

The association of HIV infection with plasma clot characteristics in black South Africans

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DECLARATION

I, Shams Bakali hereby declare that this dissertation is my own work and has not been submitted to any institution.

Signature of student:



Date: 25/11/2020

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ABSTRACT

INTRODUCTION AND AIM

Human immunodeficiency virus (HIV) infection is still one of the largest burdens to the world's health, and South Africa reportedly has the highest prevalence in the world. HIV, as well as antiretroviral therapy (ART) are associated with hypercoagulability, consequently increasing thrombotic risk. One of the main components of hypercoagulability is altered clot properties, which contribute to thrombosis-related cardiovascular complications, as the clots with tightly packed thin fibrin structures tend to be more prothrombotic. It is hypothesised that one of the mechanisms through which HIV-infected patients' thrombotic risk is increased is *via* altered clot structure. Therefore, the current study aimed to investigate the association of HIV status and ART respectively, with fibrinogen concentration and plasma clot properties in a sub-group of participants from the South African, North West Province arm of the PURE study.

PARTICIPANTS AND METHODS

The study made use of data collected in 2005 and 2010 in the South African North West Province arm of the international Prospective Urban and Rural Epidemiology (PURE-SA-NW) study. The study included data from 151 newly infected HIV-positive participants at baseline, 70 of whom were receiving ART by 2010 and 81 who were still untreated. These participants were systematically matched to 176 HIV-uninfected individuals. Total and γ' fibrinogen concentration, as well as fibrin clot properties obtained with turbidimetry, were cross-sectionally compared at baseline between HIV-infected and HIV-uninfected participants, and between the HIV-treated, the untreated and HIV-uninfected groups at the 2010 follow-up. Prospective comparisons, over the five-year period, were furthermore made within and between these groups.

RESULTS

At baseline, HIV-infected individuals presented with lower total fibrinogen concentration and higher percentage γ' fibrinogen compared with the controls. Plasma clots from these participants were less dense (lower maximum absorbance), but took longer to lyse than those of the HIV-uninfected participants. At the 2010 follow up, the HIV-treated and untreated groups did not differ regarding the outcome variables, but differed compared to the HIV-uninfected group. Both HIV-infected groups presented with lower total fibrinogen and maximum absorbance compared to the HIV-uninfected group, with the treated group also presenting with longer clot lysis times (CLT) than the HIV-uninfected group. Prospectively, from baseline to follow-up, fibrinogen increased (or tended to) in all three groups, with γ' fibrinogen decreasing in all three groups. The rate of lateral aggregation (slope) and maximum absorbance (clot density) increased in all three groups, with a borderline slope increase in the treated group. CLT increased significantly over the 5-year period in the HIV-treated and HIV-uninfected groups, but not in the untreated group. A significant difference in the extent of change over time, was also observed for lag time, which decreased more in the HIV-treated group. The age-related progression to formation of more prothrombotic clots was somewhat delayed in the HIV untreated group, while ART use seemed to counteract this “protective” effect.

CONCLUSION

The study showed HIV-infection to be associated with lower total fibrinogen concentration and maximum absorbance compared to HIV-uninfected participants. The cross-sectional comparisons also found HIV infection to be associated with longer CLT, which could relate to altered levels of other variables affected by the HIV status, such as LDL-C, rather than to altered plasma clot structure. Prospectively, over the five-year period, HIV-infection demonstrated a tendency to delay the age-associated progression to more prothrombotic clot properties, but ART treatment partly neutralised this “protective effect.”

KEY TERMS: HIV, African, fibrinogen, clot properties, antiretroviral therapy

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LIST OF ABBREVIATIONS AND SYMBOLS

α	Alpha
β	Beta
$^{\circ}\text{C}$	Degrees Celsius
ABCA 1	Adenosine-triphosphate binding cassette transporter A1
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ART	Antiretroviral therapy
Au	Absorbance units
Apo B	Apolipoprotein B
APS	Antiphospholipid syndrome
AT	Antithrombin
BMI	Body mass index
BSA	Bovine serum albumin
CaCl_2	Calcium chloride
CAD	Coronary artery disease
Δ	Change
CCR5	Chemokine co-receptors
CD4	Cluster of differentiation 4
CEN	Centre of Excellence for Nutrition
CLT	Clot lysis time
cm	Centimetre
cm^2	Square centimetre
CRABP-1	Cellular retinoic acid-binding protein 1
CrCl	Creatinine clearance
CRP	C-reactive protein
CVD	Cardiovascular disease
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid

DVT	Deep vein thrombosis
EC	Enteric coated
EDTA	Ethylenediaminetetraacetic
eGFR	Estimated Glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
F	Factor
FDA	Food and Drug Administration
FMD	Flow-mediated vasodilation
FPA	Fibrinopeptide A
FPB	Fibrinopeptide B
g/L	Gram per litre
GGT	Gamma-glutamyl transferase
γ'	Gamma prime
GHRP	Gly-His-Arg-Pro
GLM	Generalised linear regression
GLUT-4	Glucose transporter type 4
gp120	Envelope glycoprotein 120
GPR	Gly-Pro-Arg
H ₂ SO ₄	Sulphuric acid
HbA1c	Glycated haemoglobin
HDL-C	High-density lipoprotein cholesterol
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
IL-6	Interleukin-6
IMT	Intima-media thickness
KCl	Potassium chloride
kDa	Kilodaltons

kg/m ²	Kilogram per square metre
LDL-C	Low-density lipoprotein cholesterol
LPS	Lipopolysaccharides
M	Mole
MAP	Mitogen-activated protein
mg/L	Milligram per litre
mg/ml	Milligram per millilitre
MI	Myocardial infarction
μl	Microlitre
Min	Minute
ml	Millilitre
mM	Millimole
mm ³	cubic millimetre
mmol/L	Millimoles per litre
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
n	Population size
NaCl	Sodium chloride
NCD	Non-communicable disease
ng/ml	Nanogram per millilitre
nm	Nanometre
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRF	National research foundation
NRTI	Nucleoside reverse transcriptase inhibitor
NW	North West
NWU	North-West University
NWU-HREC	North-West University Health Research Ethics Committee.
Pa	Pascal
PAI-1 _{act}	Plasminogen activator inhibitor-1 activity

PC	Protein C
PE	Pulmonary embolism
PI	Protease inhibitor
Pol-Y	Polymerase gamma
PPAR	Peroxisomal proliferator-activated receptor
PPAR-Y	Peroxisome-proliferator activated receptor type gamma
PPP	Platelet poor plasma
PS	Protein S
PT	Prothrombin time
PTTK	Partial thromboplastin time with kaolin
PURE	Prospective Urban and Rural Epidemiology
p-value	Statistical significance
%	Percentage
PWV	Pulse wave velocity
r	Correlation coefficient
RCT	Reverse cholesterol transport
RNA	Ribonucleic acid
rpm	Revolutions per minute
RTIs	Recurrent respiratory tract infections
RXR	Retinoid X receptor
SA	South Africa
SBP	Systolic blood pressure
SEM	Scanning electron microscopy
SMC	Smooth muscle cells
SNP	Single nucleotide polymorphism
SREBP-1c	Sterol-regulatory element-binding protein
SuNReA	Supporting nutrition research in Africa
SuPAR	Soluble urokinase-type plasminogen activator receptor
T	Time

TEA	Trimethylamine
TEG	Thromboelastography
TEM	Transmission electron microscopy
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TG	Triglycerides
TLR4	Toll-like receptor 4
TNF α	Tumour necrosis factor alpha
t-PA	Tissue-type plasminogen activator
U/ml	Units per millilitre
UNAIDS	United Nations programme on HIV/AIDS
u-PA	Urokinase plasminogen activator
USA	United States of America
VCAM	Vascular cell adhesion molecule/protein
vs.	Versus
VT	Venous thrombosis
VTE	Venous thromboembolic
η	Viscosity
WC	Waist circumference
WHO	World Health Organisation

CHAPTER 1: INTRODUCTION

1.1 Background

Human immunodeficiency virus (HIV) infection is still one of the largest burdens to the world's health (WHO, 2018). This life-threatening infection is highly prevalent in sub-Saharan African countries, which include South Africa (Piot *et al.*, 2001). It is estimated that 66% of people living with HIV reside in this region (Yah & Tambo, 2019). The global HIV and AIDS statistics reported South Africa as having the highest prevalence of HIV in the world, with about 7.7 million people infected, representing approximately 20.4% of its population (Avert, 2019). Different opportunistic infections accompany HIV infection and its progression, and it is associated with a variety of cardiovascular-related complications (Crum-Cianflone *et al.*, 2008; De Gaetano Donati *et al.*, 2010; Hsue, 2019). The introduction of antiretroviral therapy (ART) has significantly reduced HIV-related complications, increased survival and improved quality of life among infected individuals (Wang *et al.*, 2015). However, the drugs have also been associated with the development of cardiovascular-related complications in patients (Deeks & Phillips, 2009; Loelius *et al.*, 2018). For example, increased inflammation, dyslipidaemias, coagulation abnormalities and endothelial lining injuries are common among HIV patients receiving treatment (Deeks & Phillips, 2009; Friis-Møller *et al.*, 2003; Hsue, 2019; Sun *et al.*, 2015).

HIV as well as ART are reportedly associated with hypercoagulability, consequently increasing thrombotic risk (Armah *et al.*, 2012; De Gaetano Donati *et al.*, 2010; Haugaard *et al.*, 2013; Jackson & Pretorius, 2019). One of the main components of hypercoagulability is altered clot properties, which may contribute to thrombotic cardiovascular complications (Jackson & Pretorius, 2019; Jacobson *et al.*, 2004), as clots with tightly packed thin fibrin fibres tend to be more resistant to lysis resulting in thromboembolic events (Cesarman-Maus & Hajjar, 2005; Undas & Ariëns, 2011). This, depending on the properties of the formed clot, may result in serious complications, such as stroke and myocardial infarction, also reported in HIV-infected patients (Kaplan-Lewis *et al.*, 2016). It was, therefore, hypothesised that HIV-infected individuals may have altered fibrin clot properties.

Although limited, a number of studies have reported that HIV infection as well as some combinations of ART are associated with increased coagulation, increased fibrinogen levels and other variables that determine or tend to predict the possible presence or formation of thrombotic clots that are resistant to lysis (De Lange *et al.*, 2012; Koppel *et al.*, 2002; Madden *et al.*, 2008; Martin *et al.*, 2010). There is to date, however, still very little direct evidence for the effect of HIV infection and/or ART on fibrin clot properties. Therefore, this study was conducted to provide further understanding of the potential association of HIV and ART with plasma clot properties.

To determine the properties of plasma clots, a number of methods can be applied, some of which measure the specific properties directly while others measure them indirectly. Direct methods of determining clot structure include scanning electron microscopy (SEM), transmission electron microscopy (TEM) and thromboelastography (TEG), while mechanical properties are measured directly by using rheometry, providing information regarding the viscoelasticity of the clots being studied (Ryan *et al.*, 1999). Turbidimetry and permeability assays are indirect methods of measuring the plasma clot properties (Morais *et al.*, 2006; Pieters *et al.*, 2012).

The present study aimed to investigate the cross-sectional as well as prospective association of HIV status and ART respectively, with fibrinogen concentration and plasma clot properties in a sub-group of participants from the South African, North West Province arm of the international Prospective Urban and Rural Epidemiology (PURE-SA-NW) study. The PURE study is an international study aimed at understanding the relationship of societal influences on human lifestyle behaviours, cardiovascular risk factors, and incidence of chronic non-communicable diseases. Newly diagnosed, hence untreated, HIV-infected individuals as well as HIV-uninfected matched controls were included at baseline. At follow-up, 5-years later, the HIV infected group was divided into those who started ART at diagnosis and those who remained treatment naïve. The plasma clot properties were analysed using a turbidity method. This method is one of the fastest and most common methods of determining the formation, structure and lysis of clots.

1.2 Aim and objectives

The aim of this study was to investigate the association of HIV status and ART respectively, with fibrinogen concentration and plasma clot properties in a sub-group of participants from the South African North West Province arm of the PURE study.

To reach the specific objectives of the planned study, a sub-group of participants of the PURE-SA-NW study, who were newly diagnosed with HIV in 2005 (baseline) and who were either ART-treated or untreated by 2010 (follow-up), as well as participants who were HIV-uninfected at baseline and at follow-up, were selected from the PURE-SA-NW database and their data analysed. The specific objectives of this study include the following:

1. To cross-sectionally compare fibrinogen concentration and plasma clot characteristics obtained with turbidity measurements of:
 - HIV-infected (n = 151) and HIV negative (n = 176) participants at baseline;
 - HIV-infected treatment-naïve (n = 81) participants, HIV-infected treated (n = 70) and HIV negative (n = 176) participants at follow-up.
2. To prospectively compare within groups, fibrinogen concentration and plasma clot characteristics obtained with turbidity measurements of:
 - participants who were HIV free at baseline and follow-up (n = 176);
 - HIV-infected participants who were treatment-naïve at baseline and follow-up (n = 81);
 - HIV-infected participants who were treatment-naïve at baseline and treated at follow-up (n = 70).
3. To prospectively compare individual haemostatic variables and plasma clot characteristics obtained with turbidity measurements between the three groups as indicated in objective 2.

1.3 Research team

A multi-disciplinary team of researchers conducted the South African, North West Province arm of the Prospective Urban and Rural Epidemiology (PURE-SA-NW) study, with the research team of this specific study listed below. All team members are

affiliated with the Centre of Excellence for Nutrition (CEN) at the North-West University (NWU).

Table 1-1: Research team members

Team member	Qualification	Role in study
Mr Shams Bakali	B.Sc. Nutrition and food science	M.Sc. student: Protocol development, writing of literature review, analysis of objectives, implementation of methods, data capturing, statistical analysis, interpretation of results, writing up of dissertation
Dr Zelda de Lange-Loots	Ph.D. Nutrition	Supervisor of M.Sc. student: guidance regarding writing of protocol, literature review and dissertation, training student in methodology, guidance in data capturing, statistical analysis and interpretation of results
Prof. Marlien Pieters	Ph.D. Dietetics	Co-supervisor of M.Sc. student: guidance regarding writing of protocol and dissertation, training student in methodology, guidance regarding interpretation of results

1.4 Structure of the dissertation

This dissertation is in chapter format, consisting of five chapters. It was prepared according to the requirements of the NWU, with references adhering to the Harvard style, as used at the NWU, and language edited by a qualified language editor. The present chapter (Chapter 1) introduces the brief concept behind the study, the aims and objectives as well the research team and their respective contributions. Chapter 2 presents an overview of the literature on the study topic, which includes a description of the coagulation system, clot-related cardiovascular complications, HIV pathophysiology, ART medications and how they relate to one another. In Chapter 3, the methods and overall design used in this study are provided. The chapter begins by providing a description of the PURE-SA-NW study population, the design for the

current study and detailed methods used to determine the outcome variables of the current study. These include the methods used for the measurement of total fibrinogen, γ' fibrinogen and clot properties. The chapter concludes with a description of the statistical analyses performed.

In Chapter 4 the results of this study are presented. This chapter begins by providing the study population characteristics and between-group differences at baseline. At baseline, the study sample was divided into two groups, the HIV-infected and HIV-uninfected individuals. For the follow-up, the samples were three groups of treated, untreated and HIV-uninfected participants. The chapter also provides the prospective differences within and between the groups from baseline to the follow-up.

In the final chapter (Chapter 5), the results are discussed by providing a detailed interpretation of the findings, linked to the relevant published literature. The chapter also acknowledges and explains the limitations of the study, and gives recommendations that will guide and improve future studies. The overall conclusion of the study, which presents the new messages being imparted or suggested by the current study, is at the end of the chapter. This dissertation ends with a list of the references used for the included literature throughout all five chapters, and addenda including the ethics approval certificate of the PURE-SA-NW study.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Human immunodeficiency virus (HIV) and the acquired immunodeficiency syndrome (AIDS) infection is still one of the largest burdens to the world's health (WHO, 2018). The estimate, according to the global HIV and AIDS statistics in 2019, was that 38 million people worldwide are living with HIV, representing 0.7% of the global population (HIV.gov, 2020). This life threatening disease is highly prevalent in sub-Saharan Africa countries, which include South Africa (Piot *et al.*, 2001), and it is estimated that 66% of the people living with HIV are in this region (Yah & Tambo, 2019). The global HIV and AIDS statistics also reported South Africa as having the highest prevalence of HIV in the world, with about 7.7 million people infected, representing 20.4% of the population (HIV.gov, 2020). Different opportunistic infections accompany HIV infection and progression, and are associated with a variety of complications including cardiovascular-related complications, such as endothelial dysfunction, atherosclerosis and dyslipidaemias (Kaplan-Lewis *et al.*, 2016; Sun *et al.*, 2015).

The introduction of antiretroviral therapy (ART) has helped to reduce HIV-related complications, increase survival and improve quality of life among the infected individuals (Samji *et al.*, 2013). However, a number of ART drugs have also been associated with the development of other complications in patients, which calls for the need to address them (Loelius *et al.*, 2018). For example, inflammation impairments, coagulation abnormalities and endothelial lining injuries are common among HIV patients, both the treated and treatment naïve (Kuller *et al.*, 2008).

HIV as well as ART are associated with hypercoagulability, consequently increasing thrombotic risk (De Gaetano Donati *et al.*, 2010). One of the main components of hypercoagulability is altered clot structural and mechanical properties, which contributes to thrombosis-related cardiovascular complications (Jackson & Pretorius, 2019; Undas & Zeglin, 2006). The clots, with tightly packed thin fibrin structures, tend to be more resistant to lysis hence may bring thromboembolic complications (Cesarman-Maus & Hajjar, 2005; Undas & Ariëns, 2011). Although limited, a number of studies have reported HIV infection is also associated with increased fibrinogen

levels and other variables that determine or predict the presence or formation of thrombotic clots that are resistant to lysis, which further poses a thrombotic risk (De Lange *et al.*, 2012; Lijfering *et al.*, 2008; Madden *et al.*, 2008). There is to date, however, very little direct evidence for the effect of HIV infection and/or ART on fibrin clot properties.

This chapter will provide a review on the blood coagulation process and the thrombotic cardiovascular complications that develop due to abnormal coagulation. It will outline the aetiology of HIV with the focus on cardiovascular complications resulting from the disease itself as well as the ART used to manage it. Finally, the chapter will discuss the available literature on the influence of HIV on fibrinogen concentration and clot properties as a potential mechanistic pathway underpinning the development of HIV-associated CVD.

2.2 Haemostasis

Blood clotting or coagulation is defined as the clumping together of blood to form a gel-like structure triggered by a complex set of enzymatic reactions in response to certain stimuli (Monroe & Hoffman, 2014; Weisel & Litvinov, 2017). Under normal circumstances, coagulation occurs whenever there is a cut to a blood vessel to prevent or minimise blood loss and allow the wound to heal; this is haemostasis. However, in some cases, a blood clot can form within the blood vessel, which blocks or interrupts blood flow causing complications downstream; this is thrombosis (Bagoly *et al.*, 2017; Jackson, 2011).

When the endothelial cells of a blood vessel are injured, collagen, a vessel wall structure protein, localises platelets towards the point of injury and activates them to release a chemical known as thromboxane A₂ that also directs or calls more platelets towards the site. These platelets then change their structure and become stickier to aggregate at the point of injury (Bever *et al.*, 1984; Monroe *et al.*, 1996; Monroe & Hoffman, 2006). Mesh-like structures known as fibrin, formed from fibrinogen, are then formed on top of the platelets to stabilise the structure and trap some red blood cells in the process (Standeven *et al.*, 2005).

The mechanism behind blood clotting uses a coagulation cascade model and/or the cell-based conceptual model, which explains the reactions that lead to the formation of fibrin and the clot itself. A discussion of the cell-based model, regarded as a thorough physiology of the blood coagulation process, follows (Hoffman & Monroe lii, 2001; Kjalke *et al.*, 1998; Monroe & Hoffman, 2006).

2.2.1 Cell-based conceptual model

It has been suggested that the coagulation cascade does not lay out the whole process of clot formation; this resulted in the development of the cell-based model to fill the gaps (Hoffman & Monroe, 2001; Monroe & Hoffman, 2006). This model recognises specific cells as having key roles in blood clotting, and it approaches the process as an overlapping series of steps other than just distinct routes of intrinsic and extrinsic pathways, as in the coagulation cascade. The model explains that different cells have different pro-coagulant and anti-coagulant properties that help in controlling the process due to the chemical and enzyme interactions that exist when stimuli trigger its initiation (Hoffman & Monroe, 2001; Monroe & Hoffman, 2006). The process is divided into three overlapping steps, namely initiation, amplification and propagation, as demonstrated by Figure 2-1 below.

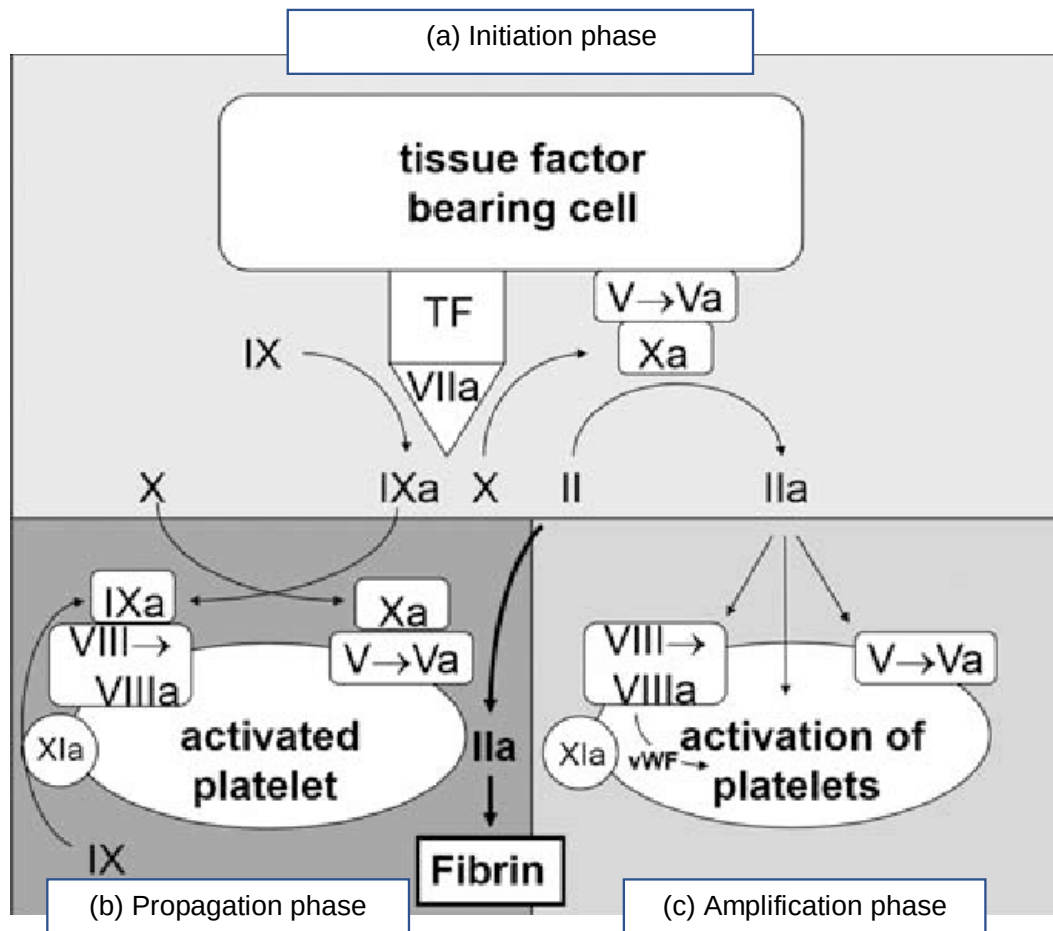


Figure 2-1: Cell-based model of haemostasis adapted from Hoffman & Monroe (2001)

2.2.1.1 Initiation phase

A series of reactions happen on tissue factor-bearing cells, which end up in the generation of small amounts of thrombin, as in the extrinsic pathway of the coagulation cascade (Hoffman & Monroe *et al.*, 2001; Monroe & Hoffman, 2006). The injury triggers exposure or release of tissue factor (TF) from the cells, which binds with plasma-derived activated factor VIIa to form a complex, which will then activate small amounts of FIX and FX into FIXa and FXa, respectively (Hoffman & Monroe *et al.*, 2001; Wildgoose & Kiesel, 1989). Factor Xa binds to FV, activates it and, in the presence of calcium ions, forms a complex known as prothrombinase, which initiates the conversion of prothrombin (factor II) into thrombin (factor IIa) (Hoffman & Monroe *et al.*, 2001; Monroe *et al.*, 1996) (Figure 2-1). At this stage, the generation of thrombin is in small amounts since the

involved factors are also only in small amounts (Bagoly *et al.*, 2017; Monroe *et al.*, 1996).

As this phase progresses, an extracellular protein known as collagen is also exposed in response to the injury, which in turn directs or attracts platelets to the site of injury and partially activates them, in the process creating a basis for the formation of a platelet plug (Armah *et al.*, 2012; Briedé *et al.*, 2001; Monroe & Hoffman, 2014; Weisel & Litvinov, 2017). The platelets interact with FVIII, which is bound to a glycoprotein known as von Willebrand factor to aggregate at that specific point of injury (Li & Gabriel, 1997; Monroe & Hoffman, 2006; Nesheim *et al.*, 1991).

2.2.1.2 Amplification phase

This phase follows on the already made traces of thrombin from the initiation phase above (Hoffman & Monroe, 2001). This thrombin activates the platelets in order to make its surface available for binding of other coagulating factors. The thrombin also activates cofactors V and VIII, and FXI on the platelet surface (Baglia & Walsh, 1998; Baglia & Walsh, 2007) (Figure 2-1).

2.2.1.3 Propagation phase

The propagation phase is characterised by the massive formation of thrombin, which converts fibrinogen to fibrin. During the amplification phase, more platelets become activated, signalling other platelets to aggregate at the point of injury, which also starts other sets of reactions (Baglia & Walsh, 2007; Monroe & Hoffman, 2014). As presented in Figure 2-1 above, the current stage begins with the interaction of FIXa (generated by TF/FVIIa complex in the initiation phase and FXIa activated by thrombin on the platelet surface) with FVIIIa on the platelet surface, to form what is called a tenase complex (Hoffman & Monroe *lii*, 2001). The tenase complex generates reasonable amounts of FXa that interact with FVa, generated by thrombin on the platelet surface, to form a complex that converts massive amounts of prothrombin into thrombin (Rawala-Sheikh *et al.*, 1990). Platelet aggregation and activation can continue until the

generation of enough thrombin is achieved (Hoffman & Monroe *et al.*, 2001; Rawala-Sheikh *et al.*, 1990).

Once an adequate amount of thrombin has been generated, the conversion of fibrinogen is initiated to form thread-like structures known as fibrin, which forms a mesh on top of the platelet aggregate (plug) to stabilise it (Jackson, 2011; Sixma & Van Den Berg, 1984). Red blood cells are trapped by the fibrin mesh (Weisel & Litvinov, 2017).

2.2.1.4 Coagulation control

The clot formation process is controlled by the action of procoagulants and anticoagulants to ensure only a reasonable extent of reactions is realised. Platelets should not keep on aggregating and fibrin should not keep on forming since it can lead to blockage of blood flow (Cesarman-Maus & Hajjar, 2005). Tissue factor pathway inhibitor (TFPI), protein C (PC), protein S (PS), antithrombin III (AT) and plasmin/plasminogen are the factors that help to control and terminate the coagulation process (Ajjan & Ariëns, 2009; Mosnier & Griffin, 2013).

TFPI is a polypeptide secreted by the endothelial cells to limit coagulation by binding to the TF/FVIIa complex in the initiation phase or the extrinsic pathway, and contains domains that inhibit FXa (Bouma & Mosnier, 2003). Another anticoagulant is PC, which is a vitamin K-dependent protease activated by thrombin (Dahlbäck & Villoutreix, 2005; Taylor Jr *et al.*, 2001). This is responsible for the inactivation of FV and FVIII of the intrinsic pathway or the amplification phase (Bagoly *et al.*, 2017; Cesarman-Maus & Hajjar, 2005; Dahlbäck & Villoutreix, 2005). The action of PC is reinforced by another vitamin K-dependent protein, PS, which acts as a cofactor of PC to ensure efficiency (Di Scipio *et al.*, 1977; Gierula & Ahnström, 2020). AT is another anticoagulant, which inhibits the activity of thrombin by binding to the thrombin's active site, thereby making it unavailable for action (Di Scipio *et al.*, 1977; Quinsey *et al.*, 2004). The AT also inhibits other coagulating factors, such as FXa, FIXa, FXIa and FXIIa, limiting their activities in coagulation (Quinsey *et al.*, 2004). The

action of AT is reinforced by an endothelial cell-produced glycosaminoglycan called heparin, which works under the influence of a cofactor called heparin cofactor II (Weisel & Litvinov, 2014).

2.2.2 Clot formation and fibrinolysis

After the aggregation of platelets at the site of injury, fibrin fibres have to form to stabilise the aggregate upon the generation and action of thrombin (White *et al.*, 2013). This fibrous protein, fibrin, is formed from a zymogen known as fibrinogen (Fuss *et al.*, 2001). Fibrinogen is a liver-synthesised glycoprotein that comprises three pairs of coiled polypeptide chains, namely $A\alpha$, $B\beta$ and γ that emerges from closely linked genes known as *FGA*, *FGB* and *FGG*, respectively (Weisel & Litvinov, 2013). The fibrinogen molecule has a mass of 340 kDa and a length of 45 nm, and consists of two outer D regions connected by a coiled-coil segment to form another region at the centre, known as the E region, linked together by 29 disulphide bonds (Huang *et al.*, 1993). Polypeptide chains are assembled and processed from their corresponding pre-pro-polypeptides in the endoplasmic reticulum (ER) and Golgi organelles of hepatic cells to produce final forms of $A\alpha$, $B\beta$ and γ chains with 610, 461 and 411 amino acids, respectively (Zhang & Redman, 1996). In the ER, progression happens from single chains to two-chain complexes to trimeric half molecules generated from the combination of $B\beta$ chains with $A\alpha$ - γ heterodimers or $A\alpha$ chains with $B\beta$ - γ dimers (Fuss *et al.*, 2001). The two are then joined at their N-termini to form a hexamer (a polymer formed from six molecules of a monomer) *via* the 29 disulphide bridges, assembling the two half-molecules (Henschen *et al.*, 1983) (Figure 2-2).

The E region is basically an interaction of N-terminal ends of the three pairs of chains from the two half-molecules, and the $A\alpha$ and $B\beta$ -chains terminals have fibrinopeptide ends designated as knob FPA and FPB (presented as the thick ends of the chains in Figure 2-2) respectively (Henschen *et al.*, 1983; Riedel *et al.*, 2011). The D region contains the carboxyl-terminal globular ends of the $B\beta$ and γ -chains, which forms a- and b-holes essential for intermolecular interactions (Fuss *et al.*, 2001; Riedel *et al.*, 2011).

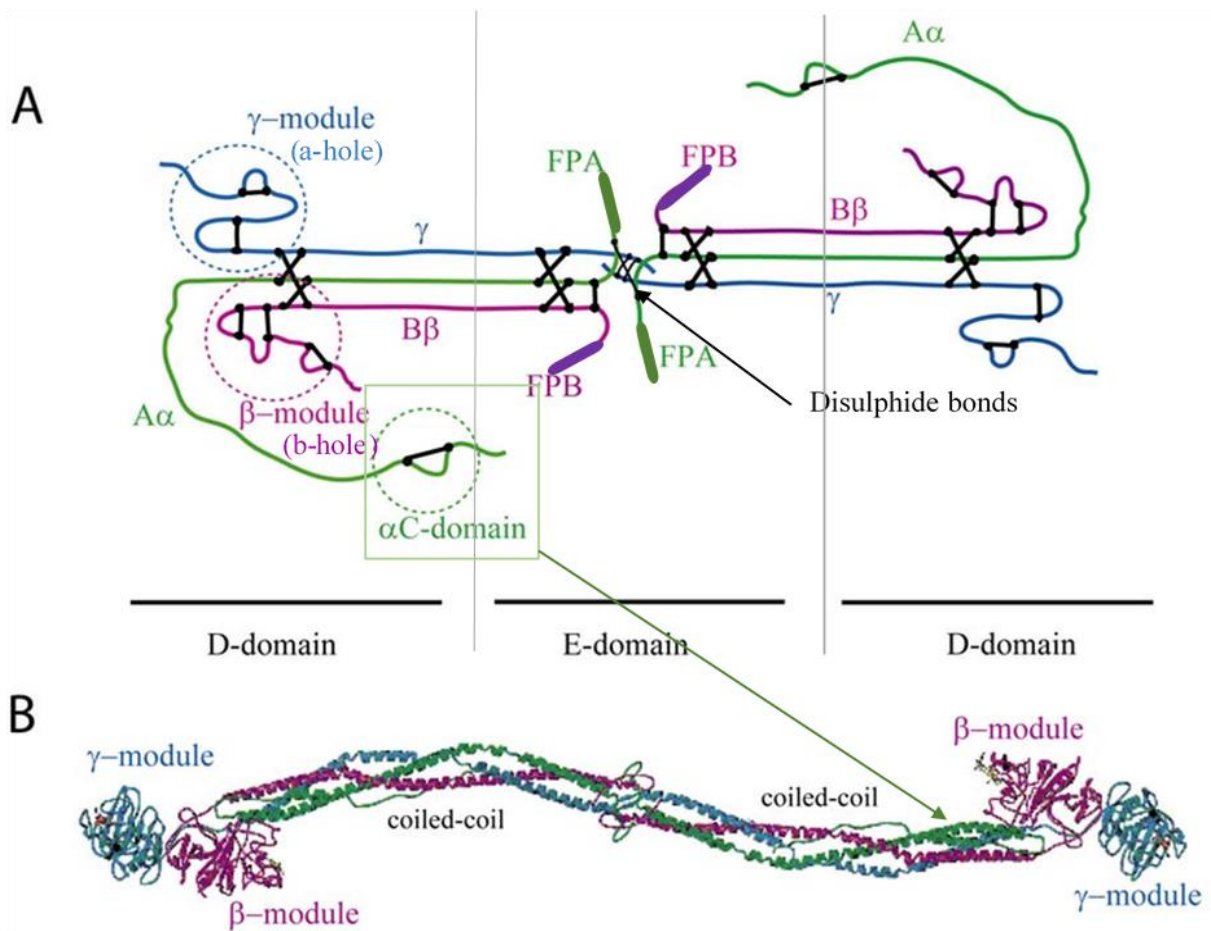


Figure 2-2: Fibrinogen molecule (A) showing all the chains and regions, while (B) shows the crystal structure of the molecule. The figure presents the A α -, B β and γ -chains in green, purple and blue, respectively (Adapted from Weisel & Litvinov, 2017; Yermolenko *et al.*, 2011).

The molecule is also comprised of the α C-region, which protrudes out as an extension from the molecule (Fuss *et al.*, 2001). As indicated in Figure 2-2, the α C-region arises from the carboxyl-end (C-terminal) of the α -chain that extends from the coiled-coil (Weisel & Litvinov, 2017; White *et al.*, 2013). The α C-region forms α C-polymers by interacting with each other, within the same or with the next molecule (Undas & Ariëns, 2011), and they are further cross-linked through the action of a plasma transglutaminase, also known as factor XIIIa, to strengthen the interaction (Weisel & Litvinov, 2017). The α C-domains interact through the N-terminal subdomains that

involve β -hairpin swapping, and the interaction of the C-terminal subdomain with α C-connector (Fuss *et al.*, 2001; Weisel, 2007). These interactions reinforce the lateral aggregation, as the protofibrils merge or interact (Fuss *et al.*, 2001; Weisel & Litvinov, 2017). The α C-regions are also important as a clot structural and mechanical determining factor, since the fibres made from fibrinogen molecules that lack the α C-regions are thinner and more tightly packed (Weisel, 2007; Weisel & Litvinov, 2017), which may also lead to thromboembolic problems as such clots are very resistant to lysis (Undas & Ariëns, 2011).

The generated thrombin targets the E region fibrinopeptides by hydrolysing the Arg-Gly bonds of the A α - and B β -chains to release the fibrinopeptides (A and B) from their respective chains (Riedel *et al.*, 2011; Weisel & Litvinov, 2013). The cleavage of the FPA results in new N-terminal sequences Gly-Pro-Arg (GPR) of the α -chains designated as knobs 'A' (Weisel & Litvinov, 2017). At this stage, fibrinogen is converted into fibrin monomers (Hoffman & Monroe *lii*, 2001; Weisel & Litvinov, 2013), and the interactions between fibrin monomers commences (Weisel & Litvinov, 2013). The β -chain also gets a new N-terminal sequence Gly-His-Arg-Pro (GHRP) making up knobs 'B' (Mosesson, 2005; Riedel *et al.*, 2011; Weisel & Litvinov, 2017). Knob A now inserts and fits into an a-hole binding pocket of the next fibrinogen molecule's D region, whilst knob B inserts and fits into a b-hole (Riedel *et al.*, 2011). These intermolecular interactions mark the beginning of fibrin fibre formation, as demonstrated in Figure 2-3.

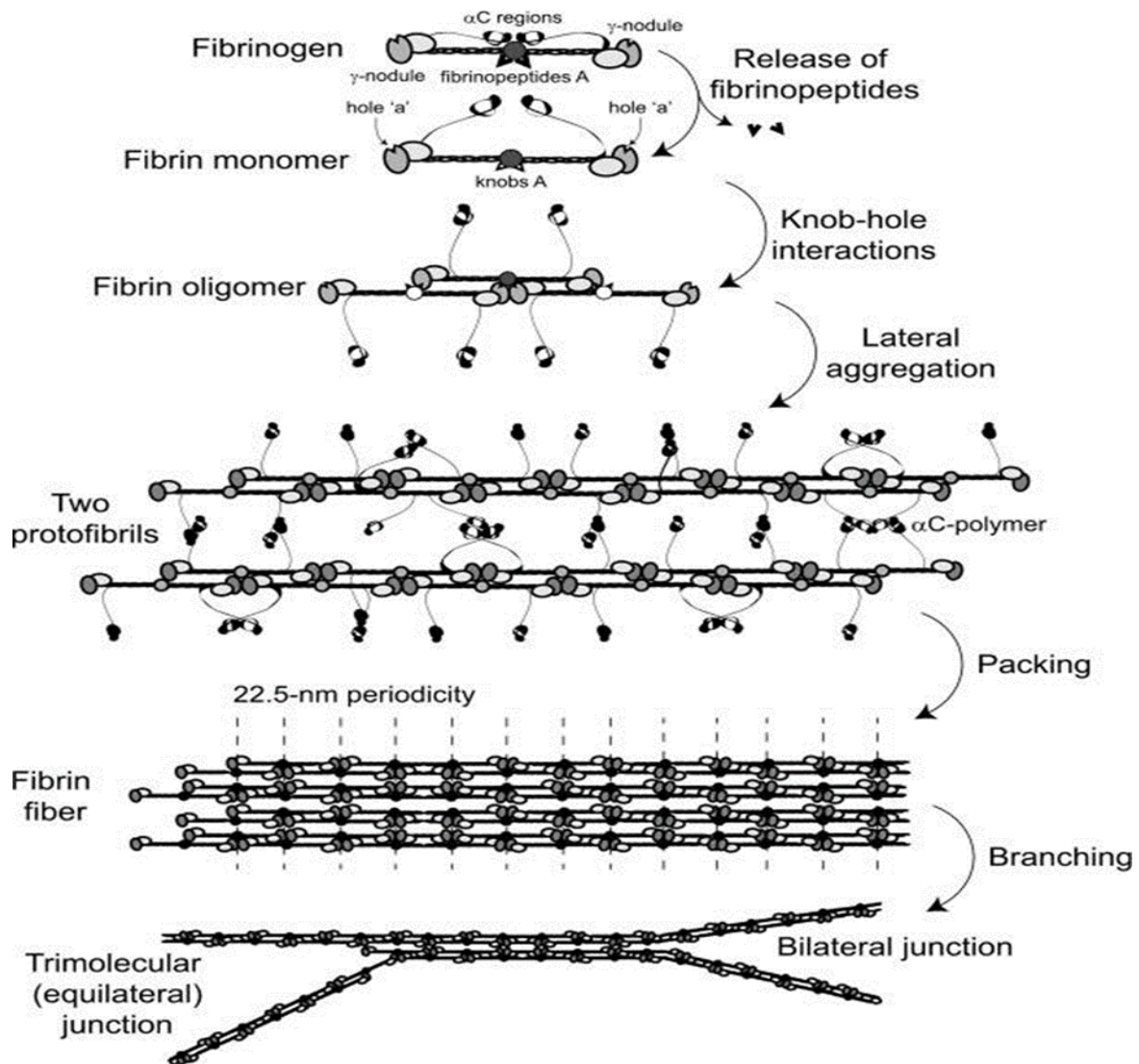


Figure 2-3: Fibrin polymerisation (Weisel & Litvinov, 2013)

Cleavage of the fibrinopeptides leads to the formation of two-stranded fibrin polymers known as protofibrils (Riedel *et al.*, 2011). Non-covalent bonding aligns the fibrin molecules in a domain overlapping pattern, and this is what keeps the protofibrils intact (Weisel & Litvinov, 2017). The protofibrils interact laterally and longitudinally to give rise to the fibres as presented in Figure 2-3 (Undas & Ariëns, 2011; Weisel & Litvinov, 2017). The mechanisms behind the inter-fibril lateral interactions are not yet fully established, but reportedly the knobs B-b-hole interactions, α C region interactions, the γ -chains c-terminals, adjacent β -nodules as well as the coiled-coils are involved in this growth of fibres in width (Weisel & Litvinov, 2017). The lateral interaction of the

protofibrils will stop after reaching a certain level where the energy required to stretch one protofibril to the next protofibril exceeds the energy of bonding (Mosesson, 2005; Weisel, 2007).

As the fibres grow in length, they also branch out of the longitudinal alignment, as presented in Figure 2-3, to form different types of branches (Fuss *et al.*, 2001). Bilateral and trimolecular or equilateral junctions are the approaches through which the fibrin fibres form their branches or junctions (Mosesson *et al.*, 1987; Weisel, 2007). The bilateral junction emerges from two protofibrils that create a divergent from the alignment due to an incomplete lateral association (Fuss *et al.*, 2001; Shainoff & Dardik, 1979), which causes them to diverge from each other, as shown in Figure 2-3 (Fuss *et al.*, 2001; Weisel & Litvinov, 2017). The equilateral junctions arise from a fibril that longitudinally interacts with one fibrin monomer of the main fibre via one knob A and one a-hole interaction, which makes the protofibrils bend away from the main fibre, as presented in Figure 2-3 (Weisel, 2007). The branching is what causes the final clot to have a three-dimensional network structure (Shainoff & Dardik, 1979; Weisel, 2007; Weisel & Litvinov, 2017).

The network is further stabilised by the action of factor XIIIa (FXIII) (Mosesson, 2005). FXIII is a transglutaminase, which comprises two catalytic A-subunits and two carrier B-subunits (Muszbek *et al.*, 2011), which naturally circulates in the blood (Muszbek *et al.*, 2011; Weisel, 2007). FXIII is activated by thrombin, which slices off a 37-residue activation peptide of the A-subunit (Mosesson, 2005). This weakens the A and B subunit interaction resulting in a calcium-mediated A subunit dissociation from the B subunits, as well as a conformational change resulting in the formation of the activated FXIII (FXIIIa) (Mosesson, 2005; Muszbek *et al.*, 2011; Weisel, 2007). FXIIIa produces covalent cross-links (γ -glutamyl- ϵ -lysine bonds) between fibrin α and γ -chain protofibrils that stabilises the fibrin network and protects it from untimely lysis (Undas & Ariëns, 2011; Weisel, 2007).

Fibrin diameter, pore size and nature of branching are all structural properties that determines the general characteristics of the clot. Fibrin structure is critical in both bleeding and thrombotic events (Bagoly *et al.*, 2017). Fibrinogen concentration is one

of the most important determinants of the final fibrin clot structure (Weisel & Litvinov, 2017); fibrin fibres made from a high fibrinogen concentration are more tightly packed and formed at a faster rate leading to formation of clots that are very resistant to lysis, since permeability of fibrinolytic factors through the clot is reduced, therefore posing a thrombotic risk (Bagoly *et al.*, 2017; Undas, 2017). Thrombin generation is another important factor determining the final fibrin clot structure (Undas, 2017). If thrombin is generated in small amounts and at a slow pace, the fibrin fibres become thicker and more loosely packed, whilst those formed by large amounts of thrombin, become thinner and more tightly packed forming a structure that is more resistant to mechanical stress and enzymatic lysis (Monroe & Hoffman, 2006; Undas, 2017).

Once a clot has formed and the vessel wound has finally healed, the system calls for a mechanism that will dissolve the clot to restore normal blood flow (Bagoly *et al.*, 2017; Weisel & Litvinov, 2014; Weisel & Litvinov, 2017). This process is known as fibrinolysis, defined as the process of degrading fibrin (Cesarman-Maus & Hajjar, 2005). The coagulation and fibrinolytic systems are counter-balanced to realise optimal formation of clots, since their tightly controlled, combined presence is necessary to achieve effective haemostasis (Cesarman-Maus & Hajjar, 2005; Weisel & Litvinov, 2017). The mechanism of fibrinolysis is accomplished by the action of a protease known as plasmin, which is formed through the activation of a liver-synthesised plasma zymogen known as plasminogen (Cesarman-Maus & Hajjar, 2005). The activation is through the action of plasminogen activators, tissue-type plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA) in the presence of fibrin (Cesarman-Maus & Hajjar, 2005; Weisel & Litvinov, 2017). The t-PA, the primary enzyme of the process in blood (Yakovlev & Makogonenko, 2000), is an endothelial-derived plasma serine protease that activates the fibrin-bound plasminogen (Chapin & Hajjar, 2015; Weisel & Litvinov, 2017). Fibrinogen has plasminogen- and t-PA-binding sites situated on each and every α C-domain (Weisel & Litvinov, 2017). As plasmin cleaves the fibrin structure, it exposes additional lysine-binding sites, which accelerates the generation of plasminogen, making it available for further degradation of fibrin (Weisel, 2007; Yakovlev & Makogonenko, 2000). The t-PA activates plasminogen by splitting the Arg561-Val 562 peptide bond of the zymogen, thereby forming plasmin (Fuss *et al.*, 2001; Weisel & Litvinov, 2017).

Plasmin cleaves both fibrinogen and fibrin into several soluble degradation products (FDPs) by binding to C-terminal lysine residues and cutting the fibrin fibres transversely (Yakovlev & Makogonenko, 2000). The degradation products of fibrinolysis include terminal fragments D and E, intermediate fragments X and Y, as well as the smallest degradation products known as D-dimers, as presented in Figure 2-4 (Weisel & Litvinov, 2013; Yakovlev & Makogonenko, 2000). The D-dimers are the smallest end-products of fibrinolysis (Yakovlev & Makogonenko, 2000), which are primarily two D-regions of adjacent fibrin molecules cross-linked together through the action of FXIIIa via the γ - γ bond. (Chapin & Hajjar, 2015; Weisel & Litvinov, 2017). The D-dimers are carried into the liver where they are further degraded (Cesarman-Maus & Hajjar, 2005).

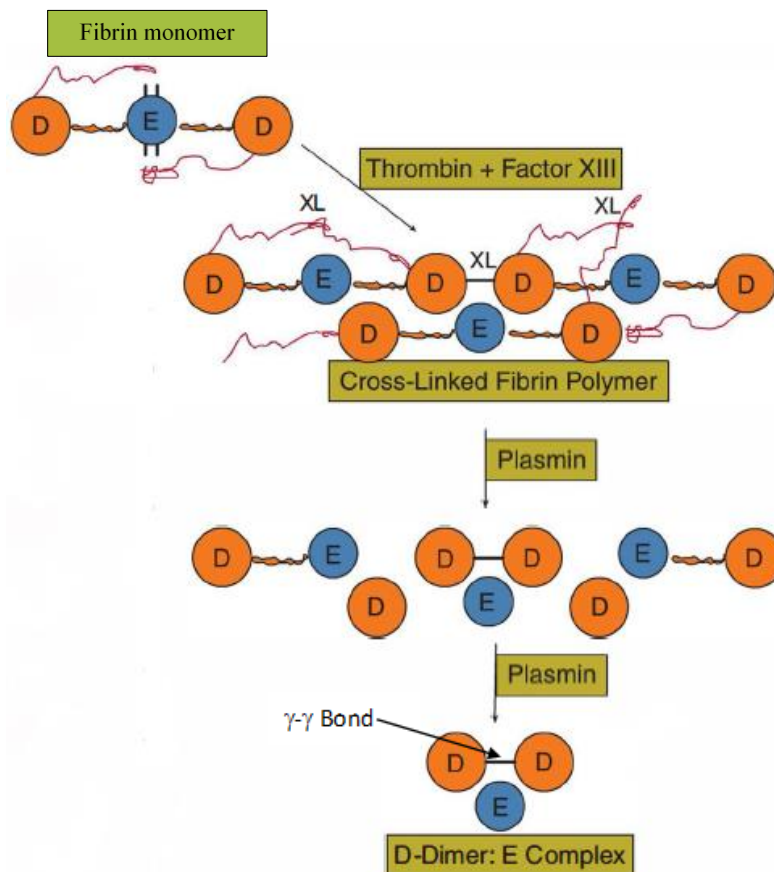


Figure 2-4: Action of fibrinolysis, adapted from Marder *et al.* (2012).

The fibrinolytic process is controlled by a number of inhibitors' different levels in order to prevent uncontrolled bleeding (Marder *et al.*, 2012), for example through the inhibition of plasminogen activation and/or inhibiting the action of plasmin (Bagoly *et al.*, 2017; Messmore, 2006). This is accomplished through the action of plasminogen activator inhibitor-1 (PAI-1), a potent inhibitor of t-PA and u-PA, α 2-antiplasmin, which binds and inhibits plasmin, and also through the action of thrombin activatable fibrinolysis inhibitor (TAFI) (Weisel & Litvinov, 2014). TAFIs suppresses fibrin lysis by removing terminal lysine residues from the fibrin (Monroe & Hoffman, 2006). These lysine residues are the plasminogen and t-PA binding sites necessary for the commencement of fibrinolysis, therefore TAFI's action suppresses the whole process (Hoffman & Monroe III, 2001; Weisel & Litvinov, 2014).

2.3 Cardiovascular complications

As mentioned in the previous sections, abnormal blood clot formation and structure leads to thrombotic and cardiovascular complications. In some cases, a blood clot formed inside a blood vessel, can occlude the vessel, which blocks or minimises blood passage to downstream organs; this is referred to as thrombosis (Kyrle & Eichinger, 2005; Mosnier & Griffin, 2013). In some situations, part of a clot may break off from the point of clotting and move with the blood to different branches of the vessel system where they can obstruct smaller vessels, again interrupting blood flow; this is called an embolus (Kyrle & Eichinger, 2005; Ząbczyk & Undas, 2017). Thrombotic disorders can be grouped into two groups, according to the vascular bed it occurs in, venous thrombosis (VT) and arterial thrombosis (Anderson Jr & Spencer, 2003).

- Venous thrombosis (VT)

This is a cardiovascular problem characterised by the formation of clots in veins, and grouped into deep vein thrombosis (DVT), which is the most common type, and pulmonary embolism (PE) (López & Chen, 2009; Mosnier & Griffin, 2013; Raskob *et al.*, 2014).

Deep vein thrombosis is a type of VT in which a clot forms in deep veins, mostly in the legs, and is generally characterised by swelling and pain at the point of

occurrence (Mosnier & Griffin, 2013; Raskob *et al.*, 2014). DVT may result from damage of the vessel lining, which will trigger the coagulation process, and it may also commence due to unbalanced or impaired levels of procoagulants and anticoagulants (López *et al.*, 2004). Pulmonary embolism can be a consequence of DVT, since it occurs when an embolus breaks off from a DVT clot and travels to another site such as the lungs, blocking its blood supply (Carson *et al.*, 1992; Furie & Furie, 2008; Tapson, 2008).

- Arterial thrombosis

Arterial thrombosis is a condition where a clot obstructs or blocks an artery. Arterial thrombosis results from arterial conditions such as atherosclerosis and may result in serious health conditions, such as myocardial infarction, peripheral artery disease, coronary artery disease (CAD), carotid stroke, renal artery occlusion or paradoxical embolism (Furie & Furie, 2008; Gawaz *et al.*, 2005).

Atherosclerosis is the accumulation of cholesterol in the sub-endothelial layer of a vessel wall to form a fatty plaque (Weber & Noels, 2011). The whole process results when the endothelial lining of a blood vessel is damaged due to factors such as hyperlipidaemia, hypertension and/or different infections (Gawaz *et al.*, 2005; Moore & Tabas, 2011; Sun *et al.*, 2015). The endothelial layer can be damaged by any of the above-mentioned factors, leading to the accumulation or aggregating and oxidation of LDL cholesterol at the site of damage to form what is known as a fatty streak (Moore & Tabas, 2011). The oxidation triggers the immune system through the recruitment of monocytes (macrophages), which attack and digest the oxidised cholesterol (Swirski *et al.*, 2007; Weisel, 2007). The macrophages will attack the cholesterol to the point where they become full and die in the process, this signals other monocytes and the accumulation continues, forming foam cells (Moore & Tabas, 2011; Weber & Noels, 2011). The accumulation also triggers the migration of smooth muscle cells of the vessel wall into the fatty aggregate to cover up the surface by creating a fibrous cap mainly made from collagen and elastin (Kearns *et al.*, 2017). The fibrous cap is there to prevent the contents of the plaque from

entering the blood stream (Ylä-Herttuala *et al.*, 2011); however, the fatty plaque narrows the blood vessel and interferes with the blood flow (Hemmat *et al.*, 2018; Weber & Noels, 2011).

In some cases, the fibrous cap may burst due to shear stress from the blood flowing through the reduced lumen, and this will trigger the coagulation factors to form a clot in an attempt to seal the site of injury (Ylä-Herttuala *et al.*, 2011; Ząbczyk & Undas, 2017). This can further narrow the vessel, worsening the situation or even leading to complete vessel occlusion (Kearns *et al.*, 2017; Weber & Noels, 2011). Part of the clot may also break off to form an embolus, which may travel and block vessels downstream (Bagoly *et al.*, 2017; Ząbczyk & Undas, 2017).

The next sections will focus on altered clotting and cardiovascular complications associated with HIV and the use of antiretroviral drugs.

2.4 Human immunodeficiency virus (HIV) pathophysiology

The human immunodeficiency virus (HIV) is a retrovirus that attacks and depletes cells of the immune system (De Cock, 2017; Gougeon, 2003). HIV transmission is through the exchange of fluids, such as blood, semen, vaginal fluid and breast milk, from an infected individual to another. HIV attacks the CD4⁺ T cells, where it enters and replicates itself to affect more cells (Friedrich *et al.*, 2011; Redfield & Burke, 1988). It has an outer envelope with embedded glycoproteins, and an inner part that comprises two viral RNA strands. It contains several enzymes used during the different stages of its life cycle (Watts *et al.*, 2009).

The virus has an envelope surface protein known as gp120 that binds to CD4 receptors and chemokine co-receptors (CCR5) of the host T-cells, to commence membrane fusion and the entry of the viral genetic material, as illustrated in Figure 2-5 (Redfield & Burke, 1988; Watts *et al.*, 2009). Only the genetic inner part of the virus, coated with matrix and capsid proteins, is inserted into the host cell, while the envelope proteins are left on the cell surface (Smith & Helenius, 2004). The matrix and capsid

proteins are dissolved releasing two strands of viral RNA and enzymes (Redfield & Burke, 1988). A viral enzyme, known as reverse transcriptase, assembles the naturally occurring host cell nucleotides to make a double-stranded viral DNA from the single-stranded RNA (Smith & Helenius, 2004; Watts *et al.*, 2009). Once two double-stranded DNAs form, another viral enzyme, known as integrase, inserts the DNA into the host cell nucleus where it incorporates the viral DNA (referred to as a provirus) into the host chromosomes (Redfield & Burke, 1988; Watts *et al.*, 2009). Next, a host cell enzyme, known as RNA polymerase, interacts with the DNA to make the HIV genomic material and messenger RNA (mRNA) that end up interacting with ribosomes of the cell ER to make viral proteins (Redfield & Burke, 1988; Smith & Helenius, 2004). The viral proteins and RNA assemble through the action of viral enzymes known as proteases, which then move out of the host cell; this is known as budding (Sundquist & Kräusslich, 2012; Von Schwedler *et al.*, 2003). As they bud out of the cell, host cell membrane coats the assembly to form an envelope embedded with glycoproteins (Huang *et al.*, 2012; Sundquist & Kräusslich, 2012). As the HIV life cycle continues, the T-cells are damaged in the process, leading to a weakened immune system and onset of different opportunistic diseases that eventually progress to what is known as acquired immunodeficiency syndrome (AIDS) (Huang *et al.*, 2012; Smith & Helenius, 2004).

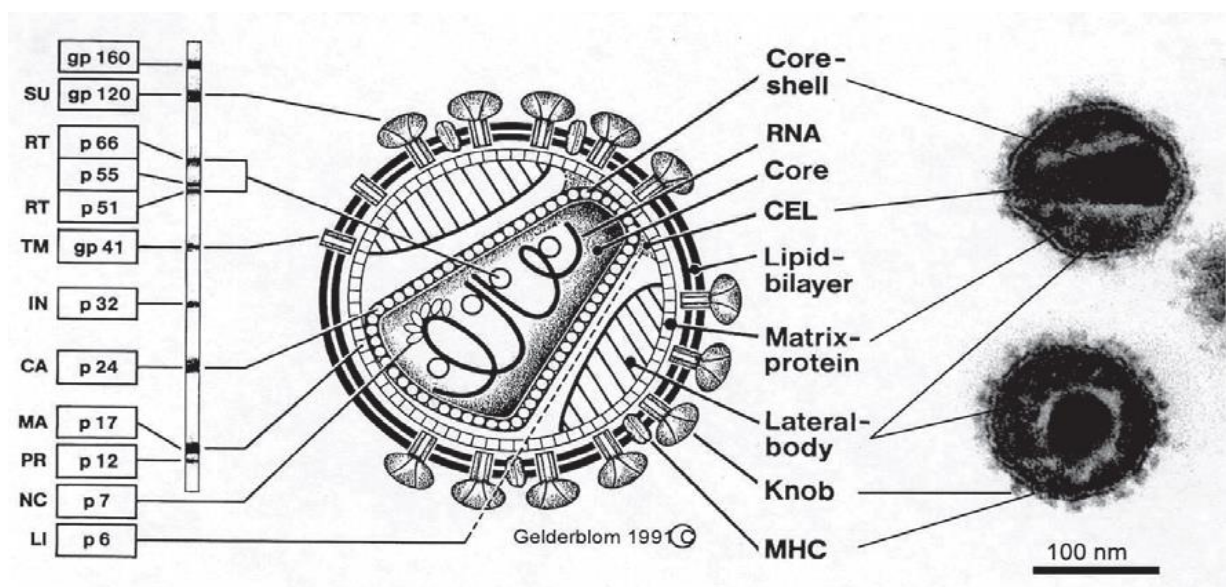


Figure 2-5: The structure of HIV (Karger, 2016)

The progression of the disease can be grouped into stages, according to either the CD4 cell count or the signs/symptoms present in an infected individual (WHO, 2007). Based on the CD4+ cell count, the WHO (2007) has classified HIV progression as detailed in Table 2-1.

Table 2-1: WHO CD4+ count-based HIV/AIDS progression

HIV immunodeficiency classification	CD4+ cell count (per mm³)
Non-significant	≥500
Mild	Between 350 to 499
Advanced	Between 200 to 349
Severe	<200

The WHO (2007) has also grouped the progression, based on the onset of specific clinical complications, as outlined in Table 2-2.

Table 2-2: WHO clinical staging of HIV/AIDS

Clinical stages	Signs and symptoms
Primary HIV infection	• Asymptomatic • Acute retroviral syndrome
Stage 1	• Asymptomatic with/without persistent generalised lymphadenopathy
Stage 2	• Recurrent respiratory tract infections (RTIs, sinusitis, bronchitis, otitis media, pharyngitis) • <10% weight loss • Herpes zoster • Recurrent oral ulceration • Angular cheilitis • Papular pruritic eruptions • Fungal nail infections • Seborrheic dermatitis

Stage 3	• > 10% weight loss • More than a month's duration of unknown cause of chronic diarrhoea • Persistent fever of unknown cause (> once / month) • Oral hairy leucoplakia • Persistent oral candidiasis • Pulmonary tuberculosis • Acute necrotising ulcerative stomatitis, gingivitis, or periodontitis • Severe bacterial infections • Unexplained anaemia, thrombocytopenia, or neutropenia
Stage 4	• Pneumonia • HIV-related wasting syndrome • Severe recurrent bacterial pneumonia • Infection with chronic herpes simplex > 1 month • Oesophageal or airway candida infection • Kaposi's sarcoma • Extra-pulmonary tuberculosis • Cytomegalovirus infection • HIV encephalopathy • Toxoplasmosis of the central nervous system • Extra-pulmonary Cryptococcus infection • Progressive multifocal leukoencephalopathy • Disseminated non-tuberculous mycobacterial infection • Chronic cryptosporidiosis (with diarrhoea) • Disseminated mycosis (coccidiomycosis or histoplasmosis) • Chronic isosporiasis • Recurrent non-typhoidal Salmonella bacteraemia • Lymphoma or other solid HIV-associated tumours • Atypical disseminated leishmaniasis • Invasive cervical carcinoma • HIV-associated nephropathy or symptomatic cardiomyopathy

2.5 Antiretroviral drugs

Highly active ART drugs were first introduced around 1995, and since then they have significantly reduced the levels of HIV-related opportunistic infections and deaths among these patients, and led to an increased quality of life (Alshahrani *et al.*, 2017). However, accessibility has been a problem especially in low- and middle-income countries such as the sub-Saharan Africa countries, South Africa included (Karim & Karim, 2002).

The drugs exist in different classes, categorised based on their specific function or approach in minimising viral invasion and immune suppression (Alshahrani *et al.*, 2017). Different types of drugs are used in combinations in order to realise maximum minimisation of viral invasion and general disease progression (Jackson & Pretorius, 2019), as discussed below.

2.5.1 Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs)

The NRTIs are the first class of ART that primarily reduce viral replication by inhibiting the action of reverse transcriptase enzymes aimed at converting the viral RNA into a double stranded DNA. NRTIs mimic the natural nucleosides required in making up the DNA, and terminates the DNA synthesis once incorporated into the nucleoside chain (Cihlar & Ray, 2010). Listed below are the currently available Food and Drug Administration (FDA)-approved NRTIs for HIV treatment; the drugs with a star are those presently used in South Africa.

- Zidovudine *
- Didanosine *
- Stavudine *
- Lamivudine *
- Abacavir *
- Didanosine EC [enteric coated]
- Emtricitabine *
- Tenofovir *

2.5.2 Nonnucleoside reverse transcriptase inhibitors (NNRTIs)

NNRTIs is another class of drugs that compromises the action of reverse transcriptase enzymes by blocking reverse transcriptase's viral RNA binding site, which prevents the commencement of reverse transcription (Merluzzi *et al.*, 1990; Spence *et al.*, 1995). Both NRTIs and NNRTI aim to inhibit the process of reverse transcription, but they differ in their approach. NRTIs inhibit full transcription of RNA (to DNA), while NNRTIs prevent the transcription from commencing by direct interaction with the enzymes (Cihlar & Ray, 2010; Merluzzi *et al.*, 1990). There are several drugs in this class available on the market:

- Nevirapine *

- Delavirdine
- Efavirenz *
- Etravirine *
- Rilpivirine

2.5.3 Protease inhibitors (PIs)

This class of drugs minimises viral replication by binding to viral proteases that are responsible for synthesis of viral particles and general maturation of the virus (Flexner, 1998; Koudriakova *et al.*, 1998). Listed below are the approved available PIs:

- Tipranavir
- Atazanavir *
- Indinavir *
- Darunavir *
- Fosamprenavir
- Saquinavir *
- Lopinavir/Ritonavir *
- Nelfinavir
- Ritonavir *
- Trapanavir

2.5.4 Integrase strand transfer inhibitors (INSTIs) (integrase inhibitors)

The INSTIs interfere with viral integration by blocking integrase enzymes as the virus tries to insert its DNA into the host CD4-cell DNA during replication (De Luca *et al.*, 2011). The following are available INSTI drugs:

- Raltegravir *
- Dolutegravir
- Elvitegravir

2.5.5 Entry inhibitors (fusion inhibitors)

Another class of drugs that block receptors that aid host cell viral entry. Maraviroc and Enfuvirtide are the available drugs in this category (Alshahrani *et al.*, 2017; Briz *et al.*, 2006).

2.5.6 ART in South Africa

Several factors affect the availability and usage of ART, such as affordability, accessibility and suitability. Some drugs have been stopped due to their side effects, and some are not available because they are not affordable (Karim & Karim, 2002). South Africa has one of the largest ART programmes in the world, covering a large population of infected individuals (Jackson & Pretorius, 2019). Table 2-3 lists the drugs available in SA.

Table 2-3: ART drugs available and used in South Africa

ART Class	Available Drugs
Nucleoside/nucleotide reverse transcriptase inhibitors	○ Zidovudine, Lamivudine, Didanosine, Abacavir, Stavudine, Tenofovir and Emtricitabine
Non-nucleoside reverse transcriptase inhibitors	○ Efavirenz, Nevirapine and Etravirine
Protease inhibitors	○ Atazanavir, Indinavir, Lopinavir/ Ritonavir, Saquinavir and Darunavir
Integrase strand transfer inhibitors	○ Raltegravir

For individuals who cannot afford to purchase ART's, the South African government has made a select number of these drugs available, free of charge, at community clinics. The South African North West arm of the Prospective Urban and Rural Epidemiology (PURE-SA-NW) study included patients who used the ART regimens available at the clinics, which consisted of a combination of two nucleoside reverse transcriptase inhibitors (Stavudine and Lamivudine) and one non-nucleoside reverse transcriptase inhibitor, either Efavirenz or Nevirapine (Botha *et al.*, 2014; Fourie *et al.*, 2015; Teo *et al.*, 2009).

2.6 Mechanisms through which HIV and ART contribute to cardiovascular complications

Cardiovascular complications are reportedly ten times more prevalent in HIV-infected patients than in the general population (Jacobson *et al.*, 2004). Although the introduction of ART has significantly improved the quality of life of HIV patients (Pastori *et al.*, 2015), studies have shown that ARTs themselves are associated with cardiovascular complications such as atherosclerosis, myocardial infarction, peripheral artery disease, ischemic stroke and other metabolic syndromes, which include dyslipidaemia, lipodystrophy and insulin resistance (Kaplan-Lewis *et al.*, 2016); different drug combinations or regimens have also shown to be contributing more than others (Aberg, 2009). This section will provide insight on the causes and possible mechanisms through which the disease itself, as well as the antiretroviral drugs, contribute to cardiovascular disease development in HIV.

2.6.1 HIV-related CVD complications

Atherosclerotic cardiovascular complications are reportedly high in individuals infected with HIV (Barnes *et al.*, 2017). Immune suppression by HIV, through the attacking of the CD4 T-cells and also the increase in viral load, as the virus replicates, have led to the onset of inflammation and immune cell activation, leading to endothelial cell damage (Brenchley *et al.*, 2006). All of these are underlying causes for atherosclerotic development, which result in cardiovascular complications (Sun *et al.*, 2015).

Altered levels of measures of atherosclerosis and arteriosclerosis, respectively, that predict disease progression and severity, such as carotid intima-media thickness (IMT), and flow-mediated vasodilation (FMD) and pulse wave velocity (PWV), have been reported in HIV patients (Sun *et al.*, 2015). The inflammatory cytokine, known as tumour necrosis factor alpha (TNF α), secreted in response to a viral attack, such as HIV, contributes to atherosclerotic conditions by influencing leukocyte attachment and transmigration into the vascular wall through inducing expression of cell adhesion molecules (E-selectin, vascular cell adhesion molecule 1 (VCAM-1) and intercellular cell adhesion molecule 1 (ICAM-1), increasing intima-media thickness and the risk of atherosclerosis (Kleinbongard *et al.*, 2010; Sun *et al.*, 2015). TNF α also induces migration, proliferation and apoptosis of vascular smooth muscle cells (SMC), resulting

in vascular damage (Kleinbongard *et al.*, 2010). The HIV surface protein, gp120, also contributes to atherosclerotic complications as it induces SMC activation and changes in its physiology through activation of signalling pathways that damage the SMC physiology (Schechter *et al.*, 2001).

Dyslipidaemia is another cardiovascular risk factor that emerges in HIV infection even before introducing anti-retroviral treatment to the patient (So-Armah & Freiberg, 2018). In the literature, dyslipidaemia, pertaining to HIV, was reported as increased levels of triglycerides and very low density lipoprotein cholesterol (VLDL-C), and decreased levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) (El-Sadr *et al.*, 2005; Liang *et al.*, 2001). The HIV accessory protein, nef, and the adenosine-triphosphate binding cassette transporter A1 (ABCA 1) interaction has been reported to contribute to these abnormal lipid profiles by compromising the removal or transporting of cholesterol from the vessel wall (Mujawar *et al.*, 2006; Non *et al.*, 2017). The HIV's nef protein complicates the normal mechanism by impairing the stimulation and redistribution of ABCA1, which affects the clearing of excess cholesterol to the liver, leading to foam cell formation as it gets engulfed by the macrophages (Mujawar *et al.*, 2006; Non *et al.*, 2017).

Microbial translocation in HIV-infected patients, is another contributing mechanism that drives the initiation and onset of inflammation (Brenchley *et al.*, 2006). HIV targets the CD4 lymphocytes of the immune system for its replication in the human body, and about 80% of these CD4 cells are in the gut mucosa, which functions as a barrier and a control for vascular system entry (Brenchley *et al.*, 2006; Dillon *et al.*, 2014). The loss of CD4 cells, as HIV replication continues, impairs the protective role of the gut mucosa, which leads to microbial translocation into the blood stream (Dillon *et al.*, 2014). This situation alerts or stimulates the immune system, as a protective measure against the microbial invasion, and some endotoxins, such as lipopolysaccharides (LPS), are produced in the blood resulting in the initialisation of inflammation (Brenchley *et al.*, 2006). LPS also contribute to insulin resistance as it circulates through the bloodstream (Pedro *et al.*, 2018). Insulin is a peptide hormone responsible for the balancing of blood sugar levels, therefore if its efficacy is altered, the risk of developing diabetes mellitus (DM) is increased (Noubissi *et al.*, 2018). Circulating LPS

stimulates the release of cytokines and activates toll-like receptor 4 (TLR4) that induces the activation of serine kinases of the liver, muscle and adipose tissue, triggering the serine phosphorylation of insulin receptors, which impairs insulin signalling (Pedro *et al.*, 2018). LPS may also result in endoplasmic reticulum (ER) stress, mitochondrial dysfunction and activation of other cytokines (Brenchley *et al.*, 2006; Diao *et al.*, 2014). It has also been reported that LPS initiates tissue factor production on monocytes, which in turn results in the activation of the coagulation process, increasing the risk of thrombotic events (Funderburg *et al.*, 2010).

HIV has shown to be associated with hypercoagulation, which poses a risk for thrombotic events. The impaired coagulation reported in HIV patients is considered to relate to the accompanying chronic inflammation (Jackson & Pretorius, 2019; Zaaqog *et al.*, 2015). In response to microbial invasion or HIV and viral proteins, the release of cytokines such as TNF- α and Interleukin-6 (IL-6) induce TF expression that can trigger coagulation via the extrinsic pathway (O'Brien, 2012). It has also been reported that in *in vitro* studies, activation of arterial smooth muscle cells by the HIV membrane glycoprotein, gp20, leads to TF expression (Jacobson *et al.*, 2004). The released TNF- α enhances the release of the fibrinolytic inhibitor, PAI-1 by the endothelial cells, and also downregulates other anticoagulants such as protein C, inhibiting the mechanism of fibrin lysis (Jacobson *et al.*, 2004); this may lead to thrombotic events since the rate at which clots are degraded becomes limited (Jackson & Pretorius, 2019; Jacobson *et al.*, 2004). Antiphospholipid syndrome (APS) is one of the contributors of increased blood clot formation in HIV, as HIV infection has been associated with elevated levels of antiphospholipid antibodies (Jacobson *et al.*, 2004). APS can lead to hypercoagulation by enhancing protein S resistance, which limits fibrinolysis (Hassoun *et al.*, 2004). Platelet activation is another pathway through which hypercoagulation presents in HIV (Khare *et al.*, 2018). Reportedly, platelets can attach to and carry around the HI-virus to different parts in the body, and this endocytosis event leads to the activation of the platelets and the release of P-selectin from their alpha granules (Chabert *et al.*, 2015). The platelet activation results in triggering of more platelet activation and aggregation, which may lead to thrombosis initiation.

Endothelial dysfunction is another CVD risk factor demonstrated in HIV patients. The onset of increased carotid intima-media thickness in patients has captured attention

since it is correlated with atherosclerotic and cardiovascular complications (De Saint Martin *et al.*, 2007). Endothelial cells have essential properties in the vascular system, such as the secretion of coagulation factors and acting as a barrier for the vascular system (Kearns *et al.*, 2017). It has been reported that virus entry into the endothelial cells may occur via CD4 antigen and galactosyl-ceramide receptors, which can lead to endothelial cell proliferation and even apoptosis, which may create room for an atherosclerotic event to initiate (Barbaro *et al.*, 2001). Since HIV requires CD4 cell receptors and co-receptors to interact with a host cell, the gp120 of its coat has shown to have an effect on non-CD4 cells by prolonging the MAP kinase pathway; it has also been seen to be contributing to mitochondrial damage, which may lead to apoptosis of the endothelial cells (Himanshu *et al.*, 2013).

The introduction of ART drugs has helped to alleviate some of the complications described above to improve the health status of the infected individuals (Samji *et al.*, 2013). However, some of these drugs are also associated with other metabolic complications that relate to cardiovascular health (De Gaetano Donati *et al.*, 2010). Some of the mechanisms through which certain ART drugs are associated with increased risk for cardiovascular complications, including hypercoagulation, are explained in the following section.

2.6.2 ART-related complications

ART, like any other medication, is associated with side effects that places an unwanted burden on the individuals using it. Cardiovascular risk factors and complications have been associated with the use of certain drug regimens, especially those containing protease inhibitors and some with nucleoside reverse transcriptase inhibitors in their combinations (Himanshu *et al.*, 2013). Discussed below are the mechanisms through which these two classes of drugs influence cardiovascular complications among HIV patients.

2.6.2.1 Nucleoside reverse transcriptase inhibitor (NRTI)

The NRTI operates in a way that inhibits the interaction of the viral DNA and the cell nucleosides through competitive binding (Himanshu *et al.*, 2013). Since NRTI resembles the real nucleosides with a few exceptions, it also affects several cell

mechanisms that involve nucleosides, since they utilise natural nucleoside pathways, which result in competing actions and eventually complications (Himanshu *et al.*, 2013). An example of such is mitochondrial dysfunction (Selvaraj *et al.*, 2014). NRTIs work through inhibiting an enzyme known as DNA polymerase gamma (Pol- γ), which results in reduced replication of mitochondrial DNA (mtDNA), and eventually mitochondrial damage, impairing the mitochondrial respiratory chain and consequently reducing cell energy (Selvaraj *et al.*, 2014; Sun *et al.*, 2015). The NRTIs also inhibit nucleoside transporters and kinases involved in phosphorylation that aid in mtDNA replication (Himanshu *et al.*, 2013).

Some studies have illustrated the occurrence of mitochondrial dysfunction during zidovudine monotherapy in selected patients to result in induced cardiomyopathy (Brinkman *et al.*, 1999; Himanshu *et al.*, 2013). A study by Dalakas *et al.* (1990) reported abnormal mitochondria with paracrystalline inclusions in the biopsy specimens of zidovudine-treated HIV patients (Brinkman *et al.*, 1999; Dalakas *et al.*, 1990). This was also positively associated with myopathy in these patients (Dalakas *et al.*, 1990). Similar findings were presented by Arnaudo *et al.* (1991), who reported severely reduced amounts (up to 78% reduction as compared to the normal controls) of mtDNA in muscle biopsy of zidovudine-treated patients (Arnaudo *et al.*, 1991).

2.6.2.2 Protease inhibitors (PI)

The PIs have been seen to have an impact on CVD risk in HIV patients through the development of dyslipidaemia (Henry & Melroe, 1998). Abnormal lipid profiles present as increased levels of plasma cholesterol, triglycerides (TG) and apolipoprotein B (Kaplan-Lewis *et al.*, 2016). PI-induced dyslipidaemia is considered the basis for the increased prevalence of atherosclerotic events among PI-treated HIV patients (Henry & Melroe, 1998; Himanshu *et al.*, 2013).

Studies have shown that PIs tend to inhibit the expression of sterol-regulatory element-binding protein (SREBP-1c) that regulates the enzymes responsible for the synthesis and uptake of cholesterol, fatty acids and LDL-C, clearance of TG and cholesterol-rich lipoproteins, and adipocyte differentiation (Goedeke & Fernández-Hernando, 2012; Non *et al.*, 2017). Impairment in the mechanisms of SREBP-1c can lead to

uncontrolled lipid profiles, which is a risk factor for cardiovascular complications (Non *et al.*, 2017). PIs reportedly have an effect on the expression of lipogenic genes of the liver cells, which enhances mechanisms that lead to the production of TGs and cholesterol (Henry & Melroe, 1998; Himanshu *et al.*, 2013).

PIs have also been reported to impair lipid metabolism through their interaction with cellular retinoic acid-binding protein 1 (CRABP-1) (Carr *et al.*, 1998; Waterlow, 1994). CRABP-1 is involved in lipid metabolism by aiding the conversion of retinoic acid to cis-9-retinoic acid, which interacts with retinoid X receptor (RXR) and peroxisome-proliferator activated receptor type gamma (PPAR- γ) that inhibits adipocyte cell apoptosis and controls differentiation as well as proliferation (Carr *et al.*, 1998; Waterlow, 1994). The PIs bind to the CRABP-1 catalytic region, inhibiting the stimulation and action of RXR leading to increased peripheral adipocyte apoptosis and impaired differentiation (Carr *et al.*, 1998). This eventually results in compromised adipocyte fat storage and uncontrolled release of fat into the bloodstream to increase lipid levels (Calza *et al.*, 2003).

The PIs further contribute to dyslipidaemia by inhibiting the elimination of nascent apolipoprotein B (Apo B) by intercellular proteasomes (Non *et al.*, 2017). The Apo B-containing lipoproteins, which include LDL-C and VLDL-C, have proved to be causal agents of atherosclerosis and atherosclerotic-related complications (Shapiro & Fazio, 2017). It is also assumed that PIs influence the expression of enzymes involved in the liver biosynthesis of TG, resulting in dyslipidaemia (Lenhard *et al.*, 2000).

PIs have furthermore been seen to be contributing to the development of insulin resistance by blocking and reducing the translocation of the glucose transporter receptor known as glucose transporter type 4 (GLUT-4), and in so doing, interfere with insulin efficiency (Pedro *et al.*, 2018). The GLUT-4 receptor is responsible for glucose uptake, hence this impairment affects sugar levels, which may result in the development of DM (Noubissi *et al.*, 2018). Indinavir and Ritonavir are the PIs that block GLUT-4. As mentioned above, PIs affect the action of CRABP-1, which, in the context of glucose metabolism, leads to reduced efficacy of the peroxisomal proliferator-activated receptor (PPAR) that plays a role in glucose metabolism and

insulin sensitivity (Lamichane *et al.*, 2018; Noubissi *et al.*, 2018). It has been noted that the activation of the innate immune system in HIV-treated patients, inhibits the action of insulin through impaired or modified insulin signalling pathways (Pedro *et al.*, 2018). Furthermore, PIs (specifically, Lopinavir and Ritonavir) impair the function of pancreatic β -cells, which are essential for insulin secretion in response to blood glucose levels. *In vitro* studies have shown a decrease in β -cell function among those who use PI-containing regimens (Flint *et al.*, 2009).

The use of PI is associated with markers of atherosclerosis, manifested in terms of increased IMT and PWV in patients on PI-containing regimens, but the mechanism behind this has not been established (Thienemann *et al.*, 2013). The assumption is oestrogen is the role player in limiting HIV- and ART-induced inflammation and cell apoptosis, and it enhances the action of enzymes involved in nitric oxide synthesis, thereby reducing severity and progression of cardiovascular complications in women of reproductive age (Lee *et al.*, 2004; Stampfer *et al.*, 1991).

2.6.3 Association of HIV infection and ART with fibrinogen concentration and clot properties

This section investigates the relationship of HIV and ART with fibrinogen concentration and blood clot properties, it being the focus of this dissertation. There were only two studies in literature that investigated the association of HIV and/or ART with clot properties (De Lange *et al.*, 2012; Ronsholt *et al.*, 2015). Table 2-4 presents studies that investigated the association of HIV and/or ART on fibrinogen concentration, being the precursor and main determinant of fibrin clot structure; the results of other coagulation markers investigated in these studies are included.

Table 2-4: Studies investigating the relationship of HIV and ART on fibrinogen concentration and clot properties

Reference	Origin	Aim of the study	Participants	Design	ART used	Measure-ments	Coagulation results in HIV+ participants	
							Fibrinogen & clot properties	Other coagu-lation factors
Koppel <i>et al.</i> , 2002	Sweden	To elucidate the role of some major thrombogenic factors in HIV-infected patients receiving or not receiving PI-containing ART	Included 363 HIV+ patients Compared: 266 receiving PI-containing ART & 97 treatment naïve with mean ages of 43±9 & 38±8, respectively Note: Those on PIs were older, had a longer HIV infection duration & a higher prevalence of AIDS than the ART naïve	Prospective study analysis	PIs and NRTIs	Fibrinogen PAI-1	↑ fibrinogen levels in those on PI-containing ART	↑ PAI-1 levels in those on PI-containing ART
Lijfering <i>et al.</i> , 2008	Netherlands	To ascertain whether progression to AIDS was associated with an increased frequency and/or severity of thrombophilic abnormalities and secondarily to determine the overall risk of venous and arterial thrombosis in HIV-infected patients	Included 109 HIV+ patients on ART Compared: Three groups of asymptomatic, early symptomatic and AIDS patients	Retrospective case control	Not specified	F VIII Fibrinogen	↑ fibrinogen in AIDS patients as compared to other groups Note: ↑ fibrinogen & other thrombophilic factors in HIV+ patients than the healthy population	↑ FVIII in AIDS patients as compared to other groups

Reference	Origin	Aim of the study	Participants	Design	ART used	Measure-ments	Coagulation results in HIV+ participants	
							Fibrinogen & clot properties	Other coagulation factors
Madden <i>et al.</i> , 2008	USA	To assess the association of antiretroviral therapy with fibrinogen levels in HIV-infection	Included: 1412 Caucasians & Black-Americans with a median age of 43.0 (38.0–49.0) years Compared: 1131 HIV+ & 281 HIV- controls	Cross-sectional analysis	PI and NNRTIs	Fibrinogen	↑ fibrinogen in HIV+ as compared to the controls. ↑ in those taking PIs compared to those taking NNRTI	
Fourie <i>et al.</i> , 2010	South Africa	To assess whether newly identified HIV-1-infected Black South Africans showed signs of endothelial dysfunction, accelerated atherosclerosis and increased coagulation	Included: 600 Black South Africans, older than 35 years Compared: 300 newly identified HIV+ participants of the baseline PURE-SA-NW study population and individually matched with 300 HIV- participants, according to age, sex, body mass index (BMI) and locality	Nested case-control	Newly diagnosed	Fibrinogen and PAI-1	NS in fibrinogen levels	NS differences in PAI-1 levels

Reference	Origin	Aim of the study	Participants	Design	ART used	Measure-ments	Coagulation results in HIV+ participants	
							Fibrinogen & clot properties	Other coagu-lation factors
Jong <i>et al.</i> , 2010	Netherlands	Markers of inflammation & coagulation in HIV+ patients on combined ART with or without Abacavir (ABC) were compared to pinpoint a pathogenic mechanism for the increased risk of myocardial infarction in patients using ABC	Included: 81 HIV+ patients on ART (with few or no active signs of infection) Compared: 27 patients who were using ABC-containing ART & 54 used non-ABC-containing ART Note: they were also stratified into risk categories for CVD according to hsCRP levels (35% fell in the low-risk category, 30% in the average-risk & 33% in the high-risk categories)	Nested case-control	ABC-containing & PI- and NNRTIs-containing	Fibrinogen FVII Pro-thrombin fragment 1+2(F1+2)	NS differences in fibrinogen levels between the 2 groups (based ABC use). ↑ fibrinogen & F1+2 levels in the hsCRP high-risk category as compared to the low-risk	NS differences in FVII and F1+2 between the 2 groups. ↑F1+2 in 37% of the whole sample (regardless of the ABC use).
De Lange <i>et al.</i> , 2012	South Africa	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	Included: 1792 participants from a large-scale cohort of apparently healthy African men and women older than 35 years of age Compare: 306 HIV+ individuals & 1486 HIV- individuals	Prospective cohort	None	Clot lysis	↑ Clot lysis time (CLT)	

Reference	Origin	Aim of the study	Participants	Design	ART used	Measurements	Coagulation results in HIV+ participants	
							Fibrinogen & clot properties	Other coagulation factors
Kaplan <i>et al.</i> 2012	USA	To identify potential cardiovascular disease risk markers among HIV-infected women initiating antiretroviral treatment	Included: 254 women with a median age of 37 years HIV+ women were systematically matched to HIV- controls Compared: Before and after ART in 127 HIV+ patients, and also compared them to 127 HIV- controls	Nested case-control	PIs, NNRTIs and NRTIs	Fibrinogen	NS differences in any of the groups (Before & ART, and with the controls)	
Jobse <i>et al.</i> , 2015	South Africa	To determine how known polymorphisms within the fibrinogen gene and environmental factors influence the change in total and γ' fibrinogen over a 5 year period in 1288 black South Africans enrolled in the PURE-SA-NW study, and to determine whether gene-environment interactions influence total and γ' fibrinogen on both a cross-sectional and longitudinal (prospective) level in Africans	Included: 1288 participants from a large-scale cohort of apparently healthy African men and women older than 35 years of age in the 2010 follow up Compared: 214 HIV+ participants to HIV- participants as the controls	Prospective cohort	Combination of NNRTIs & NRTIs	Total fibrinogen, γ' fibrinogen	↓ fibrinogen levels	NS interaction with γ' fibrinogen

Reference	Origin	Aim of the study	Participants	Design	ART used	Measure-ments	Coagulation results in HIV+ participants	
							Fibrinogen & clot properties	Other coagu-lation factors
Osime <i>et al.</i> , 2015	Nigeria	To assess coagulation & haematological indices of HIV patients on ART	Included: 100 HIV+ individuals Compared: 50 patients on ART and 50 other who have not yet commenced ART	Case-control	Not specified	Fibrinogen PT & PTTK	NS differences between the groups.	↑ PT and PTTK in those on ART
Rönsholt <i>et al.</i> , 2015	Denmark	To examine functional haemostatic properties, including fibrinolysis, in plasma samples from HIV+ patients	Included: 82 participants with a median age of 55 Compared: 67 long-term treated HIV+ patients & 15 health HIV- controls (sex- and age-matched)	Case-control	Not specified, but were on ART	Clot lysis Clot formation	↑ clot lysis (↓ CLT) Delayed clot formation	
Montoya <i>et al.</i> , 2017	USA	To compare plasma biomarkers of coagulation between HIV-infected individuals and HIV-uninfected controls and to assess the impact of disturbances in coagulation on neurocognitive functioning in HIV	Included: 100 adults above the age of 50 (mean age of 57 years) Compared: 66 HIV+ participants (on ART with undetectable plasma viral load) and 34 HIV- controls	Case-control	Not Specified	fibrinogen Tissue factor (TF)	↓ fibrinogen	NS differences in the levels of TF

Reference	Origin	Aim of the study	Participants	Design	ART used	Measure-ments	Coagulation results in HIV+ participants	
							Fibrinogen & clot properties	Other coagu-lation factors
Seyoum <i>et al.</i> , 2018	Ethiopia	To assess basic coagulation parameters of HIV-infected patients in comparison with HIV-seronegative individuals	Included: 300 adult participants above the age of 18 years, included HIV+ who were matched to HIV-controls Compared: 100 ART-naïve HIV+ adults, 100 HIV+ on ART and 100 HIV-apparently healthy (age and sex-matched) controls	A comparative cross-sectional Study case control	Not specified	PT & APTT		↑PT & APTT in ART-naïve as compare to those on ART ↑in all HIV+ (on ART & ART-naïve) patients as compared to HIV- controls

Note: HIV+, represents HIV-infected patients; ↑ symbol represent an increase in a variable; ↓symbol represent a decrease in a variable; HIV = human immunodeficiency virus; PI = protease inhibitor; ART = antiretroviral treatment; AIDS = acquired immunodeficiency syndrome; NRTI = nucleoside reverse transcriptase inhibitor; PAI-1 = plasminogen activator inhibitor type 1; FVII = factor VII; NNRTI = non-nucleoside reverse transcriptase inhibitor; NS = not significant; ABC = abacavir; CVD = cardiovascular disease; hsCRP =high sensitive C-reactive protein; CLT = clot lysis time; USA = United States of America; PURE-SA-NW = South African North West Province arm of the Prospective Urban and Rural Epidemiological study; BMI = body mass index; γ' = gamma prime; PT = prothrombin time; APTT = partial thromboplastin time; TF =Tissue factor.

Table 2-4 presents different studies that included HIV-infected patients and different measured variables that relate to clotting and clot formation. Twelve studies from different countries were included. Only two studies assessed the clot properties in the HIV-infected participants through the measuring of clot lysis (De Lange *et al.*, 2012; Ronsholt *et al.*, 2015), and the other studies measured other variables assumed to have an effect or to predict clot properties; the other included variables are fibrinogen levels, γ' fibrinogen levels, factor VIII (FVIII) levels, prothrombin fragments 1 + 2 (F1+2) levels, prothrombin time (PT) and partial thromboplastin time with kaolin (PTTK) (Fourie *et al.*, 2011; Jobse *et al.*, 2015; Jong *et al.*, 2010; Kaplan *et al.*, 2012; Koppel *et al.*, 2002; Lijfering *et al.*, 2008; Madden *et al.*, 2008; Montoya *et al.*, 2017b; Osime *et al.*, 2015; Seyoum *et al.*, 2018).

Inconsistencies have, however, been noticed in these studies as most have conflicting results. In the studies that assessed clot lysis, longer CLT in recently diagnosed HIV-infected individuals are reported compared to the controls (De Lange *et al.*, 2012). These results indicate possible thrombotic clot properties, as these types of clots are resistant to fibrinolysis. The study by Rönsholt *et al.*, (2015) also assessed clot lysis in long-term treated HIV-infected individuals and compared them to healthy HIV-uninfected controls; the study found accelerated clot lysis (lower CLT) among the HIV-infected individuals compared to the controls. The differences in these results could be because the Rönsholt *et al.* (2015) study included treated participants, thus suppressing the viral load to the lowest levels possible. The differences may also have risen from differences in genetics between the study populations, Black South Africans and Danish, as different people possess different genetic traits that tend to respond differently to certain conditions (Humphries, 1995; Soria *et al.*, 2005).

The studies conducted by Koppel *et al.* (2002), in Sweden, and Madden *et al.* (2008), in the USA, reported increased fibrinogen levels among HIV-infected individuals compared to the controls. They further reported the differences between HIV-infected individuals taking different kinds of ART, with significantly higher fibrinogen levels among those taking PI-containing ART than those not on PIs. Lijfering *et al.* (2008) also conducted a study aimed at ascertaining whether progression to AIDS was associated with an increased frequency and/or severity of thrombophilic abnormalities,

and secondly to determine the overall risk of venous and arterial thrombosis in HIV-infected patients. The study reported increased levels of fibrinogen as well as factor VIII among the AIDS patients compared to the non-AIDS HIV-infected patients (Lijfering *et al.*, 2008). The studies generally reflect increased fibrinogen levels in HIV-infected individuals compared to the uninfected controls, but they further reflect increased levels among those on PIs as well as an increase as the infection progresses.

The Jong *et al.*, (2010) study did not find any significant differences in fibrinogen and F1+2 levels when they compared HIV-infected patients using ABC-containing ART and those using non-ABC-containing ART. The study, however, found increased fibrinogen and F1+2 levels in the hsCRP high-risk category compared to those in the low-risk. It was also reported there was increased F1+2 levels in 37% of the whole sample, regardless of the ABC use (Jong *et al.*, 2010). The study supports the assumption that fibrinogen levels increase as the infection progresses, as an increase was found among those with high hsCRP levels.

Increased levels of fibrinogen are associated with the formation of clots with tightly packed fibres and very resistant to lysis, which pose a risk for thrombotic-related complications (Undas & Ariëns, 2011; Weisel, 2007). A number of studies have reported non-significant or lower levels of fibrinogen among the HIV-infected individuals, as noted in the studies by Fourie *et al.* (2010), Kaplan *et al.* (2012), Osime *et al.* (2015), Jobse *et al.* (2015), and Montoya *et al.* (2017). The studies by Fourie *et al.* (2010), Jobse *et al.* (2015) and Montoya *et al.* (2017) included clinically well HIV-infected participants, with few or no signs of sicknesses, and this could be the reason why no significant differences were found when compared to the controls. The Osime *et al.* (2015) study only compared patients on ART and those who were yet to commence ART, and never compared uninfected controls; this could be the reason why they found no significant differences because those on ART might have had their viral load suppressed, while those not on ART might have been newly diagnosed and clinically well.

2.7 Conclusion

According to the studies presented in Table 2-4, as well as the review of the general literature included, it can be hypothesised that there is a certain level of abnormality in the plasma clot properties among HIV-infected patients, which may assist in explaining the inevitable increased levels of cardiovascular diseases in these patients, both those who are on treatment and those who are treatment naïve. There is, however, limited information on plasma clot properties in HIV-infected individuals, therefore, the current study aimed to bridge this knowledge gap.

CHAPTER 3: METHODS

3.1 Introduction

Human immunodeficiency virus (HIV) infection and progression are accompanied by different opportunistic infections, and are associated with a variety of complications, including cardiovascular-related complications (De Gaetano Donati *et al.*, 2010; Wang *et al.*, 2015). The introduction of highly active antiretroviral therapy (ART) has reduced HIV-related complications and improved quality of life among infected individuals (Wang *et al.*, 2015). However, different drugs have also been associated with the development of other cardiovascular-related complications in patients (Loelius *et al.*, 2018). HIV as well as ART are associated with hypercoagulability, consequently increasing thrombotic risk (De Gaetano Donati *et al.*, 2010; Hsue, 2019). Altered clot properties, is one of the main contributors of thrombosis-related cardiovascular complications (Undas & Ariëns, 2011). There is, however, very little direct evidence for the association of HIV infection and/or ART on the properties of fibrin clots. This sub-study aimed to investigate the association of HIV and ART respectively, with plasma clot properties in a sub-group of participants from the South African North West Province arm of the Prospective Urban and Rural Epidemiological study (PURE-SA-NW).

This study included all eligible PURE-SA-NW participants and made use of previously collected data. Descriptive and comparative statistics were performed to obtain sample characteristics and distributions as well as testing the association of HIV and ART, with different haemostatic and plasma clot properties. The study aimed to achieve the following objectives:

1. To cross-sectionally compare fibrinogen concentration and plasma clot characteristics obtained with turbidity measurements of:
 - HIV-infected (n = 151) and HIV negative (n = 176) participants at baseline;
 - HIV-infected treatment-naïve (n = 81) participants, HIV-infected treated (n = 70) and HIV negative (n = 176) participants at follow-up.

2. To prospectively compare within groups, fibrinogen concentration and plasma clot characteristics obtained with turbidity measurements of:

- participants who were HIV free at baseline and follow-up (n = 176);
- HIV-infected participants who were treatment-naïve at baseline and follow-up (n = 81);
- HIV-infected participants who were treatment-naïve at baseline and treated at follow-up (n = 70).

3. To prospectively compare individual haemostatic variables and plasma clot characteristics obtained with turbidity measurements between the three groups, as indicated in objective 2.

3.2 Ethical considerations

This sub-study was conducted under the larger study titled, “Characterisation of the haemostatic profile of a Black South African Population” and all variables measured were covered in the larger study (NWU-00034-17-S1), which is affiliated with the South African North West Province arm of the international PURE study. The larger study was approved by the North-West University Health Research Ethics Committee (NWU-HREC). Ethical approval for this sub-study was obtained from the Health Research Ethics Committee of the North-West University (NWU-HREC) (NWU-00034-17-A1-04) in 2019 (Addendum A – Ethics approval letter). All members of the research team completed ethics training to ensure the study was ethically accepted and conducted in an appropriate manner.

3.3 Study population

3.3.1 Recruitment

This sub-study included data from individuals who participated in the PURE-SA-NW study. The PURE study is a large-scale international epidemiology study implemented in several countries, including South Africa. The North West Province PURE-SA-NW study included Setswana-speaking individuals residing in Ikageng, Zonderwater, Extension 7 or Extension 11 (urban settlements), Ganyesa (urban-rural town) or Tlaskgameng (rural area) using a door-to-door approach during recruitment. These

areas were chosen in an effort to equally represent urban, urban rural (transitional), and rural areas of the North West Province. Permission to access and recruit community members were granted on a judicial (national) and goodwill (tribal/community and household) basis, by the South African Department of Health, Potchefstroom and Ganyesa city mayors, tribal chiefs, community leaders (acting as gatekeepers), and household heads, respectively. Although consent was given for the study in its entirety (2005 – 2015), tribal/community permission was also sought for each follow-up data collection time period out of respect for each community's leadership structure.

Potential participants were approached, and with their verbal consent, given all the information regarding their possible participation. Individuals had one week to contemplate their participation with their family members. Participants had the option to decide whether or not they would show up for the data collection day, regardless of their initial decision to be involved or not. Individuals who made the decision not to be involved in the PURE-SA-NW study did not show up for baseline measurements and consequently not approached for any further involvement. In total, 6 000 households were approached during recruitment, with the aim of reaching a participant group of 2 000 or more participants based on a prior power calculation published by Vorster (2002). The selected communities and the included households were considered stable, with regard to movement in and out of the area.

3.3.2 Inclusion and exclusion criteria

Individuals were eligible to participate in the PURE-SA-NW study if they were apparently healthy, older than 35 years, and not using any acute or chronic medication (except for blood pressure medication). Individuals had to be willing to partake in follow-up data collection for the upcoming 10 years and by implication, had to plan on remaining residents in the area of recruitment for this time. Lastly, eligibility was ultimately determined by the individual's voluntary willingness to be involved, and by his/her ability to be of full comprehension prior to signing a voluntary written informed consent (Addendum B). Consequently, individuals who were inebriated or not capable of being fully informed at the time of consent were not allowed to participate. If the head of the approached household did not approve of household members'

participation, a non-complier questionnaire was completed and the house, as a whole, excluded. Apparently healthy volunteers could participate, however, participants diagnosed HIV-infected for the first time when tested for this study were still included. These participants received information and counselling to contact their healthcare worker and some of the individuals diagnosed HIV-infected commenced ART while others did not.

3.4 Study design

For this study, a sub-group of participants who participated in the larger PURE-SA-NW study from 2005 to 2010 were used. During the baseline data collection in 2005, a number of participants were newly identified as HIV-infected, and some subsequently commenced ART, while others did not; this was based on the CD4 count at the time (CD4 cell count of ≤ 200 cells/mm³). The participants who commenced ART received treatment under the government programme running at that point in time. They were given a combination of ART that comprised two nucleoside reverse transcriptase inhibitors (NRTIs), and one non-nucleoside reverse transcriptase inhibitor (NNRTIs).

This study compared the previously measured plasma clot properties generated from turbidimetry measurements using a selected group of the PURE-SA-NW study participants. These clot properties included lag time, slope, maximum absorbance, clot lysis time, as well total and γ' fibrinogen. The potential participants for the sub-group were identified from the PURE-SA-NW dataset based on several factors. The PURE-SA-NW dataset underwent sorting to identify HIV-infected participants who were not using ART during the 2005 baseline data collection and had data for all the variables critical to this study, including inflammation, kidney function, blood lipids, blood sugars as well as body mass index (BMI) data, collected for both 2005 and 2010 follow-up. Participants missing any of the above-mentioned variables were excluded. Potential diabetic participants were further excluded from the list. This led to the identification of 70 HIV-infected participants who were receiving ART by 2010 and 81 HIV-infected participants who were still untreated. The selected HIV-infected participants were then

systematically matched to 176 HIV-uninfected controls by considering their sex, age and BMI (Figure 3-1).

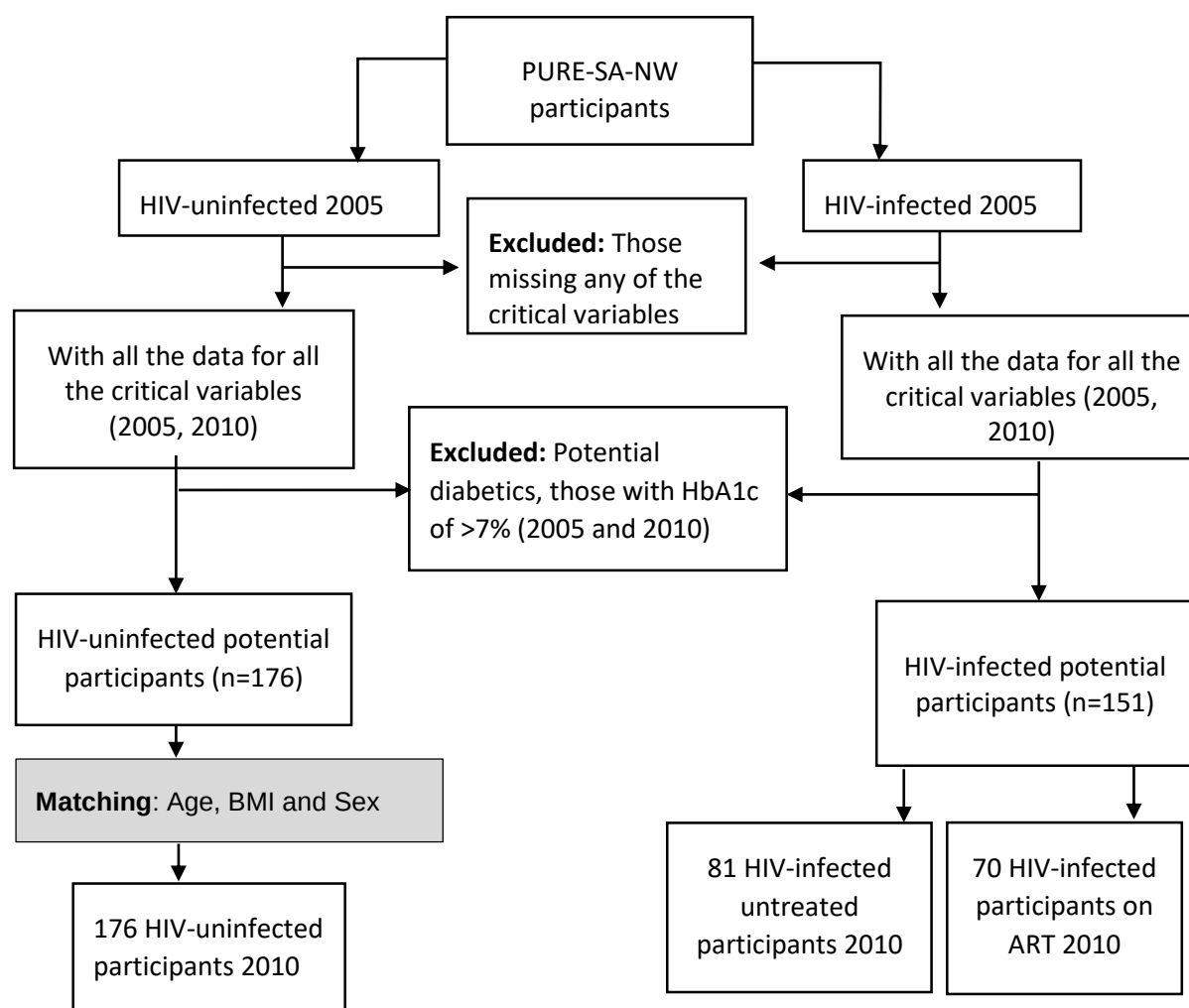


Figure 3-1: Study design and participant selection

3.5 Method of determining HIV status in PURE-SA-NW

Voluntary HIV testing was performed on the study clinic visit day with participant's consent. Registered HIV counsellors were available to perform pre- and post-counselling. Each participant could choose whether she/he wanted to, in privacy from a counsellor, receive their individual test result. HIV status was determined on-site according to the South African Department of Health protocol and the UNAIDS/WHO Policy Statement on HIV-testing (UNAIDS/WHO: 2004). The First Response (PMC

Medical, India) rapid HIV card test using whole blood was used. If positive, results were conformed using the Pareeshak card test (BHAT Bio-tech, India). Follow-up plans and referrals depended on the test results.

3.6 Determination of plasma clot properties

Platelet poor plasma was prepared from fasting blood samples, collected by professional registered nurses in appropriate collection tubes. For the measurement of fibrinogen, γ' fibrinogen and clot properties, blood was placed into 3.8% sodium citrate tubes, centrifuged at 2 000 g for 15 minutes at 10° and stored at -80°C until analyses.

3.6.1 Total and γ' fibrinogen concentration

3.6.1.1 Total fibrinogen

The procedures used to determine fibrinogen concentration at both time points used the same principles but different analysers. For the 2005 samples, total fibrinogen concentration was measured using a modified Clauss method with the Multifibrin U-test, BCS analyser (Dade Behring, Deerfield, IL, USA). In this method citrated plasma was coagulated by adding a large amount of thrombin and the clotting time per sample is based on the amount of fibrinogen in the sample. An ACL-200 (Instrumentation Laboratories, Milan, Italy) determined the fibrinogen concentrations for the 2010 samples. This procedure is based on prothrombin time, where a high concentration of thrombin is also added to plasma and the fibrin formation is determined (Clauss, 1957; Mackie *et al.*, 2003). A calibration curve is obtained by clotting a reference plasma sample with a known fibrinogen concentration (Clauss, 1957; Mackie *et al.*, 2003). Bovine thrombin was used to trigger coagulation in a fibrinogen-C kit (HemosIL, Instrumentation Laboratory, Bedford, USA) and the clotting time was measured by the above-mentioned respective analysers. The total fibrinogen concentrations of the samples, expressed in g/L, were determined by comparing them to a standard curve generated from reference plasma samples. Owing to different analysers used for the two time-points, a subsample (n=140) of the 2005 samples were re-run when the 2010 samples were analysed and checked against the original results. This was to exclude

any possibility of differences between the 2005 and 2010 results due to the use of different analysers.

3.6.1.2 γ' fibrinogen

An enzyme-linked immunosorbent assay (ELISA) was used to measure γ' fibrinogen concentrations. The assay makes use of antibodies that act against γ' fibrinogen and fibrinogen to determine the concentration. It uses a 2.G2.H9 mouse monoclonal coating antibody (Santa Cruz, USA) that acts against γ' fibrinogen by targeting a chain peptide consisting of the carboxyterminal sequence (VRPEHPAETEDSLYPEDDL) (Lovely *et al.*, 2002b), as well as goat polyclonal horseradish peroxidase (HRP)-conjugated antibody against human fibrinogen (Abcam, Cambridge, USA).

The mouse monoclonal coating antibody, which acts against γ' fibrinogen, was coated on a 96-well microtiter plate (Merck, Darmstadt, Germany) and incubated overnight at 4°C. The following day, a blocking buffer containing bovine serum albumin was added to the plate and incubated for an hour at room temperature. A plasma sample diluted with dilution buffer (consisting of 2 M TEA, 5 M NaCl, 0.5 EDTA and 1 M Benzamidinium with a 7.5 pH) (Merck, Darmstadt, Germany) was added to the wells and incubated for another hour at room temperature. The goat polyclonal HRP-conjugated antibody, which acts against fibrinogen, was then added and incubated for a further hour at room temperature to detect the quantity of γ' fibrinogen. This was followed by the addition of a substrate buffer (TMB and UP; bioMérieux, Boxtel, Netherlands) and incubated for a further 15 minutes at room temperature. The reaction was then stopped by adding 1 M sulphuric acid (H₂SO₄) (Merck, Darmstadt, Germany) and a washing buffer, which was used after every incubation period to wash the plate wells three times. A Multiskan FC spectrophotometer (Thermo Fisher Scientific, Waltham, USA) determined the absorbance of the samples at 450 nm.

The results were compared to a standard curve generated from pool plasma, with a known γ' concentration, to quantify the concentration of each of the measured samples. Three repeats of 1:25, 1:50, 1:100, 1:200 and 1:400 pool plasma dilutions were used in setting up the standard curve. Each run included three pool plasma runs

of similar dilutions to that of the study sample plasmas. The pool plasma repeats were included as controls and to assess variations within or between samples. The same pool was used for the 2005 and 2010 samples. Finally, with reference to the standard curve, the calculation of fibrinogen γ' concentration in g/L, and ratio expressed as percentage of total fibrinogen, followed.

Plasma clot properties can be determined using different direct and indirect methods. Direct methods of determining clot structure include scanning electron microscopy (SEM), transmission electron microscopy (TEM) and thromboelastography (TEG), while mechanical properties are measured by rheometry, providing information regarding the viscoelasticity of the clots being studied (Ryan *et al.*, 1999). Turbidity and permeability measures provide indirect measures of structural clot properties (Morais *et al.*, 2006; Pieters *et al.*, 2012). In the current study, turbidimetry determined the plasma clot properties in order to determine the association of HIV and ART, respectively, with these clot properties.

3.6.2 Turbidimetry

Turbidimetry is one of the fastest and most commonly-used methods of determining the nature of clots (Weisel & Nagaswami, 1992). Turbidimetry uses light to measure the concentration of a given substance or specimen, by analysing the amount and intensity of light that is absorbed by or transmitted through the substance or any form of specimen (Pieters *et al.*, 2008; Weisel & Nagaswami, 1992). In the case of plasma clots, the light absorbance increases as the clot fibres form and grow (Carr Jr & Hermans, 1978; Carr Jr *et al.*, 1977). Clot formation and overall structure is recorded and visualised by a plotted light absorbency-against-time curve, known as the sigmoidal curve (Carr Jr & Hermans, 1978; Sjøland *et al.*, 2007). The sigmoidal curve is divided into lag time, slope, maximum absorbance and clot lysis time (CLT) (Weisel & Nagaswami, 1992). Lag time is the initial time from the addition of thrombin or tissue factor until the light absorbency curve begins to pick up, followed by the slope, which is an increase in absorbency that gives rise to the curve until maximum absorbance (highest point of the curve) is attained (Weisel & Nagaswami, 1992). Lag time indicates the amount of time required for activation of the coagulation cascade and sufficient formation as well as growth of protofibrils to allow lateral aggregation, while slope and

maximum absorbance are indicators of the rate of lateral aggregation and fibre diameter, respectively (Carr Jr & Hermans, 1978; Pieters *et al.*, 2008; Weisel & Nagaswami, 1992). In plasma clots, maximum absorbance is known to indicate fibre clot density (Pieters *et al.*, 2020). The CLT, in minutes, is calculated by obtaining the difference between the midpoint in turbidity of clot formation and midpoint of the turbidity of clot lysis, and is a measure of fibrinolysis (Lisman *et al.*, 2005).

In this study, the global fibrinolytic assay measured/determined the plasma clot properties (Lisman *et al.*, 2005)) (Addendum C). A tissue factor (TF) (Dade Innovin, Siemens Healthcare Diagnostics Inc. Marburg, Germany) and calcium chloride (CaCl_2) (Merck, Darmstadt, Germany) mixture was used to activate the coagulation process in platelet-poor plasma. Tissue plasminogen activator (tPA) (Actilyse, Boehringer Ingelheim, Ingelheim, Germany) was used to trigger clot lysis.

Frozen plasma samples were thawed in a water bath at 37°C. A multi-channel pipette pipetted the samples into wells of an untreated microtiter plate (Merck, Darmstadt, Germany), followed by the addition of assay buffer to make a 50% dilution of the samples, which was shaken at 1100 revolutions per minute (rpm) for 10 seconds using a plate-shaker. An assay mixture was prepared and consisted of 18.75 x diluted TF, 113 mM CaCl_2 , 167 ng/ml tPA, 67 μM phospholipids (Rossix, Mölndal, Sweden) and assay buffer (25 mM HEPES; 137 mM sodium chloride (NaCl); 3.5 mM potassium chloride (KCl); 1% BSA; pH 7.4) (Merck, Darmstadt, Germany). The assay mixture was added to each well of a Nunc® MaxiSorp™ microtiter plate (Merck, Darmstadt, Germany). A multi-channel pipette was then used to transfer the diluted plasma samples over to the plate containing the assay mixture. The contents, which were shaken at 1400 rpm for 10 seconds, were covered with liquid paraffin oil to prevent evaporation/drying during the measurement. The final concentration in the clots were 1750 x diluted TF, 17 mmol/l CaCl_2 , 80 ng/ml tPA and 10 mmol/l phospholipid vesicles. The plate was placed into a Multiskan Ascent spectrophotometer plate reader (Labsystems, Virginia, USA) immediately after addition of the paraffin oil, and the clot formation process was measured at an absorbance of 405 nm every nine seconds for the first two minutes, every 15 seconds up to 30 minutes and every minute until the clot is lysed or up to 270 minutes. The data obtained from the curves were analysed using Origin® software version 8.5 (Origin lab®, 2010). To ensure comparability

between the 2005 and 2010 turbidimetry results, the same control (pool) plasma was used for the 2005 and 2010 measurements and the turbidity data at each time point was normalised according to the pool sample data.

3.6.3 Previously-measured covariables

The study also investigated several additional covariables that were previously measured. These variables can vary over time and have the potential to influence the clot properties being investigated in this study. The following variables were used in the systematic matching of the groups being investigated in this study, and some of them were entered as covariates in different statistical models. Table 3-1 lists the co-variables and the methods, as well as the tools used to measure them.

Table 3-1: Co-variables included in the study

Variables	Method of measurement and apparatus used
Age	Age=(exam date-date of birth)/365 (years)-2005, 2010
Sex	Questionnaire: 0= Female; 1= Male-2005, 2010
CD4 count	Whole blood “Flow cytometric analysis” determined by the National Health Laboratory service. Beckman COULTER® EPICS® XITM, Fullerton, USA- 2005, 2010
Smoking	Questionnaire: Use of any tobacco product (cigarette, cigar, snuff, chew, beedies) (0: Not answered; 1: Formerly used; 2: Currently use; 3: Never used)-2005, 2010
Alcohol use	- PURE-SA-NW Adult questionnaire: Consumption of any alcohol products (1: Formerly used; 2: Currently use; 3: Never used)-2005 - Questionnaire: Currently consuming any alcohol product (0 = No, 1=Yes) -2010
Waist circumference (WC)	Holtain unstretchable metal tape: Using standard procedures (Marfell-Jones <i>et al.</i>, 2006)-2005, 2010
Weight	Precision Health Scale (A & D Company, Japan): Using standard procedures (Marfell-Jones <i>et al.</i>, 2006)-2005, 2010
Body mass index (BMI)	Calculated: weight in kg/(height in cm*height in cm)-2005, 2010

Blood pressure	• OMRON HEM-757 device (Omron Healthcare, Kyoto, Japan)-2005, 2010
Creatinine Clearance (CrCl)	• Calculated - Cockcroft-Gault formula (Cockcroft & Gault, 1976)-2005, 2010 $[(140 - \text{age}) * (\text{weight in kg}) * (0.85 \text{ if female})] / (72 * \text{creatinine in } \mu\text{mol/l})$
Glomerular filtration rate (GFR)	• Calculated - MDRD Formula (Levey <i>et al.</i>, 1999)-2005, 2010 $32788 \times \text{serum Cr}^{-1.154} \times \text{age}^{-0.203} \times 0.742 \text{ (if female)} \times 1.21 \text{ (if black)}.$
Gamma-glutamyl transferase (GGT)	• Konelab20i™ auto-analyzer, Thermo Fisher Scientific Oy, Vantaa, Finland-2005 • Cobas Integra 400 plus Roche®, Basel, Switzerland-2010
Glucose	Enzymatic reference method with hexokinase using: • Vitros DT6011 Chemistry Analyser; Ortho-Clinical Diagnostics, Rochester, New York, USA-2005 • Cobas Integra 400 Roche Clinical System, Roche Diagnostics, Indianapolis-2010
Glycated haemoglobin (HbA1c)	• D-10 Haemoglobin testing system from Bio-Rad (#220-0101) Ion-exchange high-performance liquid chromatography-2005, 2010
C-reactive protein (CRP)	• Analysed serum samples using high sensitivity, particle-enhanced turbidimetric assay. Konelab20i™ auto-analyser, Thermo Fisher Scientific Oy, Vantaa, Finland-2005, 2010
Interleukin 6 (IL-6)	• Enzyme-linked immunosorbent assay and Human electrochemiluminescence immunoassay. Quantikine® HS ELISA, R&D Systems, Minneapolis, USA and Elecsys 2010, Roche, Basel, Switzerland-2005, 2010
Soluble urokinase-type plasminogen activator receptor (SuPAR)	• Enzyme immunoassay. suPARnostic® ELISA kit (ViroGates, Copenhagen, Denmark)-2005, 2010
Intercellular adhesion molecule (ICAM)	• Human sICAM-1 sandwich ELISA assay, IBL, Hamburg, Germany sandwich ELISAs-2005, 2010
Vascular cell adhesion molecule/protein (VCAM)	• Human sVCAM-1 sandwich ELISA assay, IBL, Hamburg, Germany-2005, 2010
Plasminogen activator inhibitor (PAI-1)	• Spectrolyse®/pl PAI-1 (Trinity Biotech plc, Bray Co, Ireland), Chromogenic assay kit-2005

Total cholesterol, HDL-C, Triglycerides	- Konelab20i™ auto-analyser, Thermo Fisher Scientific Oy, Vantaa, Finland- 2005 - Cobas Integra 400 Roche® Clinical System, Roche Diagnostics, Indianapolis enzymatic colorimetric method- 2010
LDL-cholesterol	Calculated - Friedewald formula (Johnson <i>et al.</i>, 1997)-2005, 2010 $LDL-C = TC - HDL-C - VLDL-C$ where $VLDL-C = 0.456 * TG$
Oral contraceptive use in women	PURE-SA-NW Adult questionnaire: 0 = No, 1=Yes-2005, 2010

3.7 Statistical analysis

The statistical analysis of this study was performed using IBM-SPSS software version 26, and a p-value of 0.05 was used to indicate statistical significance. Continuous data was described using means and standard deviation or median and interquartile range according to variable skewness assessed by visual inspection of the histogram and Q-Q plots. Categories were reported as percentages. Not normally distributed data was log-transformed to improve the normality and used in further analyses. Scatterplots identified any outliers, which were checked by evaluating the raw data to ensure it was correctly entered. The decision to remove or keep the outliers in the dataset depended on whether the value was higher or lower than expected physiological ranges.

To identify the potential covariates likely to influence the results, t-test and Chi-square comparisons were performed to determine the baseline differences between the HIV-infected and HIV-uninfected participants in terms of their inflammation, kidney function, blood lipids, blood sugars as well as body mass index (BMI) data (Table 3-1). This was followed by Pearson's correlation test to identify the variables that correlated with the clot properties as well as fibrinogen.

The covariates that differed significantly between HIV-infected and HIV-uninfected participants, and at the same time correlated with fibrin clot properties, were entered as covariates into a Generalised Linear Regression (GLM) analysis to determine

whether plasma clot properties independently associated with HIV status or whether these associations were driven by differences in the covariates between HIV-infected and uninfected participants. To cross-sectionally compare the differences between the HIV-infected treated, untreated and HIV-uninfected groups at the 2010 follow-up, analysis of variance (ANOVA) followed by Tukey's HSD test and GLMs for fibrinogen and clot properties as outcomes, adjusted for covariates, were performed. Next, paired t-tests were used to determine which covariates changed over the 5-year period in each of the three groups, followed by mixed linear models, which determined whether the degree of change over time in fibrinogen concentration and clot properties differed between the three groups. The interaction p-value was used to interpret the changes over time between the groups. These models were adjusted for covariates that demonstrated differences in changes between the three groups to determine whether any observed changes in clot properties over time between the three groups were independently associated with HIV and/or ART, or whether it was driven by changes in the covariates over time.

The results of this study will be reported in Chapter 4.

CHAPTER 4: RESULTS

4.1 Introduction

The focus of this study was to investigate the association of HIV status and antiretroviral therapy (ART) respectively, with plasma clot properties in a sub-group of participants from the South African North West Province arm of the Prospective Urban and Rural Epidemiology (PURE-SA-NW) study. It has been reported that HIV and ART are associated with hypercoagulability, therefore with increasing thrombotic risk (De Gaetano Donati *et al.*, 2010). One of the main components of hypercoagulability is altered clot properties, which may contribute to thrombosis-related cardiovascular complications (Jackson & Pretorius, 2019; Jacobson *et al.*, 2004), as clots with tightly packed thin fibrin fibres tend to be more resistant to lysis resulting in thromboembolic manifestation (Cesarman-Maus & Hajjar, 2005; Undas & Ariëns, 2011). This may result in serious complications such as stroke and myocardial infarction, which are also common in HIV-infected patients (Kaplan-Lewis *et al.*, 2016). We therefore hypothesised that the relationship between HIV and thrombosis may possibly be due to altered fibrin clot properties among HIV-infected patients.

To achieve the aim and reach the specified objectives as set out in Chapter 1, previously collected data of all eligible PURE-SA-NW participants were analysed. The performing of descriptive and comparative statistical analyses was to obtain the characteristics and distributions of the study sample, and to test the association of HIV status and ART with different haemostatic and plasma clot properties.

The first objective of this study was to determine whether plasma clot properties differed between newly diagnosed (treatment naïve) HIV-infected and uninfected participants in 2005; then, as some but not all the HIV-infected participants commenced ART after diagnosis, to compare the clot properties at 5-year follow-up of HIV-infected participants using ART with treatment naïve participants and HIV uninfected participants. After determining the distribution of the data, independent t-tests determined which variables differed between HIV-infected and uninfected participants in 2005. Next, Pearson correlation analyses determined which covariates were associated with the plasma clot properties. Thirdly, variables that differed

significantly between HIV-infected and uninfected participants, as well as those that correlated with fibrin clot properties were entered as covariates into a Generalised Linear Regression (GLM) analysis to determine whether plasma clot properties were independently associated with HIV status or whether these associations were driven by differences in the covariates between HIV-infected and uninfected participants.

The 2010 data used a similar approach, except that analysis of variance, instead of independent t-tests compared the HIV-uninfected and HIV-infected, treated and untreated participants. Paired t-tests determined which variables changed over the 5-year period in the three groups, and lastly, mixed linear models determined whether changes over time in fibrinogen concentration and clot properties differed between the three groups. These models were adjusted for covariates that demonstrated differences in changes over time between the three groups to determine whether any observed changes in clot properties between the three groups were independently associated with HIV and/or ART, or whether it was driven by changes in the covariates.

4.1.1 Baseline comparisons (2005)

4.1.1.1 Population characteristics

The study group of eligible PURE-SA-NW study participants included 151 newly diagnosed HIV-infected participants, 70 of whom were receiving ART by 2010 and 81 who were still untreated. Matching these participants to 176 HIV-uninfected individuals used their sex, age and body mass index (BMI) values. Table 4-1 presents the characteristics of this study group. The table presents normally distributed data as mean [standard deviation], and median (interquartile ranges) for not normally distributed data.

The ages of these two female-dominated groups (70.2 % in the HIV-infected, 60.8 % in the HIV-uninfected) differed significantly, with the uninfected group being older with a median age of 44 years (40-50.5) compared to the 42 years (38-48) mean age of their counterparts ($p=0.021$). Sex distribution, tobacco use and alcohol consumption did not differ between the two groups.

Baseline body mass index (BMI) and C-reactive protein (CRP) did not differ between the two groups. BMI ranged from 15.1 to 43.8 kg/m², with median values of 21.2 (18.7-24.6) kg/m² for HIV-infected group and 21.5 (19.3-25.9) kg/m² for the uninfected group. The HIV-infected participants had a CRP median of 2.54 (0.69-8.43) mg/L and the uninfected group a median of 2.21 (0.71-6.5) mg/L.

The HIV-infected participants had significantly lower systolic blood pressure (SBP) (p=0.002), estimated glomerular filtration rate (eGFR) (p=0.026), gamma-glutamyl transferase (GGT) (p=0.029), glucose (p=0.009), total cholesterol (TC) (p < 0.001), high-density lipoprotein cholesterol (HDL-C) (p=0.025) and low-density lipoprotein cholesterol (LDL-C) (p<0.001), compared to the HIV-uninfected participants. This group also had significantly higher levels of soluble urokinase-type plasminogen activator receptor (SuPAR) (p=0.003), intercellular adhesion molecule (ICAM) (p=0.001) and vascular cell adhesion molecule/protein (VCAM) (p<0.0001) compared to the HIV-uninfected group.

Table 4-1: Descriptive characteristics of study population at baseline

Variable	HIV-infected n=151	HIV-uninfected n=176	p-value
Age (years)	42.0 (38.0-48.0)	44.0 (40.0-50.5)	0.021
Men/Women (%)	29.8 / 70.2	39.2 / 60.8	0.075*
Smokers/non-smokers	96 / 55	107 / 68	0.651*
Alcohol consumption/not (%)	49.7 / 50.3	46.6 / 53.4	0.612*
WC (cm)	75.0 [9.71]	76.7 [10.3]	0.130
Weight (kg)	58.4 [12.9]	59.4 [14.4]	0.499
BMI (kg/m ²)	21.2 (18.7-24.6)	21.5 (19.3-25.9)	0.610
Systolic blood pressure (mmHg)	122 [17.8]	129 [22.3]	0.002
Diastolic blood pressure (mmHg)	83.6 [12.6]	86.4 [14.2]	0.062
CrCl (ml/min)	94 [33]	100 [32.3]	0.084
eGFR (mL/min/1.73m ²)	121 [39.5]	132 [40.4]	0.026
GGT (U/L)	40.6 (26.1-71.7)	46.6 (30.6-87)	0.029
Glucose (mmol/L)	4.66 [0.85]	4.91 [0.85]	0.009

HbA1c (%)	5.50 [0.44]	5.53 [0.51]	0.477
CRP (mg/L)	2.54 (0.69-8.43)	2.21 (0.71-6.5)	0.342
IL-6 (pg/ml)	4.24 (2.48-6.94)	3.64 (2.18-6.25)	0.171
SuPAR (ng/ml)	3.69 (2.93-4.64)	3.29 (2.74-4.07)	0.003
ICAM (ng/ml)	564 (335,844)	432 (268-720)	0.001
VCAM (ng/ml)	917 (511-1644)	496 (314-898)	<0.0001
PAI-1 _{act} (U/ml)	3.93 (1.40-7.08)	4.29 (1.25-7.72)	0.662
TC (mmol/L)	4.43 [1.19]	5.13 [1.34]	<0.001
HDL-C (mmol/L)	1.26 [0.53]	1.74 [0.74]	<0.001
LDL-C (mmol/L)	2.61 [0.90]	2.89 [1.14]	0.025
TG (mmol/L)	1.08 (0.81-1.38)	1 (0.76-1.38)	0.616

WC = waist circumference; BMI = body mass index; HbA1c = glycated haemoglobin; eGFR = estimated glomerular filtration rate; GGT = gamma-glutamyl transferase; ICAM = intercellular adhesion molecule; VCAM = vascular cell adhesion molecule; BMI = body mass index; CRP = C-reactive protein; IL-6 = interleukin 6; SuPAR = soluble urokinase-type plasminogen activator receptor; TC = total cholesterol; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; TG = triglycerides; CrCl = creatinine clearance; PAI-1_{act} = plasminogen activator inhibitor-1 activity.

- The normally distributed data are presented as mean [standard deviation], while not normally distributed data are presented as median (interquartile range).
- * = Chi-square comparison.

4.1.1.2 Correlations between the covariates and plasma clot properties at baseline

Table 4-2 presents the correlations between all the covariates and the specific targeted clot property variables at baseline. Total fibrinogen correlated positively with CrCl ($r=0.14$, $p=0.042$), CRP ($r=0.38$, $p<0.001$) and IL-6 ($r=0.27$, $p<0.001$). Percentage γ' fibrinogen demonstrated a negative correlation with CrCl ($r=-0.18$, $p=0.007$) and eGFR ($r=-0.23$, $p=0.001$), but correlated positively with BMI ($r=0.12$, $p=0.05$), ICAM ($r=0.13$, $p=0.032$) and PAI-1_{act}. ($r=0.15$, $p=0.009$).

Lag time had a significant positive correlation with age only ($r=0.21$, $p<0.001$), whilst slope correlated positively with CRP ($r = 0.26$, $p < 0.001$), and negatively with diastolic blood pressure (DBP) ($r=-0.14$, $p=0.035$), PAI-1_{act} ($r=-0.17$, $p=0.003$) and triglycerides (TG) ($r=-0.12$, $p=0.033$). Maximum absorbance also correlated positively with CRP, as well as with TC and LDL-C, but demonstrated a negative correlation with PAI-1_{act}. ($r=-$

0.15, $p=0.006$). There was a significant positive correlation found between CLT and WC, weight, BMI, HbA1c, CRP, PAI-1_{act}, LDL-C as well as TG. Clot lysis time additionally showed negative correlations with SBP, eGFR, GGT and HDL-C, indicating a decrease in CLT with respect to an increase in these variables.

Table 4-2: Correlations between covariates and plasma clot properties at baseline

Variables	Total fibrinogen (g/L)		γ' fibrinogen (%)		Lag time (min)		Slope (au/s)		Maximum absorbance (Δ au)		CLT (min)	
	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
Age (years)	0.02	0.792	-0.03	0.57	0.21	<0.001	-0.02	0.749	0.07	0.202	-0.07	0.219
WC (cm)	0.06	0.357	0.02	0.712	-0.05	0.428	-0.01	0.915	-0.01	0.890	0.27	<0.001
Weight (kg)	0.06	0.402	-0.01	0.922	-0.04	0.507	-0.01	0.934	-0.01	0.929	0.23	0.001
BMI (kg/m ²)	0.08	0.188	0.12	0.05	0.03	0.655	-0.03	0.626	0.04	0.497	0.34	<0.001
SBP (mmHg)	-0.10	0.153	-0.06	0.376	0.07	0.314	-0.08	0.219	-0.05	0.415	-0.19	0.004
DBP (mmHg)	-0.07	0.326	-0.05	0.449	0.08	0.263	-0.14	0.035	-0.10	0.138	-0.07	0.327
CrCl (ml/min)	0.14	0.042	-0.18	0.007	-0.08	0.246	-0.01	0.930	-0.05	0.472	-0.02	0.821
eGFR (mL/min/1.73 m ²)	0.12	0.085	-0.23	0.001	-0.06	0.337	0.04	0.557	-0.03	0.655	-0.27	<0.001
GGT (U/L)	-0.06	0.320	-0.04	0.518	-0.01	0.848	-0.04	0.471	-0.10	0.072	-0.21	<0.001
Glucose (mmol/L)	-0.06	0.408	-0.04	0.593	0.02	0.823	-0.07	0.290	-0.02	0.733	-0.03	0.664
HbA1c (%)	0.08	0.206	0.10	0.126	-0.07	0.269	0.09	0.161	0.13	0.056	0.28	<0.001
CRP (mg/L)	0.38	<0.001	-0.08	0.158	0.003	0.962	0.26	<0.001	0.28	<0.001	0.20	0.001
IL-6 (pg/ml)	0.27	<0.001	-0.11	0.063	0.076	0.180	0.08	0.18	0.10	0.091	-0.10	0.084
SuPAR (ng/ml)	-0.02	0.781	-0.02	0.775	0.02	0.734	-0.06	0.311	-0.09	0.125	-0.06	0.29

ICAM (ng/ml)	-0.04	0.468	0.13	0.032	-0.11	0.061	0.06	0.291	-0.04	0.523	-0.07	0.253
VCAM (ng/ml)	-0.04	0.540	0.09	0.118	-0.06	0.301	0.06	0.338	-0.07	0.199	-0.06	0.297
PAI- 1 _{act} (U/ml)	-0.01	0.860	0.15	0.009	0.03	0.649	-0.17	0.003	-0.15	0.006	0.44	<0.001
TC (mmol/L)	-0.02	0.750	-0.01	0.862	0.03	0.659	0.04	0.560	0.19	0.005	0.02	0.741
HDL-C (mmol/L)	-0.12	0.063	-0.03	0.664	-0.00	0.974	0.04	0.598	0.07	0.332	-0.33	<0.001
LDL-C (mmol/L)	0.09	0.202	-0.02	0.788	0.06	0.388	0.08	0.234	0.23	0.001	0.14	0.041
TG (mmol/L)	-0.05	0.400	0.02	0.687	-0.07	0.247	-0.12	0.033	-0.10	0.096	0.32	<0.001

WC = waist circumference; BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure; HbA1c = glycated haemoglobin; eGFR = estimated glomerular filtration rate; GGT = gamma-glutamyl transferase; ICAM = intercellular adhesion molecule; VCAM = vascular cell adhesion molecule; BMI = body mass index; PAI-1_{act} = plasminogen activator inhibitor-1 activity; CLT = clot lysis time; CRP = C-reactive protein; IL-6 = interleukin 6; SuPAR = soluble urokinase-type plasminogen activator receptor; TC = total cholesterol; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; TG = triglycerides; CrCl = creatinine clearance; γ' = gamma prime.

4.1.1.3 Differences in plasma clot properties between the HIV-infected and uninfected groups

Table 4-3 presents the differences in the plasma clot property variables, at baseline, between the HIV-infected and uninfected groups, obtained from t-tests (unadjusted) and Generalised Linear Models (GLMs). Covariates adjusted for in the GLM included those that both differed between the two groups and correlated with the outcome haemostasis variables, as reported in Tables 4-1 and 4-2. Two consecutive GLMs were performed; the first, Model 1, adjusted for age, sex, BMI, SBP, HDL-C, GFR, GGT, CRP and ICAM, whilst the second, Model 2, additionally included fibrinogen to determine whether potential differences in clot properties were due to differences in fibrinogen concentration between the two groups.

The HIV-infected group had higher % γ' fibrinogen ($p = 0.010$) and longer CLT ($p < 0.001$) but lower total fibrinogen ($p=0.038$) and maximum absorbance values ($p<0.001$). There were no significant differences found for lag time and slope between the two groups. The HIV-infected and uninfected groups had % γ' fibrinogen mean values of 14.3 ± 7.39 % and 12.0 ± 8.18 %, and CLT mean values of 58.7 ± 9.57 and 54.6 ± 10.8 mins, respectively. The mean maximum absorbance values for the HIV-infected and HIV-uninfected participants were 0.37 ± 0.13 and 0.44 ± 0.15 Δau , respectively. The differences remained after adjusting for the covariates and fibrinogen in the first and second generalised regression models, which demonstrates that lower maximum absorbance and longer CLT among the HIV-infected individuals, were not the result of the differences in fibrinogen concentration or differences in the other covariates between the HIV-infected and uninfected participants.

Table 4-3: Plasma clot properties of HIV-infected and HIV-uninfected participants.

Variables	HIV-infected n=151	HIV- uninfected n=176	t-test p-value	Model 1 p-value	Model 2 p-value
Total fibrinogen (g/L)	3.03 [1.71]	3.47 [1.98]	0.038	0.013	-
γ' fibrinogen (%)	14.3 [7.39]	12.0 [8.18]	0.010	0.05	-
Lag time (min)	4.61 [2.06]	4.99 [2.01]	0.101	0.080	0.246
Slope (au/s)	6.18 [4.48]	7.08 [4.18]	0.068	0.092	0.212
Maximum absorbance (Δ au)	0.37 [0.13]	0.44 [0.15]	<0.001	<0.001	0.001
CLT (min)	58.8 [9.57]	54.7 [10.8]	0.001	0.002	0.003

CLT = clot lysis time.

- The data have been presented as mean [standard deviation]
- Model 1 = Generalised linear model that adjusted for age, , BMI, SBP, HDL-C, GFR, GGT, CRP and ICAM
- Model 2 = Generalised linear model additionally adjusted for fibrinogen

4.1.2 Follow-up comparisons (2010)

The 2010 data used a similar approach to that for the baseline data, but compared three groups: the HIV-infected participants treated with ART with those who were not on treatment and with the HIV-uninfected participants. Firstly, the data on the covariates are reported (Table 4-4) to show which covariates were included in the GLM models for the plasma clot properties (Table 4-5).

4.1.2.1 Differences in covariates

The covariates were compared at the 2010 follow-up between the three groups (treated, untreated and HIV-uninfected), and the results presented in Table 4-4. Systolic blood pressure (SBP) was significantly lower in both HIV-infected groups compared to the HIV-uninfected group ($p < 0.001$), with no difference between the two HIV-infected groups, whilst DBP was significantly lower in the untreated group

compared to the uninfected group ($p=0.032$). Both these groups demonstrated non-significant differences with the treated group. Glycated haemoglobin was significantly lower in the HIV treated group compared to the untreated ($p=0.004$) and the uninfected ($p<0.001$) group, while SuPAR was significantly higher in the HIV-treated group compared to the untreated and HIV uninfected groups ($p<0.001$). There were no significant differences found between the untreated and uninfected groups for SuPAR.

The ICAM and VCAM levels of the three groups also differed. ICAM was significantly higher in the two HIV-infected groups compared to the HIV-uninfected group (treated $p<0.001$; untreated $p<0.001$), while VCAM was the highest in the untreated group ($p<0.001$) with no difference between the HIV treated and the HIV-uninfected groups. The HIV-treated group had significantly higher GGT and PAI-1_{act} compared to the untreated group ($p=0.014$ for GGT and $p=0.014$ for PAI-1_{act}), but did not differ from the HIV-uninfected group. There were differences also found in the lipid profiles between the groups. The TC levels were significantly higher in the treated and uninfected ($p<0.001$) groups, with mean values of 4.98 ± 1.30 mmol/L and 4.84 ± 1.16 mmol/L, respectively, compared to the untreated group, which had a mean value of 4.16 ± 1.02 mmol/L. The same was also found for LDL-C, with the HIV treated group having the highest mean LDL-C of 2.95 ± 1.06 mmol/L, followed by the HIV-uninfected group with a mean of 2.77 ± 1.08 and the HIV untreated group with a mean of 2.44 ± 0.84 mmol/L. There were no significant differences between the groups for WC, BMI, CrCl, GFR, CRP, fasting glucose and IL-6. In 2010, the CD4 counts were available and compared between the treated and untreated HIV-infected groups, but there were no significant differences found between these two groups.

Table 4-4: Differences between the three groups at 2010 follow-up

Variable	HIV-treated n=70	HIV-untreated n=81	HIV-uninfected n=176	ANOVA p-value
Age (years)	48 (44-53)	47 (42-51) ^a	48 (45-55) ^a	0.047
Men/Women (%)	28.6 / 71.4	31 / 69	39.2 / 60.8	0.197 [#]
WC (cm)	75.5 [9.89]	76.4 [10.0]	78.3 [10.9]	0.110
BMI (kg/m ²)	20.2 (18.5-24.7)	22.1 (19.2-27.5)	22.5 (19.6-27)	0.127
SBP (mmHg)	125 [20.3] ^a	122 [16.3] ^b	132 [21.2] ^{a, b}	<0.001
DBP (mmHg)	83.8 [12.8]	83.2 [11.1] ^a	86.9 [11.6] ^a	0.032
CrCl (ml/min)	104 [33.1]	113 [39.6]	104 [36.8]	0.212
eGFR (mL/min/1.73m ²)	145 [37.4]	145 [33.2]	138 [36.0]	0.209
GGT (U/L)	55.7 (30.7-104) ^a	35.1 (19.7-66.9) ^a	38 (22.8-84.4)	0.020
Glucose (mmol/L)	4.89 [0.61]	4.82 [1.52]	5.05 [1.45]	0.416
HbA1c (%)	5.50 [0.47] ^{a, b}	5.93 [0.92] ^a	6.01 [0.89] ^b	<0.001
CRP (mg/L)	4 (1.43-8.95)	3.21 (1.09-8.55)	3.47 (1.09-7.34)	0.405
IL-6 (pg/ml)	3.4 (2.45-5.2)	4.11 (2.36-6.16)	3.66 (2.31-6.5)	0.688
SuPAR (ng/ml)	4.52 (3.63-5.82) ^{a, b}	3.84 (3.12-4.84) ^a	3.68 (3.03-4.41) ^b	<0.001
ICAM (ng/ml)	355 (281-470) ^a	378 (298-468) ^b	293 (244-362) ^{a, b}	<0.001
VCAM (ng/ml)	886 (720-1041) ^a	1058 (883-1370) ^b	671 (577-801) ^{a, b}	<0.001
PAI-1 _{act} (U/mL)	5.43 (3.82-9.13) ^a	3.75 (0.80-6.81) ^a	4.69 (2.39-8.35)	0.016

TC (mmol/L)	4.98 [1.30] ^a	4.16 [1.02] ^{a, b}	4.84 [1.16] ^b	<0.001
HDL-C (mmol/L)	1.31 [0.54] ^a	1.16 [0.43] ^b	1.52 [0.57] ^{a, b}	<0.001
LDL-C (mmol/L)	2.95 [1.06] ^a	2.44 [0.84] ^{a, b}	2.77 [1.08] ^b	0.007
TG (mmol/L)	1.34 (0.81-2.02) ^{a, b}	0.96 (0.76-1.21) ^a	0.98 (0.73-1.38) ^b	0.014
CD4 (cells/mm ³)	400 [222]	331 [188]	-	0.052*

WC = waist circumference; SBP = systolic blood pressure; DBP = diastolic blood pressure; HbA1c = glycated haemoglobin; eGFR = estimated glomerular filtration rate; GGT = gamma-glutamyl transferase; ICAM = intercellular adhesion molecule; VCAM = vascular cell adhesion molecule; BMI = body mass index; CRP = C-reactive protein; IL-6 = interleukin 6; SuPAR = soluble urokinase-type plasminogen activator receptor; TC = total cholesterol; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; TG = triglycerides; CrCl = creatinine clearance; PAI-1_{act} = plasminogen activator inhibitor-1 activity.

- ^{a, b} = Means with the same symbol differ significantly
- # = Chi-square comparison
- * = t-test comparison
- The normally distributed data are presented as mean [standard deviation], while not normally distributed data are presented as median (interquartile range)

4.1.2.2 Differences in plasma clot properties

Table 4-5 presents the differences in plasma clot property variables at follow-up between the HIV-treated, untreated and HIV-uninfected groups. Covariates adjusted for in the GLM included those that differed between the groups, as reported in Table 4-4. Performed were two consecutive GLMs; the first, Model 1, adjusted for age, sex, SBP, GGT, HbA1c, SuPAR, VCAM, PAI-1, HDL-C and LDL-C whilst the second, Model 2, included fibrinogen to determine whether potential differences in clot properties were due to differences in fibrinogen concentration between the groups. Where more than one variable from a common biological pathway differed, for example the different inflammatory or lipid markers, then only the variable demonstrating the biggest differences between the groups was included to limit co-linearity and overfitting of the models.

Both the HIV treated ($p=0.005$) and untreated ($p=0.028$) groups had significantly lower fibrinogen concentrations than the HIV-uninfected group, and this significance remained after adjustment for the covariates. There was, however, no difference in fibrinogen concentration between the treated and untreated HIV groups. The HIV groups also had a lower mean slope ($p=0.002$ for the treated group, $p=0.038$ for the untreated) and maximum absorbance ($p<0.0001$ for the treated group, $p=0.001$ for the untreated) than the HIV-uninfected group. The differences in maximum absorbance remained after adjustment for covariates in both Model 1 ($p<0.0001$) and Model 2 ($p=0.015$), while the differences in slope disappeared after additionally adjusting for fibrinogen in Model 2. The HIV treated group had a significantly longer CLT than the HIV-uninfected group in the unadjusted model. However, after addition of the covariates in Model 1, the difference was no longer significant, but became significant again after additionally adjusting for fibrinogen in Model 2 ($p=0.026$). The mean CLT of the HIV untreated group did not differ significantly from the other two groups. There were no significant differences in lag time or $\% \gamma'$ fibrinogen between the three groups.

Table 4-5: Between-group differences in plasma clot properties at 2010 follow-up

Variables/Groups	HIV-treated n=70	HIV-untreated n=81	HIV-uninfected n=176	ANOVA p-value	Model 1 p-value	Model 2 p-value
Fibrinogen (g/L)	3.32 [0.88] ^a	3.42 [0.90] ^b	3.77 [1.09] ^{a, b}	0.002	<0.0001	-
γ' fibrinogen (%)	10.3 [3.76]	10.9 [4.23]	10.1 [3.23]	0.265	0.180	-
Lag time (min)	4.88 [0.91]	4.88 [0.94]	4.69 [0.82]	0.147	0.114	0.157
Slope (au/s)	7.27 [2.72] ^a	7.64 [2.66] ^b	8.51 [2.53] ^{a, b}	0.001	<0.0001	0.140
Maximum absorbance (Δ au)	0.48 [0.15] ^a	0.51 [0.16] ^b	0.59 [0.18] ^{a, b}	<0.0001	<0.0001	0.015
CLT (min)	64.1 [10.1] ^a	62.4 [10.2]	60.6 [8.76] ^a	0.026	0.161	0.026

CLT = clot lysis time.

- The data are presented as mean [standard deviation]
- ^{a, b} = means with the same symbol differ significantly
- Model 1: Generalised linear model adjusted for age, sex, SBP, HbA1c, GGT, SuPAR, VCAM, PAI-1_{act}, HDL-C and LDL-C.
- Model 2: Generalised linear model additionally adjusted for fibrinogen

4.1.3 Changes over time

The performing of paired t-tests was to determine which variables changed within the groups over the 5-year period, followed by mixed linear models to determine whether changes in plasma clot properties differed over the same period between the groups. The addition of covariates that changed over the five years to the mixed linear models, particularly those for which the changes were different between the three groups, allowed the investigation of whether changes in plasma clot properties over time were independently associated with HIV and/or ART, or whether driven by changes in the covariates.

4.1.3.1 Changes over time within groups

For several variables, the changes within the three groups over the 5-year period were similar; for example, CrCl, eGFR and SuPAR increased significantly in all three groups, while ICAM decreased. Others, such as SBP and DBP showed no significant changes over time, while the remainder of the variables demonstrated different changes over time in the three groups.

Regarding anthropometry, WC increased significantly both in the HIV treated and the HIV-uninfected group, with a similar increase in BMI, although not significant in the HIV treated group. Neither WC nor BMI changed significantly in the HIV untreated group. GGT levels demonstrated a significant increase in the HIV treated group ($p=0.028$) and a decrease in the HIV untreated ($p=0.03$), as well as uninfected groups ($p<0.0001$). Regarding glucose metabolism, fasting glucose tended to increase in all three groups, with the increase in the HIV treated group being significant ($p=0.01$), while the increase in HbA1c was significant for the HIV untreated ($p=0.001$) and the HIV-uninfected group ($p<0.0001$). In terms of inflammatory markers, CRP increased significantly only in the HIV treated group ($p=0.002$) and HIV-uninfected group ($p=0.021$), while IL-6 demonstrated a borderline significant increase in the HIV treated group ($p=0.085$), with SuPAR increasing significantly in all the three groups. VCAM decreased significantly in the HIV-treated group ($p<0.0001$) but increased significantly in the HIV untreated ($p<0.0001$) and uninfected groups ($p=0.004$). The HIV treated group also demonstrated a worsening of the lipid profile compared to the untreated and the HIV-uninfected groups. LDL-C increased significantly in the HIV

treated group ($p=0.02$), while TG demonstrated a borderline increase in the same group. There were no observed changes in LDL-C and TG in the HIV untreated and the HIV-uninfected groups. However, HDL-C significantly increased in the HIV treated group ($p=0.004$), but decreased in both the HIV untreated (0.002) and the uninfected ($p<0.0001$) groups. Furthermore, PAI-1_{act} increased significantly in the HIV treated ($p=0.0007$) and uninfected ($p=0.042$) groups, while no significant change was observed in the HIV untreated group.

Total fibrinogen tended to increase over time in all three groups, with the increase reaching significance in the HIV untreated group ($p=0.03$). Percentage γ' fibrinogen decreased significantly in all three groups. Both slope (albeit borderline in the HIV treated group) and maximum absorbance increased over time in all three groups. CLT increased significantly in both the HIV treated and the uninfected group ($p<0.0001$, both), with no change in the HIV untreated group. Table 4-6 presents these results.

Table 4-6: Comparison of baseline with follow-up data within the HIV treated, untreated and uninfected groups

Variables	HIV-treated n=70			HIV-untreated n=81			HIV-uninfected n=176		
	2005 Mean [SD]	2010 Mean [SD]	p-value	2005 Mean [SD]	2010 Mean [SD]	p-value	2005 Mean [SD]	2010 Mean [SD]	p-value
Age (years)	43.5 (40-48)	48 (44-53)	-	42 (37-47)	47 (42-51)	-	44 (40-50.5)	48 (45-55)	-
WC (cm)	73.3 [7.28]	75.5 [9.89]	0.045	76.5 [11.2]	76.4 [10]	0.905	76.7 [10.3]	78.4 [10.9]	<0.0001
BMI (kg/m ²)	20.4 (18.3-23.6)	20.2 (18.5-24.7)	0.223	21.9 (19.1-27.3)	22.1 (19.2-27.5)	0.912	21.5 (19.3-25.9)	22.5 (19.6-27)	<0.0001
SBP (mmHg)	121 [17.6]	125 [20.3]	0.184	124 [18.0]	122 [16.3]	0.247	130 [22.3]	132 [21.2]	0.350
DBP (mmHg)	82.8 [12.5]	83.9 [12.8]	0.771	84.3 [12.7]	83.2 [11.1]	0.478	86.4 [14.2]	86.9 [11.6]	0.752
CrCl (ml/min)	87.1 [28.1]	104 [33.1]	0.001	100 [35.8]	113 [39.6]	0.004	101 [32.3]	104 [36.8]	0.036
eGFR (mL/min/1.73m ²)	118 [40.7]	145 [37.4]	<0.0001	126 [38.4]	145 [33.2]	<0.0001	133 [40.4]	138 [36]	0.060
GGT (U/L)	39.6 (30-65)	55.7 (30.7-104)	0.028	42.9 (25-74.3)	35.1 (19.7-66.9)	0.03	46.6 (30.6-87)	38 (22.8-84.4)	<0.0001
Glucose (mmol/L)	4.62 [0.78]	4.89 [0.61]	0.011	4.69 [0.90]	4.82 [1.52]	0.491	4.91 [0.85]	5.05 [1.45]	0.223
HbA1c (%)	5.43 [0.43]	5.50 [0.47]	0.264	5.55 [0.43]	5.93 [0.92]	0.001	5.53 [0.51]	6.01 [0.89]	0.0001
CRP (mg/L)	1.87 (0.61-5.76)	4 (1.43-8.95)	0.002	3.03 (0.89-9.02)	3.21 (1.09-8.55)	0.291	2.21 (0.71-6.5)	3.47 (1.09-7.34)	0.021
IL-6 (pg/ml)	4.13 (2.50-7.81)	3.4 (2.45-5.2)	0.085	4.69 (2.41-6.76)	4.11 (2.36-6.16)	0.686	3.64 (2.18-6.25)	3.66 (2.31-6.5)	0.323
SuPAR (ng/ml)	3.93 (3.12-5.47)	4.52 (3.63-5.82)	0.01	3.55 (2.79-4.34)	3.84 (3.12-4.84)	0.033	3.29 (2.74-4.07)	3.68 (3.03-4.41)	<0.0001
ICAM (ng/ml)	513 (313-844)	355 (281-470)	0.0004	575 (433-839)	378 (298-468)	<0.0001	432 (268-720)	293 (244-362)	<0.0001
VCAM (ng/ml)	1007 (528-1913)	886 (720-1041)	<0.0001	878 (451-1429)	1058 (883-1370)	<0.0001	496 (314-898)	671 (577-801)	0.004

PAI-1 _{act} (U/ml)	3.82 (0.92-6.75)	5.43 (3.82-9.13)	0.0007	4.11 (1.68-7.68)	3.75 (0.80-6.81)	0.429	4.29 (1.25-7.72)	4.69 (2.39-8.35)	0.042
TC (mmol/L)	4.30 [1.15]	4.98 [1.30]	<0.0001	4.54 [1.23]	4.16 [1.02]	0.011	5.13 [1.34]	4.84 [1.16]	0.012
HDL-C (mmol/L)	1.14 [0.46]	1.31 [0.54]	0.004	1.36 [0.57]	1.16 [0.43]	0.002	1.74 [0.74]	1.52 [0.57]	<0.0001
LDL-C (mmol/L)	2.63 [0.86]	2.95 [1.06]	0.020	2.60 [0.93]	2.44 [0.84]	0.143	2.89 [1.14]	2.77 [1.08]	0.267
TG (mmol/L)	1.11 (0.84-1.39)	1.34 (0.81-2.02)	0.054	1.03 (0.80-1.37)	0.96 (0.76-1.21)	0.209	1 (0.76-1.38)	0.98 (0.73-1.38)	0.96
Total fibrinogen (g/L)	3.04 [1.86]	3.32 [0.88]	0.220	3.01 [1.59]	3.42 [0.90]	0.028	3.47 [1.98]	3.77 [1.09]	0.085
γ' fibrinogen (%)	14.3 [7.68]	10.3 [3.76]	0.001	14.3 [7.20]	10.9 [4.23]	0.001	12.0 [8.18]	10.1 [3.23]	0.008
Lag time (min)	4.34 [1.96]	4.88 [0.91]	0.078	4.85 [2.12]	4.88 [0.94]	0.704	4.99 [2.01]	4.69 [0.82]	0.058
Slope (au/s)	5.93 [4.80]	7.27 [2.72]	0.056	6.38 [4.22]	7.64 [2.66]	0.015	7.08 [4.18]	8.51 [2.53]	<0.001
Max absorbance (Δ au)	0.34 [0.11]	0.48 [0.15]	<0.0001	0.39 [0.13]	0.51 [0.16]	<0.0001	0.44 [0.15]	0.59 [0.18]	<0.0001
CLT (min)	57.5 [9.72]	64.1 [10.1]	<0.0001	60.0 [9.34]	62.4 [10.2]	0.080	54.7 [10.8]	60.6 [8.76]	<0.0001

WC = waist circumference; BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure; HbA1c = glycated haemoglobin; eGFR = estimated glomerular filtration rate; GGT = gamma-glutamyl transferase; ICAM = intercellular adhesion molecule; VCAM = vascular cell adhesion molecule; BMI = body mass index; PAI-1_{act} = plasminogen activator inhibitor-1 activity; CLT = clot lysis time; CRP = C-reactive protein; IL-6 = interleukin 6; SuPAR = soluble urokinase-type plasminogen activator receptor; TC = total cholesterol; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; TG = triglycerides; CrCl = creatinine clearance; γ' = gamma prime.

- The normally distributed data are presented as mean [standard deviation], while the data not normally distributed are presented as median (interquartile range).

4.1.3.2 Changes in plasma clot properties over time between groups

The study also determined the differences in the degree of change over time between the three groups, using mixed linear models. Firstly, these changes were determined using an unadjusted model. Next, to investigate whether changes in plasma clot properties over time were independently associated with HIV and/or ART, or whether driven by changes in the covariates, the mixed linear models were adjusted for covariates that demonstrated different changes over time between the three groups. Model 1 adjusted for age, sex, PAI-1_{act}, WC, HbA1c, SUPAR, VCAM, HDL-C, LDL-C, TG and GGT, whilst Model 2, additionally adjusted for fibrinogen concentration. Where more than one variable from a common biological pathway differed, for example both the glucose metabolism markers, HbA1c and fasting glucose, or the different inflammatory markers, then only the variable demonstrating the biggest differences between the groups were included to limit co-linearity and overfitting of the models. The presentation of the results was in the form of graphs visualising the changes from one time point to the next. The interaction effect p-value indicates whether the changes over time differed between the three groups.

4.1.3.2.1 Changes in total fibrinogen concentration

While fibrinogen concentration tended to increase over the 5-year period, there was no significant difference in this increase between the three groups, in the unadjusted ($p=0.915$) or the adjusted models ($p=0.829$). These results are presented in Figure 4-1 and Figure 4-2 for the unadjusted model and Model 1 (fully adjusted model), respectively.

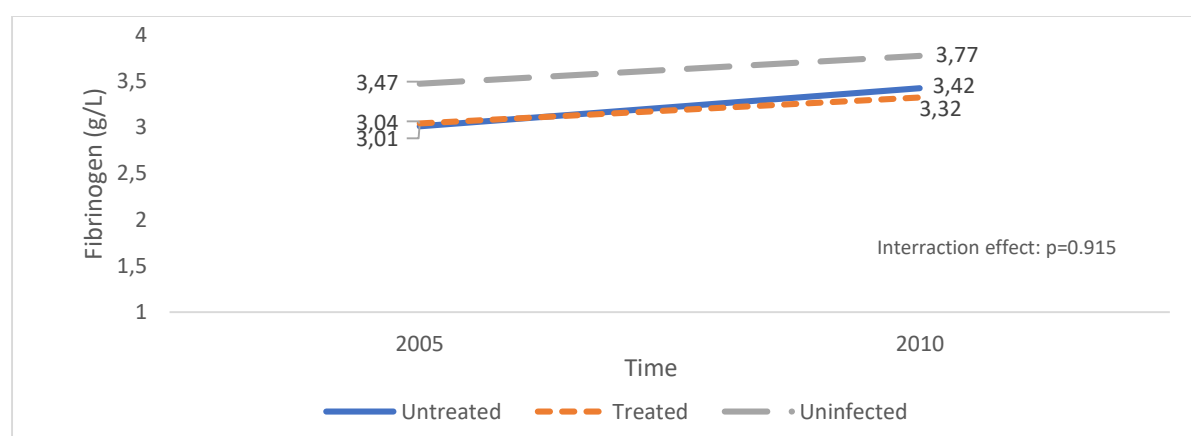


Figure 4-1: The between-group differences in the degree of change over time (unadjusted)

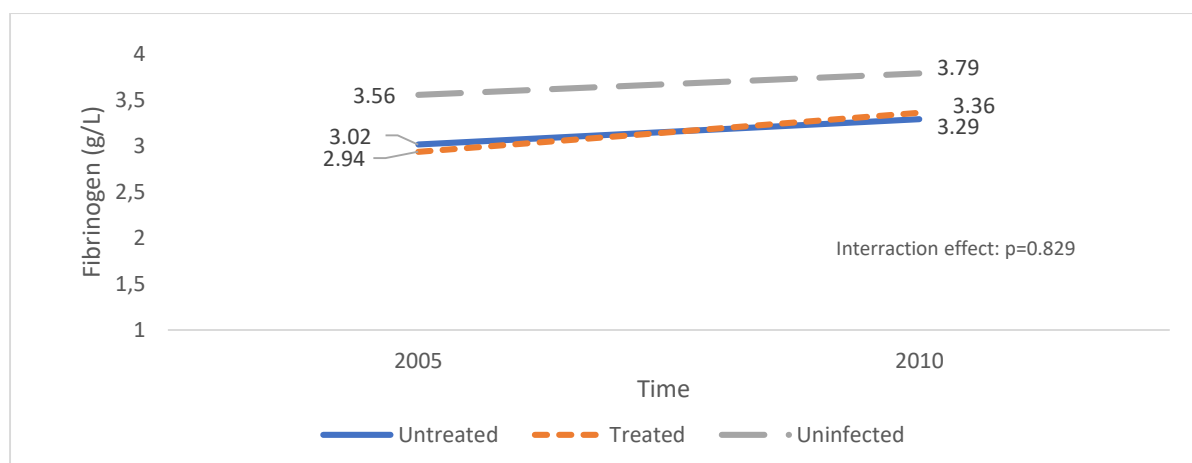


Figure 4-2: The between-group differences in the degree of change over time (Model 1)

4.1.3.2.2 Changes in total % γ' fibrinogen

Percentage fibrinogen γ' decreased in all three groups over the 5-year period, with no significant difference in the rate of decrease between the three groups, before ($p=0.175$) or after adjustment for covariates ($p=0.134$). Figures 4-3 and 4-4 graphically present these results.

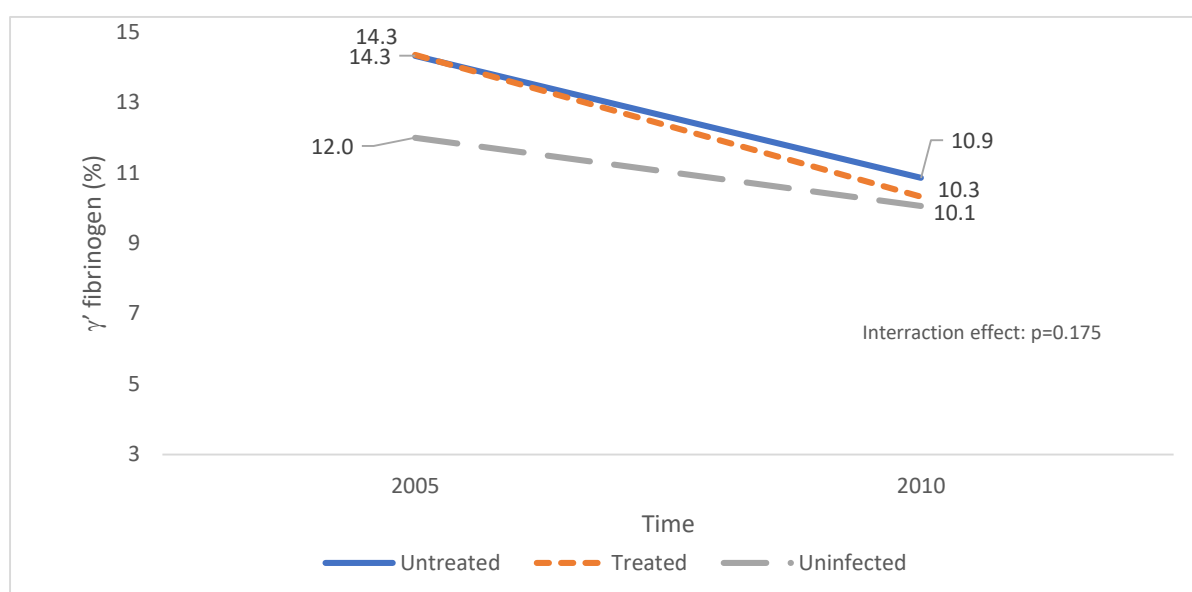


Figure 4-3: The between-group differences in the degree of change in % γ' fibrinogen over time (unadjusted)

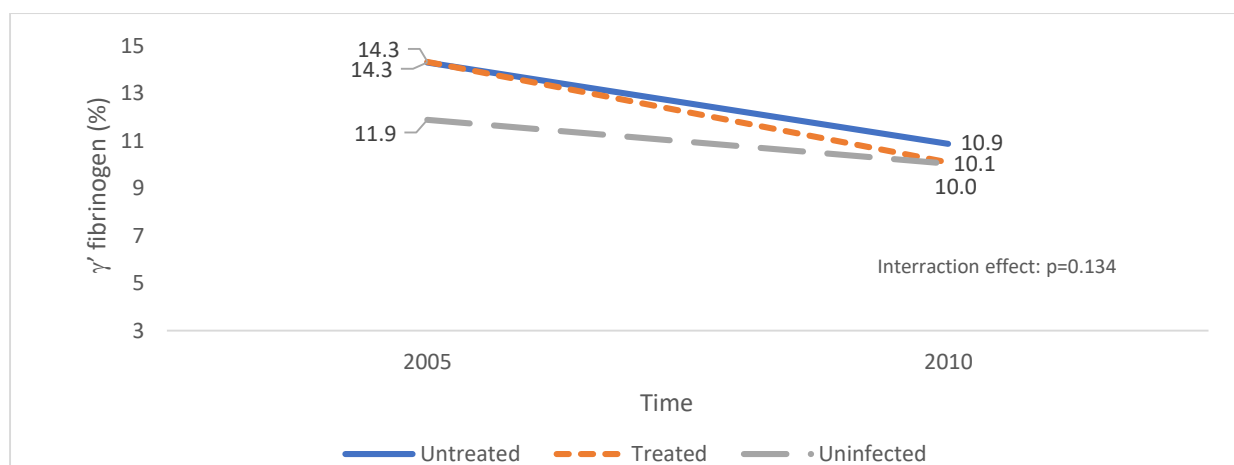


Figure 4-4: The between-group differences in the degree of change in % γ' fibrinogen over time (Model 1)

4.1.3.2.3 Changes in lag time

The same approach was applied in analysing the between-group differences in the degree of change over time for the lag time. Firstly, unadjusted mixed linear model analysis was performed followed by a model that adjusted for age, sex, PAI-1_{act}, WC, HbA1c, SUPAR, VCAM, HDL-C, LDL-C, TG and GGT, referred to as Model 1. Model 2, which additionally adjusted for total fibrinogen concentration, followed this. Figures 4-5, 4-6 and 4-7 present the results for the three models respectively, and illustrate the changes in lag time over time between the three groups differed significantly, also after full adjustment. Lag time increased in the HIV treated group, with little change over time observed in the HIV untreated and the uninfected groups.

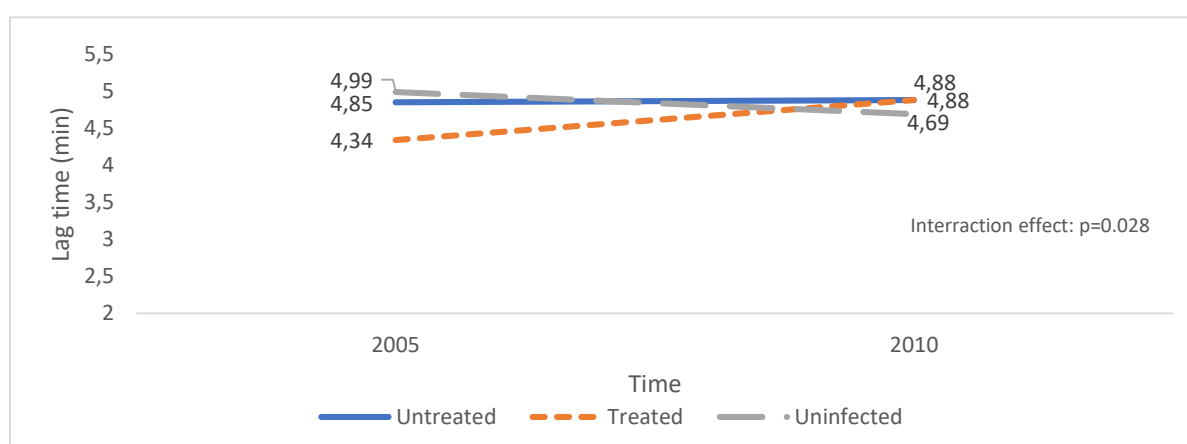


Figure 4-5: The between-group differences in the degree of change in lag time over time (unadjusted)

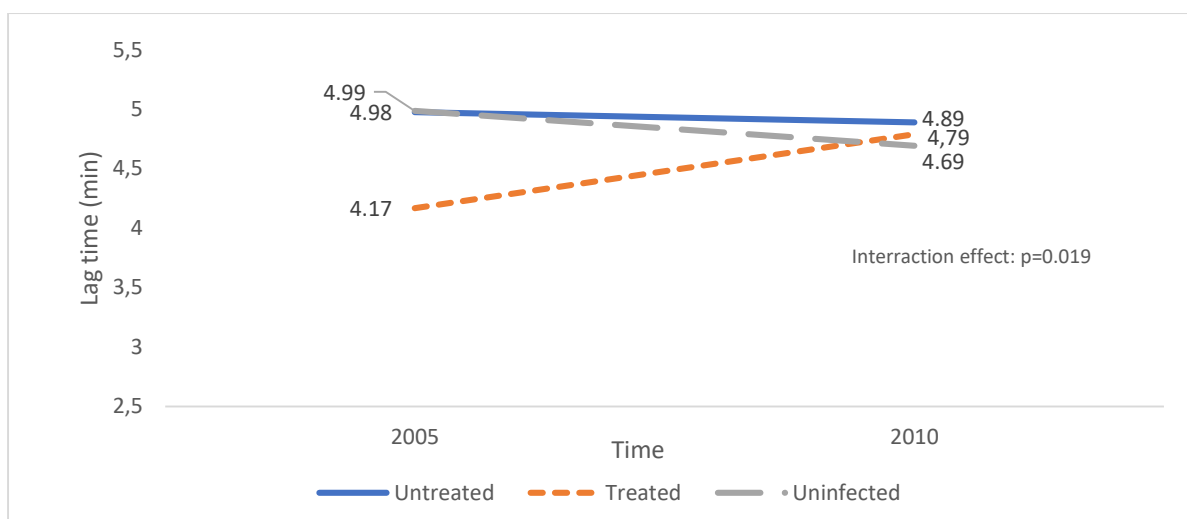


Figure 4-6: The between-group differences in the degree of change in lag time over time (Model 1)

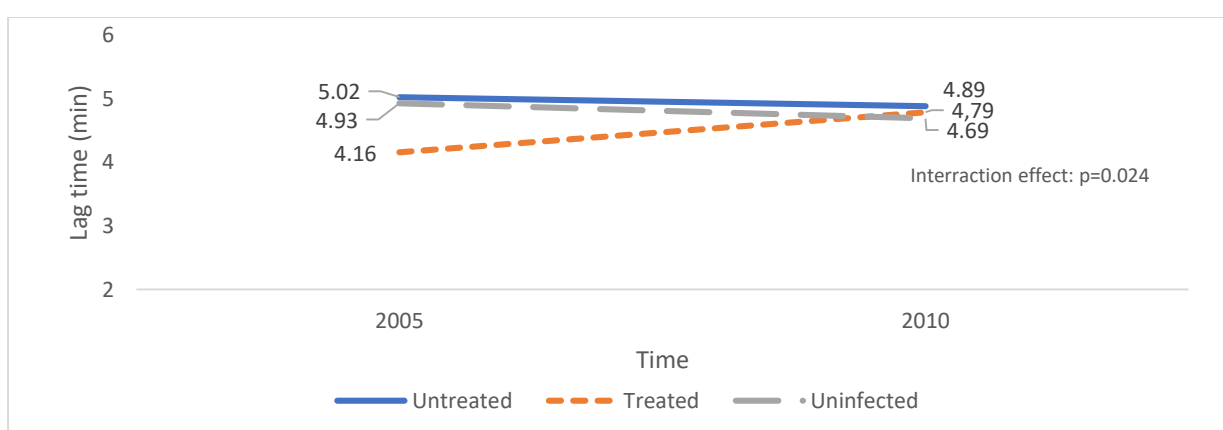


Figure 4-7: The between-group differences in the degree of change in lag time over time (Model 2)

4.1.3.2.4 Changes in slope

The results in Figures 4-8, 4-9 and 4-10, for the unadjusted and adjusted analyses, illustrate that while slope increased over time, there were no significant differences in the degree or extent of change in slope between the three groups over the 5-year period. Although not statistically significant, it does appear the slope increased to a lesser extent in the HIV untreated group, compared to the HIV treated and the uninfected group.

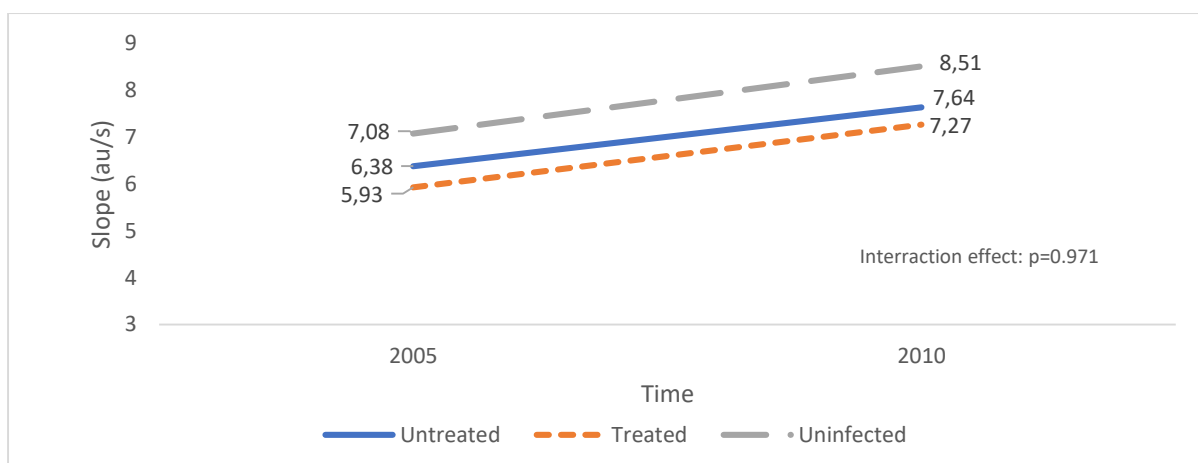


Figure 4-8: The between-group differences in the degree of change in slope over time (unadjusted)

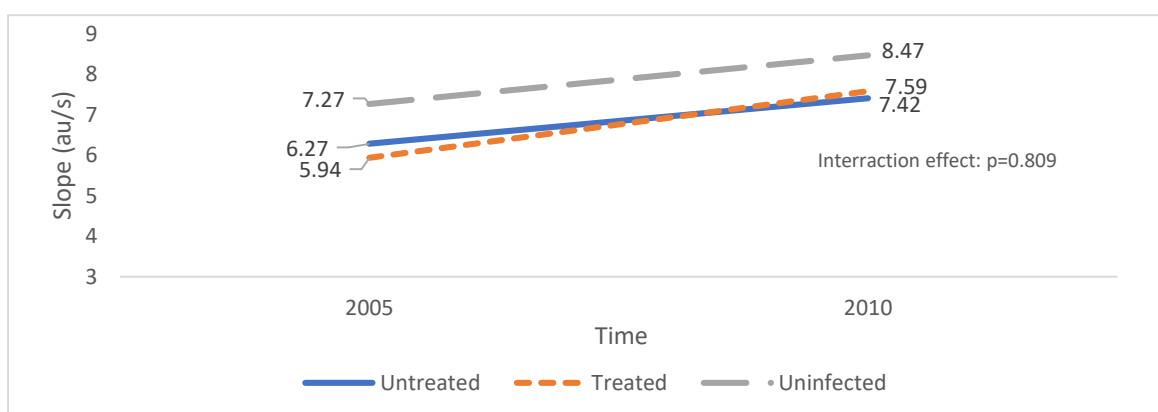


Figure 4-9: The between-group differences in the degree of change in slope over time (Model 1)

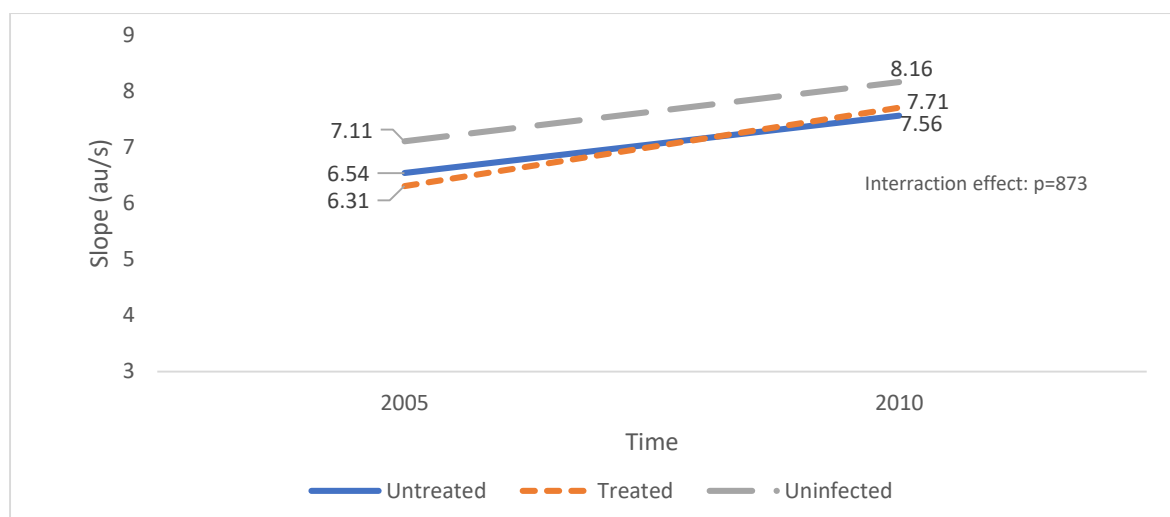


Figure 4-10: The between-group differences in the degree of change in slope over time (Model 2)

4.1.3.2.5 Changes in maximum absorbance

Maximum absorbance increased over time, but again the results illustrate there were no significant differences between the treated, untreated and uninfected groups in terms of their degree of change over the 5-year period for maximum absorbance. The results were the same before and after adjusting for all the listed covariates. However, similar to slope, the increase in maximum absorbance although not statistically significantly so, appeared to be less than the increases observed in the HIV treated and uninfected groups. Figures 4-11, 4-12 and 4-13 present the results for the unadjusted, Model 1 and Model 2 analyses, respectively.

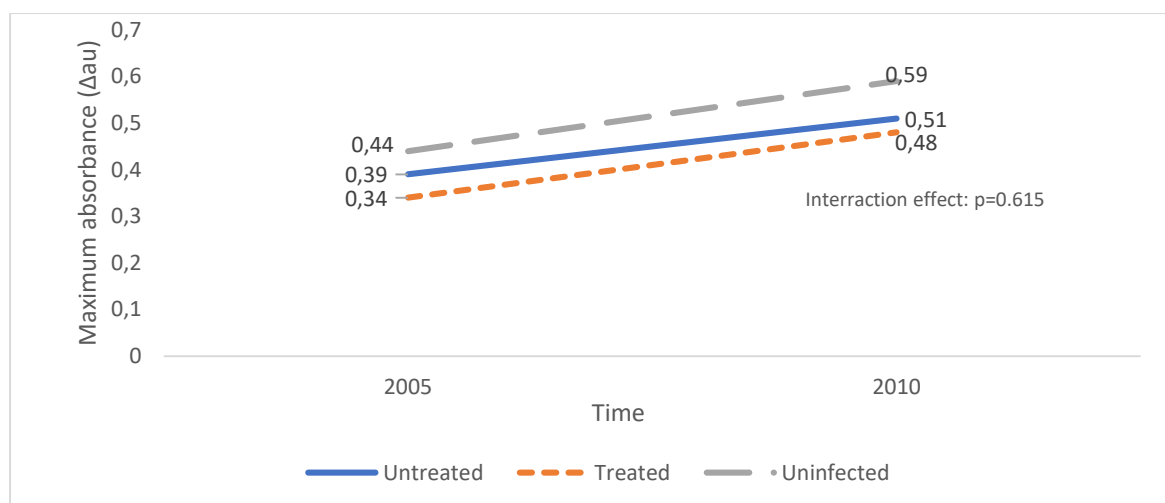


Figure 4-11: The between-group differences in the degree of change in maximum absorbance over time (unadjusted).

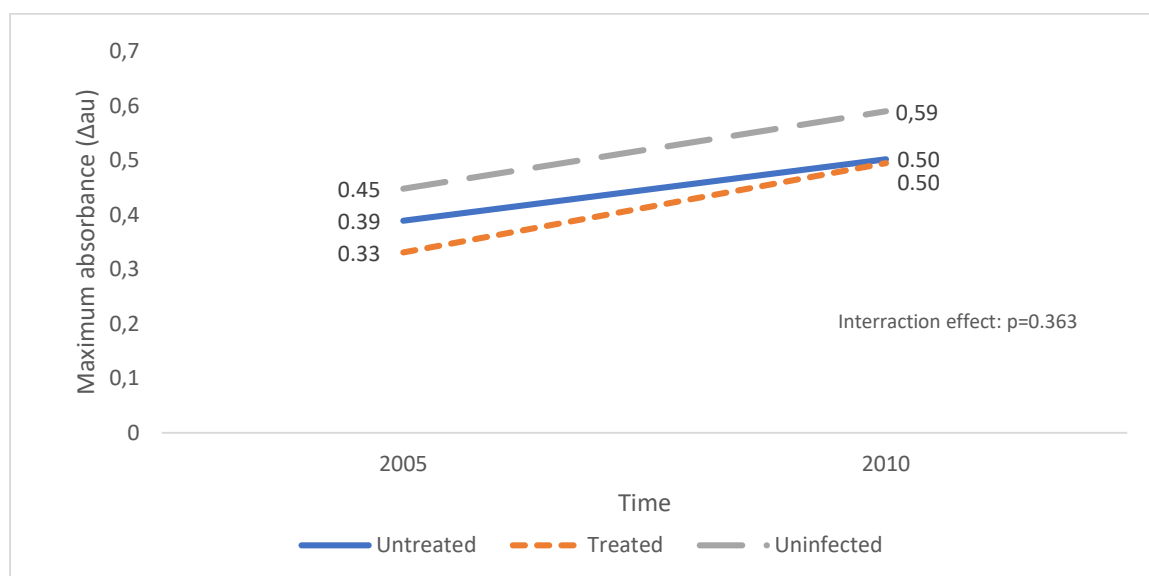


Figure 4-12: The between-group differences in the degree of change in maximum absorbance over time (Model 1)

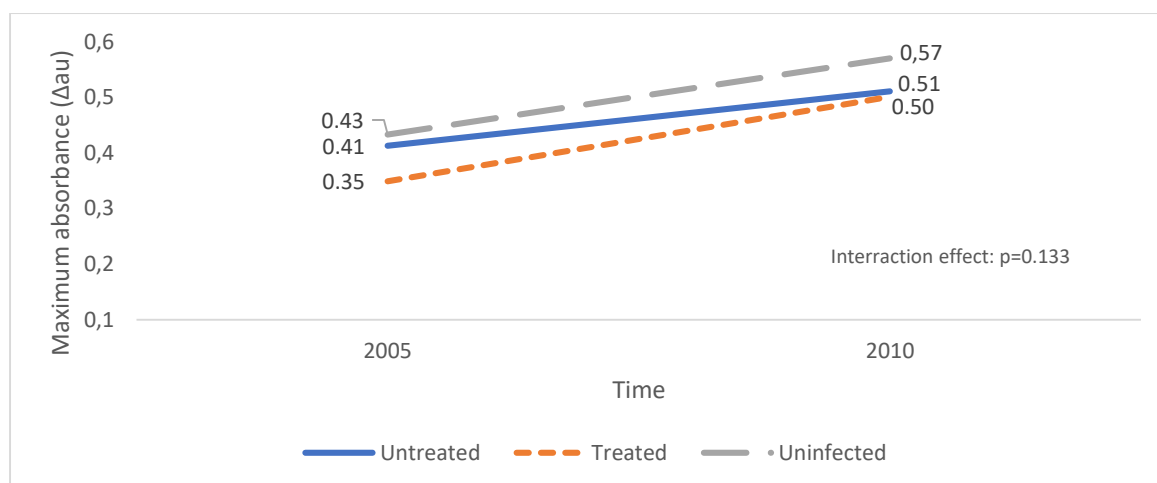


Figure 4-13: The between-group differences in the degree of change in maximum absorbance over time (Model 2)

4.1.3.2.6 Changes in clot lysis time

Clot lysis time increased (or tended to) in all three groups over time. Again, similar to slope and maximum absorbance, although no statistical significant difference was observed in the degree of increase over time between the three groups in any of the models ($p \approx 0.1$ for all three), the increase in the HIV untreated group appeared to be less than that observed in the HIV treated and the uninfected groups. This was consistent in all three models.

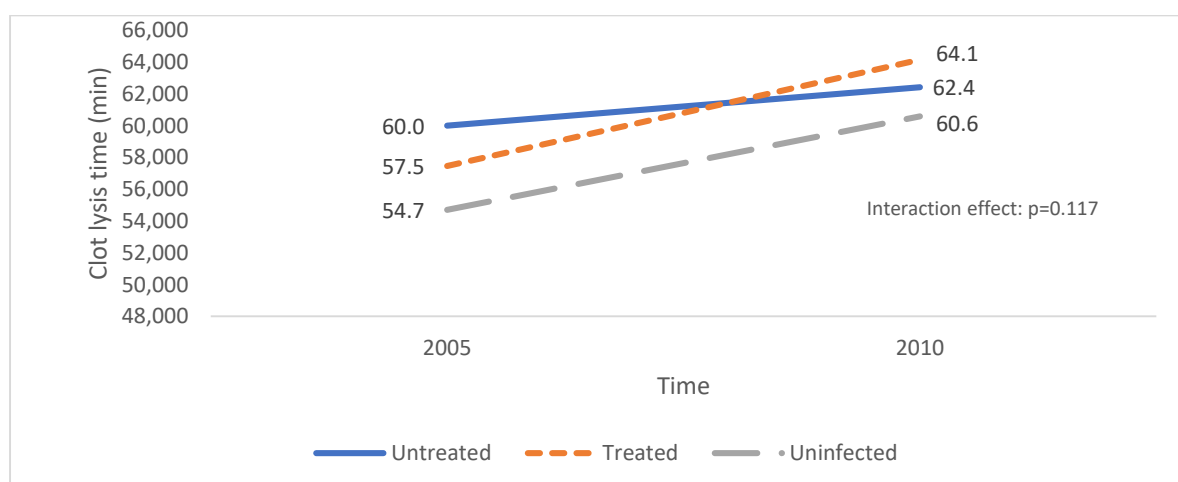


Figure 4-14: The between-group differences in the degree of change in CLT over time (unadjusted)

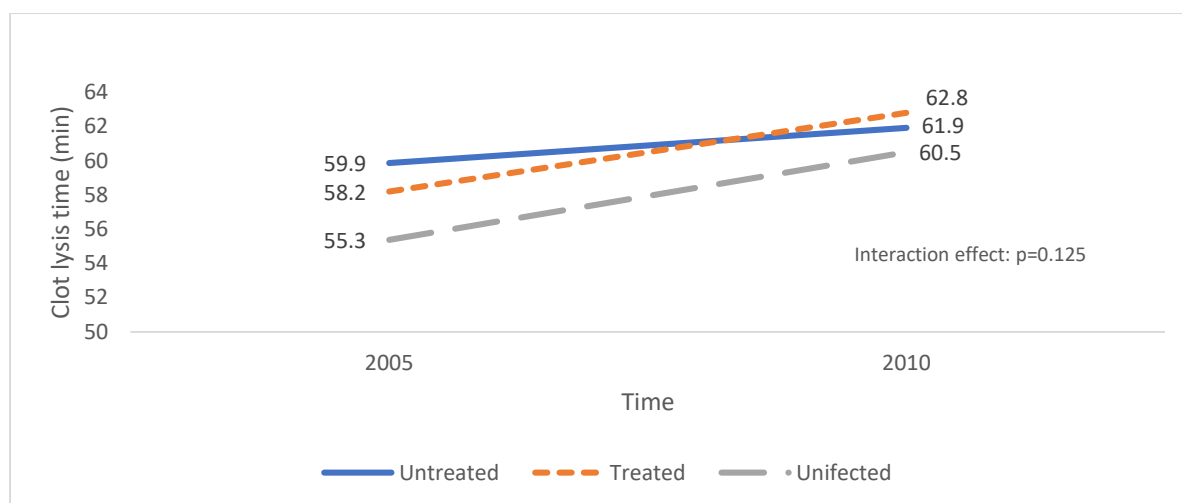


Figure 4-15: The between-group differences in the degree of change in CLT over time (Model 1)

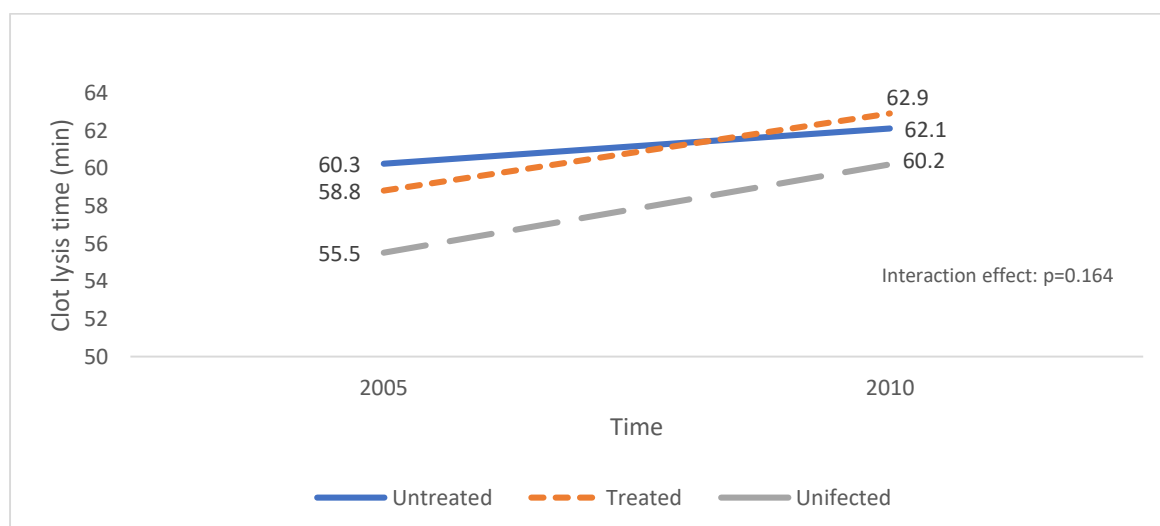


Figure 4-16: The between-group differences in the degree of change in CLT over time (Model 2)

Chapter 5 will discuss the results presented in this chapter.

CHAPTER 5: DISCUSSION AND CONCLUSION

5.1 Introduction

This prospective study aimed to investigate the association of HIV status and antiretroviral therapy (ART) respectively, with plasma clot properties in a sub-group of participants from the South African, North West Province arm of the Prospective Urban and Rural Epidemiology (SA-NW-PURE) study. Both HIV and ART are reportedly associated with hypercoagulability, and therefore increased thrombotic risk (De Gaetano Donati *et al.*, 2010). It is hypothesised that this relationship might, at least in part, be due to altered fibrin clot properties among HIV-infected patients, since altered clot properties is considered one of the main contributors of hypercoagulability and thrombosis-related cardiovascular complications (Jackson & Pretorius, 2019; Jacobson *et al.*, 2004).

This study analysed data of 151 HIV-infected PURE-SA-NW study participants newly diagnosed in 2005, 70 of whom were receiving ART by 2010 and 81 who were still untreated. These participants were systematically matched to 176 HIV-uninfected individuals by considering their sex, age and body mass index (BMI). The study aimed to achieve the following objectives:

1. To cross-sectionally compare fibrinogen concentration and plasma clot characteristics obtained with turbidity measurements of:
 - HIV-infected (n = 151) and HIV negative (n = 176) participants at baseline;
 - HIV-infected treatment-naïve (n = 81) participants, HIV-infected treated (n = 70) and HIV negative (n = 176) participants at follow-up.
2. To prospectively compare within groups, fibrinogen concentration and plasma clot characteristics obtained with turbidity measurements of:
 - Participants who were HIV free at baseline and follow-up (n = 176);
 - HIV-infected participants who were treatment-naïve at baseline and follow-up (n = 81);
 - HIV-infected participants who were treatment-naïve at baseline and treated at follow-up (n = 70).

3. To prospectively compare individual haemostatic variables and plasma clot characteristics obtained with turbidity measurements between the three groups, as indicated in objective 2.

In this chapter the results presented in Chapter 4 will be discussed to achieve the main aim of the study as well as the above-specified objectives. Discussed first are the cross-sectional between-group comparisons, both at baseline and at the 2010 follow-up, followed by the within-group changes from baseline to follow-up. Lastly, the differences in change between the three groups will be discussed. Possible limitations of this study and conclusions drawn from this work, as well as suggestions for future studies will be provided at the end of this chapter.

5.2 The association of HIV and ART with plasma clot properties

In this section, the results on fibrinogen concentration and plasma clot properties in relation to HIV as well as ART will be discussed. First the cross-sectional differences and then the prospective differences are addressed in the sections below.

5.2.1 The cross-sectional association of HIV and ART with fibrinogen concentration and plasma clot properties

5.2.1.1 The association of HIV with fibrinogen concentration and plasma clot properties at baseline: Comparison between HIV-uninfected and newly diagnosed untreated HIV-infected individuals

As only individuals with newly diagnosed HIV (and not previously known status) were included in the PURE-SA-NW study in 2005, the baseline comparison was between newly diagnosed untreated HIV-infected and HIV-uninfected controls. From the results, there were a number of differences observed for different variables, including fibrinogen concentration and clot properties.

The results show that total fibrinogen concentration was lower, while percentage γ' fibrinogen was higher in the HIV-infected individuals. These differences remained even after adjusting for age, sex, body mass index (BMI), systolic blood pressure

(SBP), high-density lipoprotein cholesterol (HDL-C), estimated glomerular filtration rate (eGFR), gamma-glutamyltransferase (GGT), C-reactive protein (CRP) and intercellular adhesion molecule (ICAM), which all differed between the HIV-infected and uninfected individuals, and which correlated with fibrinogen concentration and the clot properties. It is known that increased total and γ' fibrinogen are factors that contribute to altered clot properties (Danesh *et al.*, 2005; Lovely *et al.*, 2002a; Lovely *et al.*, 2010a). The results from the present study indicate that although total fibrinogen concentration was lower in the HIV-infected group, it was still within the normal range of 1.5 to 4.5 g/L in both groups (De Moerloose *et al.*, 2010). Despite having correlated with a number of other potential covariates, such as CrCl and IL-6, the difference in total fibrinogen concentration between the two groups remained significant even after additionally adjusting for these covariates. This suggests that the lower fibrinogen concentration in the HIV-infected group might have potentially arisen from other (not measured) mechanisms attributed or related to HIV. These results concur with a previous publication that reported on a larger sample of the same study population. The study aimed to determine how known polymorphisms within the fibrinogen gene and environmental factors influence the change in total and γ' fibrinogen over the same 5-year period and also showed the lower total fibrinogen concentration in the HIV-infected individuals compared to the uninfected group (Jobse *et al.*, 2015). Looking at known fibrinogen polymorphisms, the researchers found a higher prevalence of the common allele of the single nucleotide polymorphism (SNP) at rs4220 among the HIV-infected group, which was associated with lower fibrinogen concentration, suggesting this genotype-HIV interaction as a possible reason behind the observed results (Jobse *et al.*, 2015). This SNP is situated in exon 8 of the *FGB* gene. The data from this study population contradicts previous findings of studies that have reported higher total fibrinogen concentration in HIV-infected compared to uninfected individuals (Jahoor *et al.*, 1999; Koppel *et al.*, 2002; Lijfering *et al.*, 2008; Madden *et al.*, 2008). However, most of these studies were conducted in HIV-infected participants who were already on ART treatment, suggesting that their results may have been influenced by the ART treatment, especially since certain regimens of ART, such as PIs, which were used by the participants in those studies, are known to be associated with higher fibrinogen concentration (Koppel *et al.*, 2002; Madden *et al.*, 2008).

The HIV-infected group presented with higher % γ' fibrinogen compared to the HIV-uninfected group, however, for both groups the levels still fell within the normal range of 8-15% (Chung & Davie, 1984). Gamma prime fibrinogen is a γ -chain splice variant produced by alternative mRNA processing through the substitution of 4 C-terminal amino acids with a 20-amino acid peptide resulting in a γ -chain with a 16 amino acid extension (Chung & Davie, 1984; Lovely *et al.*, 2010b). Increased % γ' fibrinogen, above normal levels, is associated with the formation of clots that are resistant to fibrinolysis due to its effect on fibrin clot structure (Duval & Ariëns, 2017; Falls & Farrell, 1997). Since % γ' fibrinogen remained higher in the HIV-infected group, also after full adjustment, it can be hypothesised that other HIV-related mechanisms (not investigated here) may have contributed to the formation of more γ' chain splice variants.

Moving to the clot properties, the results indicate maximum absorbance to be lower in the HIV-infected compared to the HIV-uninfected group. The results remained the same even after adjusting for all the potential covariates that differed between the groups, and at the same time correlated with maximum absorbance and other clot properties. These include fibrinogen, which, as discussed above, was lower in the HIV-infected group and is known to be positively associated with maximum absorbance (Pieters *et al.*, 2020). Maximum absorbance is indicative of overall clot density and fibre diameter (Pieters *et al.*, 2020). Increased maximum absorbance is associated with increased fibrin fibre diameter, increased clot density and may be indicative of prothrombotic clots (Jahoor *et al.*, 1999; Pieters *et al.*, 2020). Therefore, the lower maximum absorbance of the fibrin clots in the HIV-infected individuals may indicate the formation of plasma clots that are less dense and consequently with lower thrombotic risk than those of healthy controls. Studies have shown that LDL-C can bind to fibrin fibres, increasing the clot density and eventually the maximum absorbance (Pieters *et al.*, 2020; Skrzydlewski, 1976). In the current study, LDL-C correlated with maximum absorbance and was significantly lower in the HIV-infected than the HIV uninfected group. However, even after adjusting for LDL-C, the difference in maximum absorbance remained, indicating the existence of additional HIV-related mechanisms or other mechanisms that were not measured in this study. There were no significant differences in lag time and slope between the HIV-infected and HIV-

uninfected individuals at baseline. Lag time is indicative of the duration of time required for sufficient formation and growth of protofibrils, from thrombin addition to the beginning of lateral aggregation, while slope is an indicator of the rate of lateral aggregation (Carr Jr & Hermans, 1978; Pieters *et al.*, 2008; Weisel & Nagaswami, 1992).

Contrary to the decreased maximum absorbance, clot lysis time (CLT) was longer in the HIV-infected individuals than the controls. The results remained significant after adjusting for the known covariates, including fibrinogen. Longer CLT is a known indicator of prothrombotic (fibrinolysis resistant) clots and is associated with cardiovascular diseases (De Lange *et al.*, 2012; Lisman *et al.*, 2005). The current results concur with the findings from a De Lange *et al.* (2012) paper that analysed the baseline data of the larger PURE-SA-NW study population and found longer CLT among the HIV-infected individuals at baseline. As previously discussed, γ' fibrinogen is associated with the formation of prothrombotic clots that are lysed at a slower rate (longer CLT), and the current study also found higher γ' fibrinogen in the HIV-infected group. The longer CLT in the HIV-infected group might therefore, be related to the higher γ' fibrinogen concentration. The difference remained, however even after adjusting for γ' fibrinogen. It is likely that the increased CLT in the HIV-infected individuals is not the result of altered clot properties, as is evident by the fact that adjustment for γ' fibrinogen did not explain the difference in CLT between the two groups, and also since the HIV-infected individuals had less dense clots than the controls, which in theory would have resulted in faster clot lysis. CLT, particularly when measured using the turbidity assay, is not affected by clot structure only, but also by a number of other proteins in the coagulation and fibrinolytic pathways; these include plasminogen, the protein responsible for lysing fibrin fibres, as well as its activator, tissue plasminogen activator (tPA) and inhibitors of the fibrinolytic pathway, including thrombin activatable fibrinolysis inhibitor (TAFI), tissue plasminogen activator inhibitor-1 (PAI-1) and α 2-antiplasmin (Mutch *et al.*, 2007; Voros *et al.*, 2015). Of all these, only PAI-1_{act} was measured in this study, and additional adjustment for PAI-1_{act} in the regression models did not significantly alter the results.

In conclusion, at baseline, the HIV-infected individuals had lower total fibrinogen and higher % γ' fibrinogen concentration than the HIV-uninfected group. However, the levels were within the normal ranges in both groups. Regarding plasma clot properties, the HIV-infected group formed less-dense clots, which however, had longer CLT. The longer CLT may be the result of the altered levels of other coagulation and fibrinolytic proteins, not measured in this study, rather than altered fibrin clot structure. Since the difference in maximum absorbance and CLT remained after full adjustment, the data suggests that these results could be attributable to other HIV-related mechanisms not investigated in this study.

5.2.1.2 The association of HIV and ART with fibrinogen concentration and plasma clot properties at follow-up: comparison between HIV-infected (ART treated and untreated) and HIV-uninfected individuals

In this section, the association of HIV and ART, respectively, with fibrinogen concentration and plasma clot properties at 2010 follow up will be discussed. For the 2010 follow-up, the HIV-infected participants were grouped into treated and untreated, and then compared to the HIV-uninfected individuals. An approach similar to the baseline analysis was followed for this time point. Two GLM analyses, following an ANOVA, differentiated the three groups while adjusting for covariates that differed between the groups, as the differences in these variables may have contributed to the differences in clot properties observed. The first GLM (Model 1) adjusted for; age, sex, SBP, HbA1c, GGT, SuPAR, VCAM, PAI-1_{act}, HDL-C, and LDL-C, whilst Model 2 additionally included fibrinogen. The treated and untreated HIV-infected groups differed in a number of potential covariates. The treated group demonstrated significantly higher GGT, SuPAR, PAI-1_{act}, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG), but also presented with significantly lower HbA1c than the untreated group. The differences in the variables have the potential to, at least in part, contribute to the observed levels of the outcome variables.

At follow-up (2010), the HIV-infected participants had been living with HIV for five years longer than at baseline, therefore, particularly in the untreated group, their risk for HIV-related complications had potentially increased (Mellors *et al.*, 2007). The CD4 count of the HIV treated group, although not significantly so, was 20% higher (400

cells/mm³) than that of the HIV-untreated group (331 cells/mm³), suggesting that the weakened immunity among the untreated individuals may result in HIV-related opportunistic infections, including cardiovascular complications (Deeks & Phillips, 2009; Schillaci *et al.*, 2008).

Similar to baseline, at follow-up, total fibrinogen concentration was significantly lower in the two HIV-infected groups compared to the HIV-uninfected group, with fibrinogen concentration being lowest in the HIV-treated group. The differences remained after adjusting for the covariates in Model 1, suggesting that the lower fibrinogen concentration in the HIV-infected groups was likely not attributed to mechanisms related to the aforementioned covariates. As explained in Section 5.2.1.1, the lower total fibrinogen concentration among the HIV-infected groups, could be attributable to the *FGB* rs4220 SNP genotype-HIV interaction, as suggested in the Jobse *et al.* (2015) paper. In addition, the Nonnucleoside reverse transcriptase inhibitors (NNRTIs) used by the HIV-treated group may have also contributed to the trend of a lower mean total fibrinogen concentration in the HIV-treated compared to the untreated group. NNRTIs have previously been reported to be associated with lower total fibrinogen Concentration (Madden *et al.*, 2008; Martin *et al.*, 2010). However, this could not be statistically replicated in this study, which may be attributed to the sample size. At follow-up, no significant differences were observed between the groups for γ' fibrinogen or lag time. The results for lag time are in agreement with the baseline data, demonstrating no difference between HIV-infected and uninfected individuals. Fibrinogen γ' was however, significantly higher in the HIV-infected than the uninfected group at baseline. While the difference was no longer significant at follow-up, the mean γ' fibrinogen of the HIV-untreated group was still higher than that of the controls, with the HIV-treated group having a mean level in between the HIV-untreated and the uninfected group.

Similar to that observed at baseline, slope (rate of lateral aggregation) was lower in the HIV-infected individuals than in the controls, with no difference in the treated compared to the untreated group. This difference however, disappeared after adjustment for fibrinogen concentration, indicating that the lower slope in the HIV-infected individuals were largely attributed to the lower fibrinogen concentration. It

does however, not appear as if ART resulted in an altered rate of lateral aggregation compared to individuals not on ART, apart from being associated with a somewhat lower fibrinogen concentration. Similarly, maximum absorbance (clot density) was lower in both the HIV groups compared to the control group, with no significant difference between the treated and untreated groups. Adjustment for the covariates as well as fibrinogen concentration did not significantly alter these findings. The results indicate the possible contribution from other, as of yet unidentified mechanisms related to HIV, but not to ART use *per se*.

CLT was longer in the HIV-treated group compared to the HIV-uninfected group. While the HIV-treated group had a longer mean CLT than the untreated group, the difference was not significant. The mean CLT of the untreated group was also not different from the HIV-uninfected. The differences disappeared after adjusting for age, sex, SBP, HbA1c, GGT, SuPAR, VCAM, PAI-1_{act}, HDL-C and LDL-C. Most of these variables are factors known to influence different clot properties, including CLT (Puccetti *et al.*, 2002; Van Meijer & Pannekoek, 1995). In particular, PAI-1_{act}, SuPAR and LDL-C were all significantly higher in the treated compared to the untreated group. PAI-1 inhibits fibrinolysis by binding t-PA, thus preventing it from binding to plasminogen to form plasmin. Higher concentrations of PAI-1 are furthermore, known to be associated with increased thrombotic events (De Pergola & Pannacciulli, 2002; Zhou *et al.*, 2003). LDL-C is reported to decelerate clot degradation by forming hydrogen and ion bonds with fibrin fibres, which eventually result in reduced clot permeation and longer CLT (Puccetti *et al.*, 2002; Skrzydlewski, 1976). SuPAR, a marker of inflammation and immune activation, is known to influence plasminogen-activation (Mondino & Blasi, 2004; Pawlak *et al.*, 2007), and also atherosclerosis and thromboembolism (Arbel & Strauss, 2015; Engström *et al.*, 2016), which are conditions strongly influenced by fibrinolysis-resistant clot structures (Libby & Simon, 2001). These factors could therefore have contributed to the longer CLT observed in the HIV-treated individuals. However, after adjusting for fibrinogen, the differences in CLT between the groups reappeared. This effect could be attributable to the differences in fibrinogen concentration between the treated and HIV-uninfected group, since fibrinogen was significantly higher in HIV-uninfected group and correlated positively with CLT, thereby rendering the differences significant again.

In conclusion, based on the cross-sectional comparison, ART use, for a period of 5-years, was not associated with significantly altered clot properties compared to untreated HIV-infected individuals. While fibrinogen tended to be lower in the group receiving ARTs, the difference was not significant. ART use was also associated with increased CLT. This longer CLT was, however, more strongly associated with higher PAI-1_{act}, SuPAR and LDL-C concentration in the ART group than with altered clot structure.

5.2.2 The prospective association of HIV and ART with fibrinogen concentration and plasma clot properties

In this section, the within-group changes over time and most importantly the differences in the extent of change between the groups will be discussed to determine the prospective association of HIV with fibrinogen and fibrin clot properties. Over time, changes in the different covariates, which were measured and compared prospectively within the groups, could relate to the differences observed in the clot properties. Waist circumference (WC), HbA1c, CRP, VCAM, PAI-1_{act} and LDL-C behaved differently over time between the three groups and were consequently added in a mixed model as covariates, alongside fibrinogen in Model 2. Total fibrinogen tended to increase in all three groups over time, while % γ' fibrinogen decreased, with no difference in the extent of change between the three groups. Lag time increased in the HIV-treated group, with little change over time observed in the HIV-untreated and the uninfected groups. Slope, maximum absorbance and CLT all increased to some extent over time in all three groups. However, for all three variables this increase appeared to be consistently less (though not statistically significant) in the HIV-untreated compared to the HIV-treated and control groups.

While there is cross-sectional evidence for both increased (Koppel *et al.*, 2002; Lijfering *et al.*, 2008; Madden *et al.*, 2008) and decreased (Jobse *et al.*, 2015; Montoya *et al.*, 2017a) fibrinogen concentration with HIV infection and ART use (depending on the type of ART), limited evidence exists for the change in fibrinogen levels over time. In a prospective study by Koppel *et al.* (2002), fibrinogen concentration increased over a 12-month period in both HIV-untreated and HIV individuals treated with PIs, but no comparisons to HIV-uninfected individuals. The current results demonstrate that in this

study population, fibrinogen concentration increased over time, but this increase was not affected by HIV status or ART use. The same can be said for % γ' fibrinogen, as there was no difference in the change (decrease) in % γ' fibrinogen between the three groups.

There is no known prospective evidence on how HIV or ART alters fibrin clot properties. In the current study, a significantly larger increase in lag time was observed in the HIV-treated compared to the other groups. None of the known adjusted covariates, including fibrinogen, significantly altered this finding. This suggests the possibility of an ART-related mechanism prospectively influencing lag time, or alternatively, this may have been due to factors not investigated in this study. Lag time is the time required for protofibrils to reach sufficient length to allow lateral aggregation (Carr Jr & Hermans, 1978; Weisel & Nagaswami, 1992), and hence, increased lag time indicates delayed clot formation. In agreement with our results, Rönsholt *et al.* (2015), reported delayed clot formation using thromboelastography (TEG) in Type 1 HIV patients on long-term combination ART compared to healthy controls. According to the literature, clots that take longer to form tend to have thicker and more loosely packed fibres that are easily broken down during fibrinolysis, compared to those formed at a faster rate (Bagoly *et al.*, 2017; Undas, 2017). However, this altered clot structure was not observed in the current study with the methodology used (turbidity) to determine clot structure and lysis, nor did Rönsholt *et al.* (2015) find differences in clot strength between the HIV-treated and control individuals, using TEG.

While slope, maximum absorbance and CLT all increased to some extent over time in all three groups, the increase appeared to be consistently less (though not statistically significant) in the HIV-untreated compared to the HIV-treated and control groups. This trend in change may be attributed to the natural age-associated increase in prothrombotic risk, where clot structures tend to become more prothrombotic as one grows older (Kurachi & Kurachi, 2000). It appears as though this progression was somewhat hampered in the HIV-untreated group, while in the HIV-treated group, the progression was more similar to that observed in HIV-uninfected individuals. The within-group changes over the five-year period indicate the HIV-treated group demonstrated an increase in WC, CRP, PAI-1_{act} and LDL-C, which may have

contributed to the comparatively faster rate of progression to a prothrombotic clot profile in the HIV-treated vs the untreated group (*via* the mechanisms previously discussed).

Overall, HIV infection and ART use demonstrated little effect on prospective changes in total and % γ' fibrinogen concentration, over a 5-year period, compared to changes observed in HIV-uninfected individuals. Furthermore, the natural age-associated progression of clot structure becoming more prothrombotic over time was somewhat delayed in the HIV group. However, ART use seems to neutralise this “protective effect,” partly because of the concomitant increase in co-variables known to be associated with hyper coagulation.

5.3 Limitations and recommendations

Turbidimetry is an indirect method of determining clot formation and structural properties. The use of only turbidity data might, therefore, have limited the potential of this study to unveil the association of HIV status and ART use with fibrinogen concentration and fibrin clot properties. Consequently, there is a need to investigate these associations further with additional direct methods, such as microscopy, permeability measurements and rheometry, to complement the measurements used in this dissertation. While the prospective nature of the study can help to provide mechanistic insight based on the sequence of events, such as how clot properties change over time in untreated HIV patients and those on ART, its use cannot determine causality. Although the study population was well characterised, it did not include measures of a number of coagulation and fibrinolytic variables that would have been useful for more detailed investigation, particularly regarding the CLT results. Furthermore, the duration of HIV infection, prior to inclusion in the study, was not documented nor was the viral load determined. CD4 count data was also only available in 2010. The current study also does not have information on treatment duration as well as treatment compliance among the HIV-treated participants included.

Lastly, there is a need to further study the clot properties of HIV-infected individuals who are using different ART regimens, as individuals in this study population all received the same standard government-approved ART regime.

5.4 Conclusion

In conclusion, the study has improved the understanding of and supports the current knowledge on fibrinogen concentration and fibrin clot properties in ART-treated and untreated HIV-infected individuals. The study has further provided a foundation and direction for other related studies to work from.

Overall, the study results and discussion have highlighted the following:

- The study found that HIV-infection is associated with lower total fibrinogen concentration and maximum absorbance when cross-sectionally compared to the HIV-uninfected group among the Black South Africans who took part in the PURE-SA-NW study.
- Cross-sectionally, both at baseline and 2010 follow-up, the study found HIV-infection to be associated with longer CLT. The longer CLT was likely not resulting from altered clot properties, but rather related to other variables affected by the HIV status, such as LDL-C and also by other pathways not measured in this study, such as potentially altered levels of coagulation and fibrinolytic proteins.
- Although not statistically proven, the data may also be suggestive of a possible association between Nonnucleoside reverse transcriptase inhibitors (NNRTIs) and reduced fibrinogen concentration.
- Over the five-year period, ART use was prospectively associated with longer lag times, which is indicative of slower growth of protofibrils to a sufficient length that allows lateral aggregation.
- HIV status and ART use did not significantly alter the age-associated increase in fibrinogen concentration, nor did it influence change in % γ' fibrinogen concentration over a 5-year period.

- HIV-infection demonstrated a tendency to delay the age-associated progression to more prothrombotic clot properties, while ART treatment, partly neutralised this “protective effect” over the 5-year period.

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ADDENDA

Addendum A: Ethics approval letter



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North-West University Health Research Ethics
Committee (NWU-HREC)

Tel: 018 299-1206
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studies)

26 November 2019

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Health Research Ethics Committee (NWU-HREC) on 26/11/2019, the NWU-HREC hereby approves your study as indicated below. This implies that the NWU-HREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: The association of HIV infection with plasma clot characteristics in black South Africans									
Principal Investigator/Study Supervisor/Researcher: Dr Z de Lange									
Student: S Bakali-33436487									
Ethics number:									
N	W	U	-	0	0	0	3	4	-
Institution			Study Number			Year		Status	
<u>Status:</u> S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation									
Application Type: Sub-study Commencement date: 26/11/2019 Expiry date: 30/11/2020									
Risk:					Minimal				
Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is due at the end of November annually until completion.									

General conditions: <i>While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:</i> - The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-HREC: - Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and - without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study. - The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-HREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited. - Annually a number of studies may be randomly selected for active monitoring. - The date of approval indicates the first date that the study may be started. - In the interest of ethical responsibility, the NWU-HREC reserves the right to:
--

- request access to any information or data at any time during the course or after completion of the study;
- to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;
- withdraw or postpone approval if:
 - any unethical principles or practices of the study are revealed or suspected;
 - it becomes apparent that any relevant information was withheld from the NWU-HREC or that information has been false or misrepresented;
 - submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or
 - new institutional rules, national legislation or international conventions deem it necessary.
- NWU-HREC can be contacted for further information via Ethics-HRECApply@nwu.ac.za or 018 299 1206

The NWU-HREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-HREC for any further enquiries or requests for assistance.

Yours sincerely,



Digitally signed by
Prof Petra Bester
Date: 2019.11.28
14:29:39 +02'00'

Chairperson NWU-HREC

Current details (25239522) G:\My Drive\9. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2_NWU-HREC_EAL.docm
20 August 2019

File Reference: 9.1.5.4.2

Addendum B: PURE-SA-NW 2005/2010 informed consent forms



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)



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NORTH-WEST UNIVERSITY
YUNIBESITHI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

PURE-SA Project (Prospective Urban and Rural Epidemiology)

INFORMED CONSENT FORM (including the PRIMER-study)

I, the undersigned(full names and surname)

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 understand that I have the right to withdraw at any time from the study.

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. DNA damage due to environmental factors such as cooking methods and smoke will be tested for.
 - c. Testing for liver function by determining liver enzymes such as AST, GGT,
 - d. Analyses of other than genetic parameters for Diabetes Mellitus such as HbA_{1c}, Blood glucose and Insulin
 - e. Analyses of the clotting profile and hypertension markers
 - f. Analyses of bone health, iron and nutrition status

And may be stored until such time as the above measurements/analyses will be done.
2. Body measurements such as height, weight, skin fold thicknesses, arm and leg circumferences will be taken
3. Vascular sonar will be done
4. Blood pressure to be taken
5. Pulse wave velocity measurements will be made
6. A Spirometer test to be performed to determine lung function
7. Bone density will be determined with an osteometer to detect possible osteoporosis

.....
(Signature of the subject)

Signed at ... Potchefstroom / Ganyesa ... (delete not applicable option) on/...../ 2010

Witnesses

1. 2.

Signed at ... Potchefstroom / Ganyesa ... (delete not applicable option) on/...../ 2010

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 health knowledge obtained from the results may be beneficial to you and 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.

Prof. ANNAMARIE KRUGER

Contact details: 062 771 5776 / 016 200 2065 (Office)

PART 1

1. Research Unit and Faculty:
Africa Unit for Transdisciplinary Health research (AUTHeRi); 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:
Prof. Annamarie Kruger, Ph.D. (Nutrition)

4. Rank/position of project leader:
Research Director and project 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, psychology questionnaire) with the help of field workers. A blood 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 bone density will be performed.

6. 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 8 [six] hours) to complete all the tests and questionnaires. 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, haemoglobin, HIV status and blood pressure, the information will be communicated to the individual to seek professional help during an individual feedback session. Since this study is a longitudinal study, subjects that are high at risk will be identified over time and personal feedback and counselling will be given. Also, an amount of R60 will be paid to each participant to cover transport costs and enable each person to go for follow-up at a clinic of choice.

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 neither tests positive, post counselling will also be given.

Addendum C: Plasma fibrinolytic potential protocol

Plasma fibrinolytic potential protocol (as performed on the PURE 2005/2010 samples at the NWU, Potchefstroom)

(Modified set-up for Erasmus MC, Rotterdam – Method D.C. Rijken & J. Malfliet – Z. de Lange & M. Pieters)

1. Start the microplate reader (Multiskan Ascent) and set the temperature to 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).

CaCl₂: make a stock of 425 mM in MQ-H₂O 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 μ l Assay Mixture (μ l)	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 μ M	10 μ M
Assay buffer	597.3		

- Dilution of plasma samples at room temperature:** to each well of a separate microtiter plate (untreated plates) add 71.4 μ l of citrated plasma sample followed by 50.0 μ l of assay buffer, using a multi-channel pipette, and shake on a plate shaker for 10 seconds at 1100 rpm. (Use 120 μ l citrated plasma and 84 μ l assay buffer for a measurement in duplicate).
- Make Assay Mixture. Add 15 μ l of the Assay Mixture per well of a microtiter plate (NUNC- Maxisorp).
- Check if the plate reader 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).
- Then, pipette using a multi-channel 85 μ l of the diluted plasma samples over to the plate containing the Assay Mixture (because we also want to determine lag time, clotting time and max absorbance, a timer is set to determine the time from pipetting the plasma dilution to the assay mix to the start of the measurement).
- Shake on a plate shaker 10 seconds at 1100 rpm.
- Cover each well with 50 μ l liquid paraffin oil (Merck, Darmstadt, Germany) (Note: The tips of the multi-channel have to be cut to facilitate pipetting the viscous liquid OR use wide-bore tips from Lasec).
- Place the plate into the plate reader and start measuring the A405nm every 9 seconds for the first two minutes, every 15 seconds up to 30 minutes and every minute until the clots have broken down or up to 270 minutes (according

to protocol by Lisman *et al* (2005)).

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

- Using a more diluted Assay Mixture and undiluted plasma might also be possible. The final plasma concentration should be 50%.
- A final tPA concentration of 100 ng/ml was selected to induce a clot lysis time of between 60
- 100 minutes using normal plasma. When samples with a low fibrinolytic potential are measured, one could consider using a higher tPA concentration.

References

Lisman, T., de Groot, P.G., Meijers, J.C. & Rosendaal, F.R. 2005. Reduced plasma fibrinolytic potential is a risk factor for venous thrombosis. *Blood*, 105(3):1102-1105.