glucose and HbA1c. In agreement with these results, Guimarães et al. [7] and Meltzer et al. [6] found white diabetic subjects to have longer CLT than non-diabetic subjects. Another study, however, found no association between CLT and diabetes in control subjects [8]. CLT was also longer in participants diagnosed with the metabolic syndrome, than those without. These results are to be expected since most of the criteria for the metabolic syndrome were all found to be associated with longer CLTs. These results are in agreement with the results of Carter et al. [25], who found CLT to be longer in white patients with the metabolic syndrome, although a different classification system was used to diagnose metabolic syndrome and their clot lysis assay differs from the assay we used.

Although CLT was increased across fibrinogen quartiles, fibrinogen concentration explained less than 0.5% of the variance in CLT and correlated only weakly with CLT ($r = 0.18$, $p<0.0001$), indicating that fibrinogen concentration was not one

of the main contributing factors of CLT variance in this population. Theoretically fibrinogen could influence fibrin lysis rates through its effect on clot structure as has been demonstrated in purified models [26–28]. This effect, however, is less prominent in plasma models likely due to the presence and interaction of other plasma components that also affect clot lysis. It is also possible that other factors included in the model influenced the prediction value of fibrinogen. We also saw a significant increase in CLT across CRP quartiles, also after adjustment for PAI-1 and fibrinogen indicating an independent positive association between inflammation and CLT. These results are in agreement with associations between CLT, fibrinogen and CRP data from the literature [5,8].

We found no association between CLT and age and between CLT and blood pressure whereas studies in populations of European descent found trends of increased CLT with increased age [5,7,8] and increased systolic and diastolic blood pressure [8]. In agreement with this, no association was found between PAI-1_{act} and age in the PURE study population [20].

Our results also show women to have significantly longer CLTs than men, while other studies found, although not significantly, longer CLTs in white men than in women [5,6], or no differences between men and women [7]. Possible factors that might have explained the longer CLT in women in our study, are PAI-1_{act} and BMI, which were both found to be higher in the women than in the men [20]. Adjustment for PAI-1_{act} and BMI did, however, not significantly affect the results and CLTs remained longer in women than in men, indicating a possible real sex difference in this study population.

We found significantly shorter CLTs for moderate and heavy drinkers than for non-drinkers, while studies in populations of European descent reported no apparent differences between regular users of alcohol and participants who do not use alcohol or do so only occasionally [7,8]. Three factors that differed significantly between drinkers and non-drinkers [20] and that were found to be associated with CLT were considered in order to explain the association between CLT and alcohol consumption. These factors were sex, waist circumference and PAI-1_{act} [20]. Significant differences in CLT between drinkers and non-drinkers, remained, however after separate adjustment for these three factors. The fact that CLT is shorter in drinkers in this population, while a main factor determining CLT, PAI-1_{act} was found to be increased, suggests that alcohol affects CLT at least in part, in a PAI-1_{act} unrelated manner.

HIV+ participants had longer CLT's than HIV- participants after adjustments for BMI differences. One would expect the HIV-group, who had the higher BMI values to have longer CLTs due to the link between PAI-1_{act} and adipose tissue or overall body fat. Positive HIV status has on the other hand been shown to be associated with increased PAI-1 antigen and therefore probably impaired fibrinolysis. This increase may be attributed to fat redistribution in patients infected with the HIV virus [29]. However, in the PURE population PAI-1_{act} did not differ between the HIV+ and HIV- participants [30].

Due to the fact that many haemostatic factors play a role and/or influence CLT, the fact that only PAI-1_{act} and fibrinogen concentration were measured in this study population may be a limitation to the interpretation of the results of this study and could potentially lead to residual confounding. Additionally, this being a cross-sectional study, causality could not be determined for CLT. 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 CLT in black Africans associated significantly with many known CVD risk factors. Differences in these associations were however observed, compared to available data from white populations, suggesting possible ethnic differences in the association of CLT with CVD risk. Of the variables measured, CLT was most strongly related to PAI-1_{act} and BMI. CLT seems to be strongly affected by total body fat, but it seems only partly through the PAI-1-visceral fat link. Additional research is required to determine which factors associated with obesity influences CLT. Alcohol consumption in this population was significantly associated with shorter CLT, despite increased PAI-1_{act}. This also deserves further attention. Urbanisation per se is not associated with hypofibrinolysis despite an increase in presence of many CVD risk factors. The effect of urbanisation on CLT is dependent on the relationship of the individual CVD risk factors with CLT and to which degree urbanisation affects these risk factors.

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Author Contributions

Conceived and designed the experiments: AK MP JCJ ZDL DCR. Performed the experiments: ZDL. Analyzed the data: MP ZDL. Contributed reagents/materials/analysis tools: MP JCJ. Wrote the paper: ZDL MP JCJ DCR.

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