The relationship between carotid intima-media thickness, serum biomarkers of cardiovascular disease, and HIV-infection in a South African study population

by

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DECLARATION

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Abstract

Background: Brachial artery flow-mediated dilatation (FMD) and carotid artery intima-media thickness (IMT) are measures of vascular endothelial function and subclinical atherosclerosis respectively. Both endothelial dysfunction and subclinical atherosclerosis have been linked with HIV and antiretroviral therapy (ART) in the developed world. Furthermore, known biomarkers of inflammation and endothelial dysfunction can be measured to predict cardiovascular outcomes in HIV-infected individuals. The exact link between endothelial dysfunction, atherosclerosis, HIV, ART and the biomarkers remains inconclusive, particularly in South African and Western Cape populations.

Aims: To investigate the putative relationship between subclinical atherosclerosis, endothelial dysfunction, biomarkers of cardiovascular disease (CVD), and HIV-infection in a Western Cape study population.

Methods: Two different epidemiological studies were performed: a cross-sectional main study (HIV-infected, n= 204 of whom on ART n=188 and without ART n= 16; and HIV-free, n= 143), and a longitudinal sub-study to assess the 12-month temporal progression of selected CVD outcomes (HIV-free, n= 57). For both studies, participants were recruited from health clinics in Cape Town and Worcester between 2017 and 2018. Lifestyle and health data were collected, and vascular (FMD% and IMT by ultrasound technology), anthropometric (body-mass-index [BMI]; waist circumference [WC]), cardiovascular (lipid profile; blood pressure [BP]; plasma glucose), renal (creatinine; urine albumin creatinine ratio [ACR]), liver (gamma-glutamyl-transferase [GGT]) and HIV-related (viral load; CD4, duration of HIV, duration of treatment) measures were obtained. Serum biomarkers were analyzed using Luminex technology, including C-reactive protein (CRP), tumour necrosis factor-alpha (TNF-α), vascular endothelial growth factor (VEGF), plasminogen activator inhibitor-1 (PAI-1) and adhesion molecules (intercellular adhesion molecule-1 [ICAM-1], vascular cellular adhesion molecule-1 [VCAM-1], e-selectin, p-selectin).

Results: In the longitudinal study (mean age: ~45 years), WC, plasma glucose, BP, and hypertension, increased at 12-months follow-up. In the cross-sectional study (mean age: ~41 years), the HIV-infected group showed a lower BMI, WC, BP, hypertension and PAI-1 versus the HIV-free group ($p<0.05$), whereas anaemia, ACR, creatinine, GGT and VCAM-1 were increased in the HIV-infected group ($p<0.05$). Additionally, viral load and VCAM-1 levels were higher in the HIV-infected without ART group versus the ART treated group ($p<0.05$). CD4 count, total cholesterol, HDL, LDL, hemoglobin, GGT, creatinine levels were higher in the ART group compared to the ART naïve group ($p<0.05$). Flow mediated dilatation (FMD) was higher and IMT was lower in the ART group versus ART naïve group ($p<0.05$). Furthermore, no significant correlations were found between biomarkers and FMD and IMT. With regards to independent associations, in the HIV-infected group, age positively associated with

IMT ($p<0.001$), while in the HIV-free group, age, high systolic BP, obesity and LDL cholesterol positively associated with IMT ($p<0.05$). In the HIV-infected group, ART positively associated, while viral load inversely associated with FMD%. In the HIV-free group, creatinine inversely associated with FMD% ($p<0.05$).

Discussion and conclusion: Overall, our study showed a higher presence and progression of cardiovascular risk parameters in the HIV-free population. In the HIV context, ART showed a more cardioprotective and immune-protective effect, where it was associated with reduced subclinical atherosclerosis and improved endothelial function. Our study confirmed that age is an independent predictor of subclinical atherosclerosis while viral load is an independent, inverse predictor of endothelial function.

Opsomming

Agtergrond: Bragiale arterie vloei-gemedieerde dilatasie (FMD) en karotis arterie intima-media dikte (IMD) verteenwoordig die meting van vaskulêre endoteelfunksie en subkliniese aterosklerose onderskeidelik. Voorheen het studies in ontwikkelde lande aangetoon. Daar is voorheen tudies in ontwikkelde lande aangetoon dat beide endoteeldisfunksie en subkliniese aterosklerose ‘n verband met MIV en antiretrovirale terapie (ART) toon. Verder kan verskeie inflammatoriese en endoteeldisfunksie biomerkers gemeet word om kardiovaskulêre uitkomste in MIV-geïnfekteerde populasies te voorspel. Die presiese verband tussen endoteeldisfunksie, aterosklerose, MIV, ART en die biomerkers is nog nie heeltemal verklaar of duidelik nie nie, veral nie in Suid-Afrikaanse en Wes-Kaapse bevolkings nie.

Doelstelling: Om die potensiele vermoedelike verband tussen subkliniese aterosklerose, endoteeldisfunksie, kardiovaskulêre biomerkers en MIV-infeksie in ‘n Wes-Kaapse studiebevolking te ondersoek.

Metodiek: Twee verskillende epidemiologiese studies was onderneem: ‘n deuranit hoofstudie (MIV-geïnfekteer, n= 204 waarvan n=188 op ART was, en n=16 ART sonder; MIV negatief is, n= 143), en ‘n longitudinale sub-studie om die 12-maande progressie van geselekteerde kardiovaskulêre uitkomste te ondersoek (MIV negatief, n= 57). Deelnemers was by gesondheidsklinieke in Kaapstad en Worcester tussen 2017 en 2018 gewerf. Lewenstyl- en gesondheidsinligting was ingesamel, asook vaskulêre (FMD en IMD met behulp van ultraklank tegnologie), antropometriese (liggaamsmassa-indeks [LMI]; middelomtrek [MO]), kardiovaskulêre (lipiedprofiel; bloeddruk [BP]; plasma glukose), nier (kreatinien; urien albumien-kreatinienverhouding [ACR]), lewer (gamma glutamieltransferase [GGT]) en MIV-verwante (virale lading; CD4, duur van MIV-infeksie, duur van behandeling) metings was ingesamel. Serum biomerkers was met behulp van Luminex tegnologie geanaliseer, insluitende C-reaktiewe proteïen (CRP), tumor nekrose faktor-alfa (TNF-α), vaskulêre endoteel groeifaktor (VEGF), plasminogeen-aktiveerder- inhibeerder-1 (PAI-1), en aanhegtingsmolekules (intersellulêre adhesiemolekule-1 [ICAM-1], vaskulêre sellulêre adhesiemolekule-1 [VCAM-1], e-selektien, p-selektien).

Resultate: In die longitudinale studie (gemiddelde ouderdom: 45 jaar), het WC, plasma glukose, BP, en hipertensie toegeneem na 12 maande. In die deursnitstudie (gemiddelde ouderdom: 41 jaar), was BMI, WC, BP, hipertensie en PAI-1 laer in die MIV-geïnfekteerde groep in vergelyking met die geen MIV groep ($p<0.05$), terwyl anemie, ACR, kreatinien, GGT and VCAM-1 verhoog was in die MIV-geïnfekteerde groep ($p<0.05$). Verder was die virale lading en VCAM-1 vlakke hoër in die onbehandelde MIV-geïnfekteerde groep in vergelyking met die ART groep ($p<0.05$). CD4 telling, **totale** cholesterol, HDL, LDL, hemoglobien, GGT en kreatinien vlakke was hoër in die ART groep ($p<0.05$ vs onbehandeld). FMD was hoër en IMT laer in die ART groep ($p<0.05$ vs. onbehandeld). Verder kon geen betekenisvolle korrelasies tussen die biomerkers, FMD en IMT aangetoon word nie.

Wat onafhanklike assosiasies betref, het ouderdom positief geassosieer met IMT in die MIV-geïnfekteerde groep ($p<0.001$), terwyl ouderdom, hoë sistoliese bloeddruk, vetsug en LDL cholesterol positief met IMT geassosieer was in die kontrole groep ($p<0.05$). In die MIV-geïnfekteerde groep het ART positief geassosieer met FMD%, terwyl virale lading omgekeerd geassosieer met FMD%. In die geen MIV groep, was kreatinien omgekeerd geassosieer met FMD% ($p<0.05$).

Bespreking en gevolgtrekking: Oor die algemeen het ons studie ‘n hoër teenwoordigheid en progressie van kardiovaskulêre risikofaktore in die geen MIV groep getoon. In die geval van die MIV-geïnfekteerde deelnemers, het ART behandeling meer kardio- en immuunbeskermende effekte getoon, waar dit met verlaagde subkliniese atereosklerose en beter endoteelfunksie geassosieer was. Die studie het ook bevestig dat ouderdom ‘n onfhanklike voorspeller van subkliniese aterosklerose is, terwyl virale lading ‘n onafhanklike, omgekeerde voorspeller van endoteelfunksie is.

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List of abbreviations

Ach	Acetylcholine
ACR	Albumin-to-Creatinine Ratio
ACS	Acute Coronary Syndrome
ADMA	Asymmetric Dimethylarginine
AGEs	Advanced Glycation End products
AIDS	Acquired Immune Deficiency Syndrome
AIx	Augmentation Index
ANOVA	Analysis of Variance
ART	Antiretroviral Therapy
BH4	Tetrahydrobiopterin
BMI	Body Mass Index
BP	Blood Pressure
BPM	Beats Per Minute
CA	Capsid Protein
CCA	Common Carotid Artery
CCR5	CC Chemokine Receptor 5
CD4	Cluster of differentiation 4
cDNA	Complimentary DNA
CGMP	Cyclic Guanosine-3,5-Monophosphate
CHD	Coronary Heart Disease
CNS	Central Nervous System
COX-1	Cyclooxygenase-1
CPK	Creatine Phosphokinase
CRP	C-reactive Protein

CVD	Cardiovascular Disease
CXCR4	CX Chemokine Receptor 4
DBP	Diastolic Blood Pressure
EBCT	Electron Beam Computed Tomography
ECs	Endothelial Cells
EDCF	Endothelium-Derived Constricting Factor
EDHF	Endothelium-Derived Hyperpolarization Factor
EDRF	Endothelium-Derived Relaxing Factor
EDTA	Ethylenediamine Tetra-acetic Acid
eGFR	Estimated Glomerular Filtration Rate
eNOS	Endothelial Nitric Oxide Synthase
ET-1	Endothelin 1
FDC	Fixed Dose Combination
FMD	Flow Mediated Dilatation
GCP	Good Clinical practice
GGT	γ-Glutamyl Transferase
GLUT4	Glucose transporter Type 4
gp120	Glycoprotein 120
HAART	Highly Active Antiretroviral Therapy
Hb	Haemoglobin
HbA1c	Glycated Haemoglobin
HDL	High Density Lipoprotein
HIV	Human Immunodeficiency Virus
HIV-1	HIV-type 1
HIV-2	HIV-type 2

HR1 & HR2	Heptad Repeat Regions
HREC	Health Research Ethics Committee
hsCRP	High-sensitivity C-Reactive Protein
ICA	Internal Carotid Artery
ICAM-1	Intercellular Adhesion Molecule-1
IGF	Insulin-like Growth Factor
IL	Interleukin
IMT	Intima Media Thickness
INIs	Integrase Inhibitors
IQR	Interquartile Range
IVUS	Intravascular Ultrasound
JAMs	Junctional Adhesion Molecules
LDL	Low Density Lipoprotein
L-NMMA	L-NG-monomethyl Arginine
LTRs	Long Terminal Repeats
MAP	Mitogen-Activated Protein
MCP-1	Monocyte Chemotactic protein 1
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NC	Nucleocapsid Proteins
NCD	Non- Communicable Disease
NHLS	National Health Laboratory Service
NNRTIs	Non-Nucleoside Reverse Transcriptase Inhibitors

NO	Nitric Oxide
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
PAI-1	Plasminogen Activator Inhibitor-1
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PE	Phycoerythrin
PECAMS	Platelet and Endothelial Cell Adhesion Molecules
PGI2	Prostacyclin
PIs	Protease Inhibitors
PWA	Pulse Wave Analysis
PWV	Pulse Wave Velocity
RAAS	Renin–Angiotensin–Aldosterone System
RAGE	Receptor for Advanced Glycation End products
RAS	Renin Angiotensin System
REDCap	Research Electronic Data Capture
RF	Radio Frequency
RPM	Rounds Per Minutes
SANAS	South African National Accreditation System
SANC	South African Nurses council
SBP	Systolic Blood Pressure
SD	Standard Deviation
SERPINS	Serine Protease Inhibitors
SGC	Soluble Guanylate Cyclase
SMC	Smooth Muscle Cells
SSA	Sub- Saharan Africa

TB	Tuberculosis
TEE	Tenofovir Emtricitabine Efavirenz
TF	Tissue Factor
TGF	Transforming Growth Factor
TLD	Tenofovir Lamivudine Dolutegravir
TLE	Tenofovir Lamivudine Efavirenz
TLRs	Toll-like Receptors
TNFα	Tumour Necrosis Factor alpha
UNAIDS	United Nations Programme on HIV/AIDS
USAID	United States Agency for International Development
VCAM-1	Vascular Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VSMC	Vascular Smooth Muscle Cell
vWF	von Willebrand Factor
WHO	World Health Organization
WHR	Waist Hip Ratio

List of Symbols

%	Percentage
°C	Degrees Celsius
Cm	Centimetre
G	Gram
kg	Kilogram
L	Litre
m	Meter
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
mmHg	Millimetre of Mercury
mW	Milliwatt
n	Sample size
ng	Nanogram
nM	Nanomolar
α	Alpha
β	Beta
µ	Micro
µl	Microlitre
µM	Micromolar
U/L	Units per litre
µmol/L	Micromole per litre

mmol/L	Millimole per litre
Hz	Hertz
MHz	Megahertz
Pg	Picogram

Chapter 1: Introduction

1.1 Context of the PhD study

This PhD study is embedded in a larger multinational parent study named EndoAfrica, which is led by Prof Hans Strijdom (PhD supervisor). The overarching aim of the EndoAfrica parent study is to assess, in a longitudinal fashion, whether there is a link between HIV, antiretroviral therapy (ART) and cardiovascular risk factors, as well as endothelial structure and function, in South Africa. In addition to the Stellenbosch University group, the EndoAfrica research consortium consists of partners and collaborators from the Medical University of Graz (Austria), Prof Nandu Goswami (co-supervisor), Hasselt University (Belgium), Prof Tim S. Nawrot, and the University of Antwerp, (Belgium), Prof Patrick De Boever (co-supervisor). The PhD supervisor (Prof Hans Strijdom) is the national and international project coordinator and the leader of the EndoAfrica consortium.

1.2 Significance of the PhD study

Currently, South Africa is faced with a double burden of disease, with communicable diseases such as HIV on the one hand, and non-communicable diseases such as cardiovascular diseases (CVD) on the other. The underlying pathophysiological origin of many CVD is atherosclerosis and endothelial dysfunction, which can clinically and non-invasively be measured using validated gold standard techniques such as carotid artery intima-media thickness (IMT) and brachial artery flow-mediated dilatation (FMD), respectively. Both atherosclerosis and endothelial dysfunction can potentially be prevented, and endothelial dysfunction has been shown to be a predictor of future CVD in patients, especially if detected early in preclinical stage.

Interestingly, Today, CVD has become a major cause of death in the HIV population, partially due to increased lifespan of these individuals with the success of ART, as well as due to direct effects of the ART drugs. There is a lack of data from studies assessing cardiovascular risk factors in HIV infected individuals in low income countries, including in South Africa. A large body of research on this topic exists in the more affluent countries in Europe and North America, highlighting a gap in research on this topic in South Africa. Additionally, very few studies have investigated the association between HIV/ ART and, endothelial dysfunction and atherosclerosis in South Africa. The interaction between factors linked with CVD and HIV, especially with ART addition is vastly complex and their overall impact on the cardiovascular system warrants further investigation. Furthermore, there are circulating biomarkers that are associated with endothelial dysfunction and atherosclerosis, which can be screened and measured to predict cardiovascular outcomes in the HIV population. Hence the current study aims to address this gap in research especially in the South African context, where the country is known to have the highest HIV-infected population in the world. Thus, the overall aim of the PhD is to investigate

the putative relationship between subclinical atherosclerosis, endothelial dysfunction, biomarkers of cardiovascular disease, and HIV-infection in a South African population from the Western Cape Province.

1.3 Overarching aim of this PhD study

The main aim of the PhD study is to contribute in a novel and innovative way, to the scientific research body both at a national and international level in the fields of HIV and CVD. More specifically, the study aims to explore the important interaction between HIV, endothelial function and markers of subclinical atherosclerosis by shedding light on specific questions regarding HIV-associated cardiovascular risk and disease in a study cohort of adult volunteer participants living in Western Cape, South Africa. The study has an epidemiological approach with a cross-sectional design. In addition, a longitudinal sub-study was performed to explore the progression of selected CVD outcomes. Furthermore, the PhD dissertation aims to explore the relevance of specific imaging techniques and biomarkers of CVD, novel in the context of the South African research environment, and their relationship with HIV in the study population.

1.4 The structure of the dissertation

The dissertation is divided into six chapters, including this chapter 1. **Refer to figure 1.1**, which provides a schematic representation of the structure of this dissertation. The literature review (chapter 2) is divided into three sections; (i) section A, exploring CVD and underlying mechanisms, section B, focusing on HIV and its mechanisms, followed by (ii) a comprehensive literature analysis regarding the link between HIV and CVD, and (iii) concluding with a rationale as well as aims and objectives of this PhD study in section C. This is followed by the methodology applied in the study (chapter 3) and results (Chapter 4) which is divided into two sections, section A comprising of results of the main cross-sectional study and section B, reporting on results from the longitudinal sub-study. The discussion (chapter 5) is followed by a conclusion (Chapter 6).

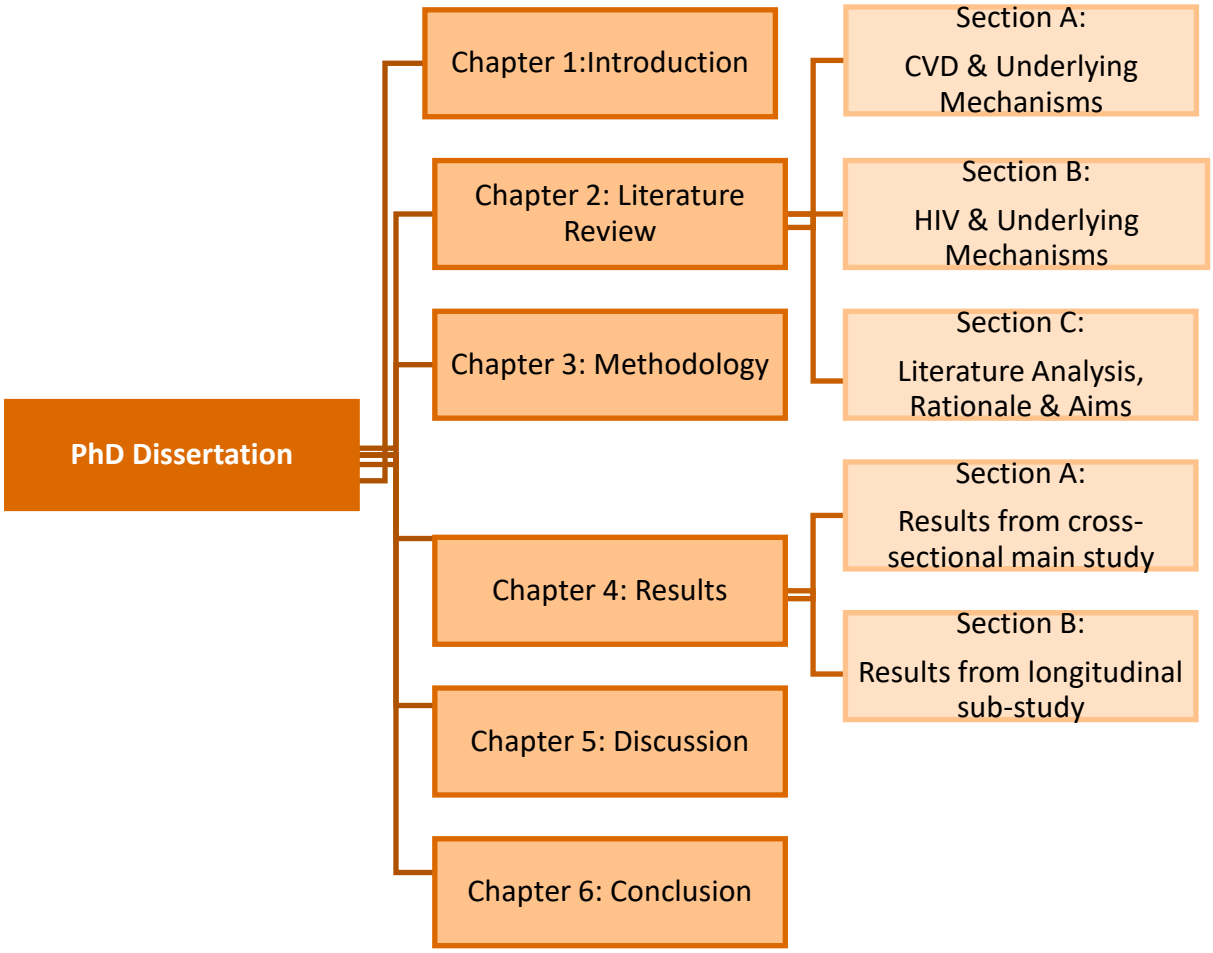


Figure 1.1: Schematic representation of the structure of this PhD dissertation.

Chapter 2: Literature review

2.1 Introduction

This chapter serves to critically appraises the current literature pertaining to the relevant themes of the PhD study. The chapter is divided into three sections (**refer to figure 2.1**), and aims to systematically describe and review each section, and conclude with the rationale and objectives of the study.

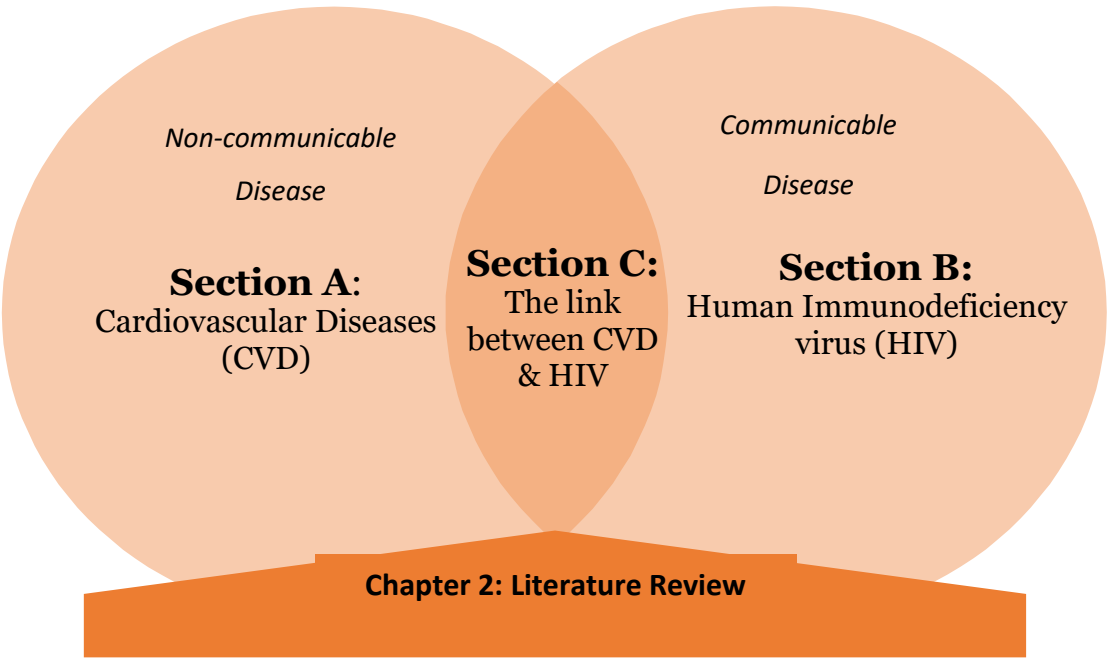


Figure 2.1: The division of the literature review chapter into three sections.

Section A: Cardiovascular disease and its underlying mechanisms

2.2 Cardiovascular disease

2.2.1 Introduction and Epidemiology

Cardiovascular disease (CVD) is a non- communicable disease (NCD) that largely represents ailments of the heart and blood vessels. At present, CVD is the leading cause of morbidity and mortality in the world. In fact, the United Nations, identified NCDs, including CVD, as a major global health concern which is increasingly hindering sustainable human development (Roth *et al.*, 2017).

Approximately 71% of all deaths globally is attributed to NCDs, with CVD representing the greatest contributor. The high CVD prevalence has been linked to increased socio-economic costs including disability, loss of productivity as well as increasing poverty and health inequality. The poor communities in the urban areas have the worst CVD outcomes, largely due to their inability to access or afford preventative services and chronic treatments (Cappuccio and Miller, 2016). The World Health Organization (WHO) reports approximately 18 million CVD related deaths annually, of which more than 75% occurs in low to middle income countries. The high-income countries are demonstrating a decline in CVD related mortality rates, possibly due to improved management strategies of CVD, including prevention and reducing risk factors pertaining to it (Cappuccio and Miller, 2016; Yusuf, Rangarajan *et al.,* 2014). In contrast, the current global CVD burden of 80% originates from low to middle income countries (Cappuccio, Miller 2016, Yusuf, Rangarajan *et al.*, 2014).

The sub-Saharan Africa (SSA) region, consisting of low to middle income countries, is predicted to show an increase in NCDs, due to epidemiological and demographical transitions. Furthermore, this region also has a high incidence of CVD, which has developed into a major health concern due to its growing mortality and morbidity rates (Dalal *et al.*, 2011). The World bank classifies South Africa as a middle-income country in the SSA region, where NCD related mortality rates have now exceeded the known burden of HIV/ AIDS and tuberculosis (infectious diseases), with CVD being the largest contributor (Schutte, 2019). In 2009, the WHO estimated that South Africa will have a two to three times higher burden of NCD (including CVDs) compared to the developed countries, with disproportionate effect on the poor in the urban settings (Mayosi *et al.*, 2009). Additionally, it was predicted that there will be a 41% increase in CVD related mortality in South Africa between the year 2000 and 2030 (Mpe 2010). Recently, the NCD Countdown 2030 (which is a collaboration between the WHO, The Lancet, NCD Alliance (a network of civil society organisations) and the Imperial College, London), further suggests that South Africa has a 51.9% probability of NCD related mortality (NCD Countdown 2030 collaborators, 2018).

2.2.2 Cardiovascular risk factors

According to the WHO, a risk factor is any characteristic or exposure that increases the probability of developing a disease or injury in an individual (WHO, 2017). The Framingham Heart Study was one of the first studies to conceptualize risk factors pertaining to CVDs (Kannel *et al.*, 1961). The World Health Federation further divided these CVD risk factors into modifiable and non-modifiable categories, which will be discussed below (World Heart Federation, 2017). A large portion of CVD risk can be attributed to modifiable risk factors such as hypertension, smoking, obesity, unhealthy diet, physical inactivity, dyslipidaemia, and diabetes mellitus, all of which account for 61% of global CVD related mortality worldwide (Cappuccio and Miller, 2016). Approximately 84% of the global burden of CVD that is caused by modifiable risk factors occur in low to middle income countries, including countries of SSA, where CVD risk factors are mostly similar in comparison to the rest of the world (Mensah, 2013). Studies indicate that eliminating these risk factors may improve the average global life expectancy by approximately five years (WHO, 2009). **Refer to table 2.1** for a summary of modifiable and non-modifiable risk factors.

Table 2.1: Summary of modifiable and non-modifiable CVD risk factors (Balakumar, Maung-u and Jagadeesh, 2016).

Modifiable Risk factors	Non- modifiable Risk factors
Hypertension	Age
Smoking	Sex
Obesity	Family history of CVD
Unhealthy diet	Ethnic or Cultural factors
Physical inactivity	Socio economic factors
Dyslipidaemia	
Type 2 Diabetes Mellitus	
Excessive use of alcohol	
Stress & Depression	

2.2.2.1 Modifiable risk factors:

Hypertension

Hypertension is regarded by many as the most common, modifiable, and independent CVD risk factor (WHO, 2016; Balakumar, Maung-u and Jagadeesh, 2016). It is defined as a systolic blood pressure reading of ≥ 140 mmHg and/or a diastolic blood pressure of ≥ 90 mmHg, following repeated examination (Seedat *et. al.*, 2011; Unger *et. al.*, 2020). Hypertension is known to be a major cause of premature death, due to its 'silent' (asymptomatic) nature. Typically, hypertension has a negative impact on the function of the heart and blood vessels and has been found to be a major risk factor for

coronary heart disease (CHD). Furthermore, hypertension has been found to cause approximately half of all ischemic strokes and an increased risk of hemorrhagic stroke (Balakumar, Maung-u and Jagadeesh, 2016). There are many behavioural characteristics that can make individuals more susceptible to hypertension, these include unhealthy diets (high salt, saturated and trans-saturated fat, low dietary potassium through reduced consumption of fruits and vegetables), lack of physical exercise, obesity and excessive intake of alcohol and tobacco. Furthermore, a family history of hypertension, age over 65 years and comorbidities with diabetes and kidney disease can also increase the risk of hypertension. The WHO has found an increase in the global hypertension prevalence from 595 million in 1975 to approximately 1.13 billion in 2015. The increase was largely seen in low to middle income countries, probably due to increase in hypertension risk factors in those regions (WHO, 2019; Antignac *et al.*, 2018). The SSA region has particularly noticed an increase in the burden of hypertension, especially due to the fact that the affected populations remain undiagnosed, untreated or inadequately treated, which, therefore leads to a higher morbidity and mortality risk (Ataklte *et al.*, 2014). The Heart and Stroke Foundation estimates that more than a third of individuals are living with hypertension in South Africa, further contributing to the CVD epidemic in this country (Heart and Stroke Foundation South Africa, 2016).

Tobacco use (smoking)

After hypertension, smoking is found to be the second leading cause of CVD. Approximately 10% of all CVDs can be attributed to tobacco use. Globally, smoking (including second hand smoking) is thought to result in approximately 6 million deaths every year, which is further predicted to increase to 8 million annually by 2030 (WHO, 2015; Balakumar, Maung-u and Jagadeesh, 2016). The use of tobacco has been shown to cause short term increases in blood pressure, reduced oxygen capacity of blood and increased clotting tendency of the blood, all of which could lead to a range of CVDs. Furthermore, smoking has been linked to increase blood triglycerides while decreasing HDL (Balakumar, Maung-u and Jagadeesh, 2016). Ultimately, smoking has been associated with an increased risk of atherosclerosis (discussed in section 2.2.3). Globally, approximately 1.1 billion people smoked tobacco in 2015 (WHO, 2015). The WHO reports that many countries today show a decline in smoking including in SSA, however, there still appears to be an increase in smoking in the Eastern Mediterranean and in some parts of African Region. At present, SSA has a considerable variation in the smoking prevalence among countries, with 26.5% in Zambia, to 60% in Sierra Leone (Owusu-Dabo *et al.*, 2009; Brathwaite *et al.*, 2015; Murphy, Liu and Parascandola, 2018). However, due to limited data, these estimations cannot be conclusive. South Africa was reported to have approximately 20% prevalence of smoking in 2016 (WHO, 2018).

Obesity

Obesity is a chronic disease affecting individuals of all ages and is sometimes referred to as 'adiposity', which refers to increased amounts of adipose (fat) tissue in the body. A benchmark for normal adiposity remains unknown, however, adiposity is essentially when there is an imbalance between the energy (calories) intake and energy (calories) used (Arboix, 2015). Increased body fat has been linked to many other CVD risk factors, such as hypertension, dyslipidaemia, type 2 diabetes and vascular diseases. Body mass index (BMI) is a well-known measure of obesity and overweight. BMI is calculated as bodyweight in kilograms divided by height in meters squared (Piché *et al.*, 2018). The WHO has classified BMI into various weight categories, **refer to table 2.2** (WHO, 2016). Waist circumference and waist-to-hip ratio are commonly used to measure abdominal obesity, which in the South African context is clinically defined by waist circumference of more than or equal to 94 cm in males and 80 cm in females (Alberti *et al.*, 2009; Seedat and Rayner, 2013). The development of many comorbidities is dependent on the BMI as well as the degree of obesity (Piché *et al.*, 2018). Research suggests that obese individuals are at higher risk of developing CVD and manifestations thereof, especially CHD, angina, myocardial infarction, heart failure and sudden cardiac death (Piché *et al.*, 2018). Studies have shown that visceral fat/ abdominal obesity is a strong risk factor for developing CVD and metabolic complications (Piché *et al.*, 2018). According to the WHO, more than 1.9 billion adults were found to be overweight with 650 million obese (WHO, 2016). At present, obesity is linked with 2.8 million deaths annually. Traditionally, being overweight or obese was more prevalent in high-income countries, however the trend has greatly moved towards low to middle income countries, with females particularly affected, in urban settings of the SSA region (Scott *et al.*, 2013). With regards to South Africa, there is an increased burden of obesity, in fact, South Africa is found to have the highest prevalence of obesity in the SSA region (Tugendhaft *et al.*, 2016).

Table 2.2: Classification of obesity according to BMI (Balakumar, Maung-u and Jagadeesh, 2016; WHO guidelines).

BMI (kg/m^2)	Categories
< 18.5	Underweight
18.5–24.9	Normal weight
25.0–29.9	Overweight
30.0–34.9	Obesity (class I)
35.0–39.9	Obesity (class II)
≥ 40.0	Obesity (class III)
≥ 50	Obesity (class IV)
≥ 60	Obesity (class V)

Unhealthy diet

Poor intake of fruits and vegetables, and high intake of saturated and trans-fat as well increased salt intake have been linked with increased weight gain and CVD risk (Balakumar, Maung-u and Jagadeesh, 2016). Reduced intake of fruits and vegetables may account for approximately 20% of CVD globally. (World Health Federation, 2018) According to the WHO, 1.7 million deaths globally are due to low fruit and vegetable consumption (WHO, 2018). High salt intake is an important determinant of hypertension and cardiovascular risk (Ha, 2018). Similarly increased consumption of saturated and trans-fat is linked with heart disease and stroke (Balakumar, Maung-u and Jagadeesh, 2016). With westernization, processed foods with elevated levels of salt and fat have slowly been infiltrating countries such as South Africa, ultimately contributing to CVD (Spires *et al.*, 2016).

Physical inactivity

Insufficient physical activity is a major CVD risk factor and places individuals at risk for developing cardio-metabolic diseases such as diabetes mellitus (Balakumar, Maung-u and Jagadeesh, 2016). Physical inactivity is a term used to identify people who do not get the recommended level of regular physical activity or exercise. The American Heart Association recommends 30-60 minutes of aerobic exercise three to four times per week to promote cardiovascular fitness. In general, individuals who achieve less than 30 minutes of moderate intensity physical activity per week are thought to be physically inactive (CDC and American Heart association, 2020). Overall, physical inactivity is thought to be amongst the top ten leading risk factors for death worldwide (World Health Federation, 2016).

Advantages of sufficient exercise include a reduction in CVD related mortality, increased exercise capacity as well as with enhanced quality of life (González and Fuentes, 2017). Furthermore, a study showed that total physical activity, running, weight training, and walking were each associated with reduced CHD risk (Tanasescu *et al.*, 2002); while the WHO states that insufficient physical activity can increase the risk of mortality by 20 - 30% in comparison to individuals who are sufficiently active (WHO, 2018). According to the WHO, physical inactivity has led to approximately 3.2 million deaths worldwide (WHO, 2015) The United States of America and the Eastern Mediterranean areas are found to have the highest prevalence of physical inactivity, with males found to be more active than females in almost every country. In the African region, many countries have reported high prevalence of physical inactivity, such as Swaziland with 49.1% and Mauritania with 52.6% prevalence in adults. As with many developing countries, South Africa is undergoing nutritional, lifestyle and socioeconomic transitions, which may in some ways be unfavourable to the health of individuals. It is estimated that 45% of South African adults are inactive (Phaswana-Mafuya *et al.*, 2013; Malambo *et al.*, 2016).

Dyslipidaemia

Dyslipidaemia is characterized by abnormal blood concentrations of one or more of the following: total cholesterol, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, and triglycerides (Arboix, 2015; Noubiap *et al.*, 2018). Dyslipidaemia is one of the leading contributors to CVD and mortality worldwide (Noubiap *et al.*, 2018). Important risk factors for CVD and stroke is high levels of LDL cholesterol and triglycerides (Tanne *et al.*, 2001). Approximately one third of ischemic heart disease is attributed to dyslipidaemia (Balakumar, Maung-u and Jagadeesh, 2016). Many studies demonstrate a decreased risk of heart disease when dyslipidaemia is controlled. High LDL cholesterol levels play a pivotal role in the initiation and progression of atherosclerosis (as described in section 2.2.3), thus directly contributing to CVD (Hansson, 2017; Pasquel and Gregg, 2019). Elevated triglyceride levels are also known to contribute to CVD. In contrast, HDL cholesterol is found to have protective characteristics in atherosclerosis and thus has an inverse relation with the development of CHD (Robert *et al.*, 2016). Overall low HDL has been associated with increased risk of developing CVD. In 2016, elevated total cholesterol levels were associated with 4.4 million deaths (Noubiap, Bigna, Nansseu, Nyaga, Balti, Justin B. Echouffo-Tcheugui, *et al.*, 2018). In 2011, approximately 25% of adults from low-income countries and more than 50% of adults from high-income countries reported elevated total cholesterol (Balakumar, Maung-u and Jagadeesh, 2016). In South Africa, diabetes as well as HIV and anti-retroviral therapies (ART) have been found to drive dyslipidaemia (Burren and Hattersley, 2004; Dave *et al.*, 2016; Ntusi, 2018). The effects of some classes of ART drugs on lipid metabolism have been described in literature and will be further explored in section B of this chapter.

Diabetes Mellitus

Diabetes Mellitus is a well-known risk factor for CVD (World Heart Federation, 2017). Many studies have shown that individuals with type 2 diabetes have a higher CVD related mortality and morbidity in comparison to individuals without Diabetes (Matheus *et al.*, 2013). In fact, CVD is the leading cause of mortality in diabetic populations (World Heart Federation, 2017). Particularly, diabetes has been associated with higher risk of CHD, stroke, and heart failure (Leon, 2015). It is well established that elevated glucose levels contribute to endothelial dysfunction (discussed later in section 2.2.4) in the arteries and atherosclerosis (Martín-Timón, 2014). Furthermore, diabetic (particularly type 2) individuals, often present with several other risk factors such as obesity, hypertension, and dyslipidaemia (Leon, 2015). In addition, evidence from literature shows that diabetic individuals often have elevated oxidative stress, vascular damage and dysfunction and a pro-clotting profile (Matheus *et al.*, 2013; Leon, 2015). All of this collectively can increase the risk of developing major CVDs such as CHD and stroke (Matheus *et al.*, 2013; Leon, 2015).

Other modifiable risk factors (excessive use of alcohol, stress and depression)

Excessive use of alcohol as well psychosocial conditions such as depression, stress and anxiety are found to be associated with increased CVD risk (Balakumar, Maung-u and Jagadeesh, 2016). Chronic, heavy alcohol consumption has been linked with increased risk of stroke, whilst light to moderate intake has been found to reduce the risk of stroke (Arboix, 2015). Alcohol has also been associated with myocardial injury and increased risk of cardiac arrhythmias (World Heart Federation, 2017). Some studies also link alcohol abuse to increased blood pressure, acute myocardial infarction, and CHD (Piano, 2017). According to WHO, alcohol abuse results in 3 million deaths annually which represents 5.3 % of all deaths worldwide (WHO, 2018). With regards to depression, some studies have demonstrated that depression may be an independent risk factor for certain CVDs (Fiedorowicz, 2014). The underlying mechanisms are related to modifications in sympathetic stimulation and alteration in lipid metabolism (Lichtman *et al.*, 2014). Depression has also been thought to indirectly contribute to CVD when it occurs concurrently with stress, physical inactivity, and medication non-adherence. According to the WHO, more than 264 million people of all ages suffer from depression, which is a major contributor to the overall global burden of disease (WHO, 2020).

2.2.2.2 Non modifiable Risk Factors

Age

It is well known in research that age is an independent risk factor for CVDs (Dhingra and Vasan, 2012). Age has been associated with arterial stiffness, and alteration of lipid profiles, all of which can contribute to the development of CVD. With increasing age, functional and structural modification can occur in the vascular wall, such as thickening of the arterial wall and stiffness. These changes could also contribute to the development of systolic hypertension and left ventricular hypertrophy (Westerman, Engberding and Wenger, 2015). Furthermore, age has been linked with elevated pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL_8, IL-13, IL-18, as well as C-reactive protein (CRP), interferons α and β, transforming growth factor β, tumour necrosis factor alpha (TNF)-α, its soluble receptors (TNF-R1 and TNF-R2), and serum amyloid, all of which play a role in the development of atherosclerosis, further contributing to CVD (Chia, Egan and Ferrucci, 2018). Due to medical advances globally, the longevity of the population is increasing, however the presence of CVD risk and related chronic complications remains in these individuals.

Sex

Differences in CVD between males and females are more prominent at a younger age. The prevalence of CVD in younger people appears to be higher in males than females; studies have attributed part of this effect on reproductive hormones (Wakabayashi, 2017). As females age, the prevalence of CVD rises almost to the level of males (Wakabayashi, 2017). In fact, females have been reported to have a more unfavourable cardiovascular risk profile, especially with regards to CHD and diabetes mellitus (Westerman, Engberding and Wenger, 2015; Balakumar, Maung-u and Jagadeesh, 2016; Wakabayashi, 2017). Although males and females do share all traditional risk factors, the impact of these factors are different in the two gender groups (Gao *et al.*, 2019). For example, some researchers have noted that age, hypertension and total cholesterol and LDL-cholesterol have a higher influence on males; while smoking, diabetes and triglycerides and HDL-cholesterol levels have a higher effect on women (Galiuto and Locorotondo, 2015; Gao *et al.*, 2019). When considering females, there are certain sex-specific factors that may influence CVD (Gao *et al.*, 2019). For instance, it is noted that pregnancy related cardiovascular complications such as preeclampsia, gestational hypertension, gestational diabetes mellitus, menopause-related hormonal changes and autoimmune diseases can also play a role (Balakumar, Maung-u and Jagadeesh, 2016; Westerman, Engberding and Wenger, 2015). Menopause specifically may impact body fat and the lipid profiles of the females, especially HDL levels as oestrogen levels significantly deplete with hormonal changes, all of which is found to independently impact HDL and body fat of females (Westerman, Engberding and Wenger, 2015).

Family history of CVD

Literature also points towards family history of CVD, and socioeconomic factors to play a part in the development of CVD. Family history of CVD can aid in predicting future CVD events in individuals (World Heart Federation, 2017). The CVD risk is dependent on the number and age of CVD affected first degree families. Studies have found that siblings of patients with CVD have approximately 40% increased risk in comparison to the siblings of individuals without CVD (Murabito *et al.*, 2005). Additionally, CVD risk increases to approximately 60-75% if the one is the offspring of parents with premature CVD (Feinleib *et al.*, 1975; Kolber, 2014). The development of CVD is not entirely dependent on family history, however, as a range of environmental and other risk factors play a role (World Heart Federation, 2017).

Other non-modifiable risk factors

Literature has also reported on genetic (e.g. ancestry), cultural and socio-economic factors that may be associated with CVD risk. Cultural factors that could increase CVD risk include dietary choices and habits (BeLue *et al.*, 2009). The so-called Western lifestyle that includes a diet high in fat and sugar, may influence metabolic and vascular function across populations (Kopp, 2019). Another study that looked at the role of ancestry showed that South Asians have a higher CVD related mortality than Europeans (Forouhi and Sattar, 2006). Lastly, socio-economic status, generally measured through education and income of an individual, is also found to be a predictor of CVD and related deaths (Rawshani *et al.*, 2015). Particularly, in high-income countries, low socioeconomic status has been shown to associate with an increased risk of CVD and mortality (Stringhini *et al.*, 2017; Antignac *et al.*, 2018; Rosengren *et al.*, 2019). Levels of education and income can impact decision making and access to healthcare which can indirectly increase the risk of CVD.

2.2.2.3 The interaction of CVD risk factors

Often the risk factors discussed above interact with each other and create a chain of mechanisms, which collectively contribute to the development of CVD. **Refer to figure 2.2** for a summary of some these pathways.

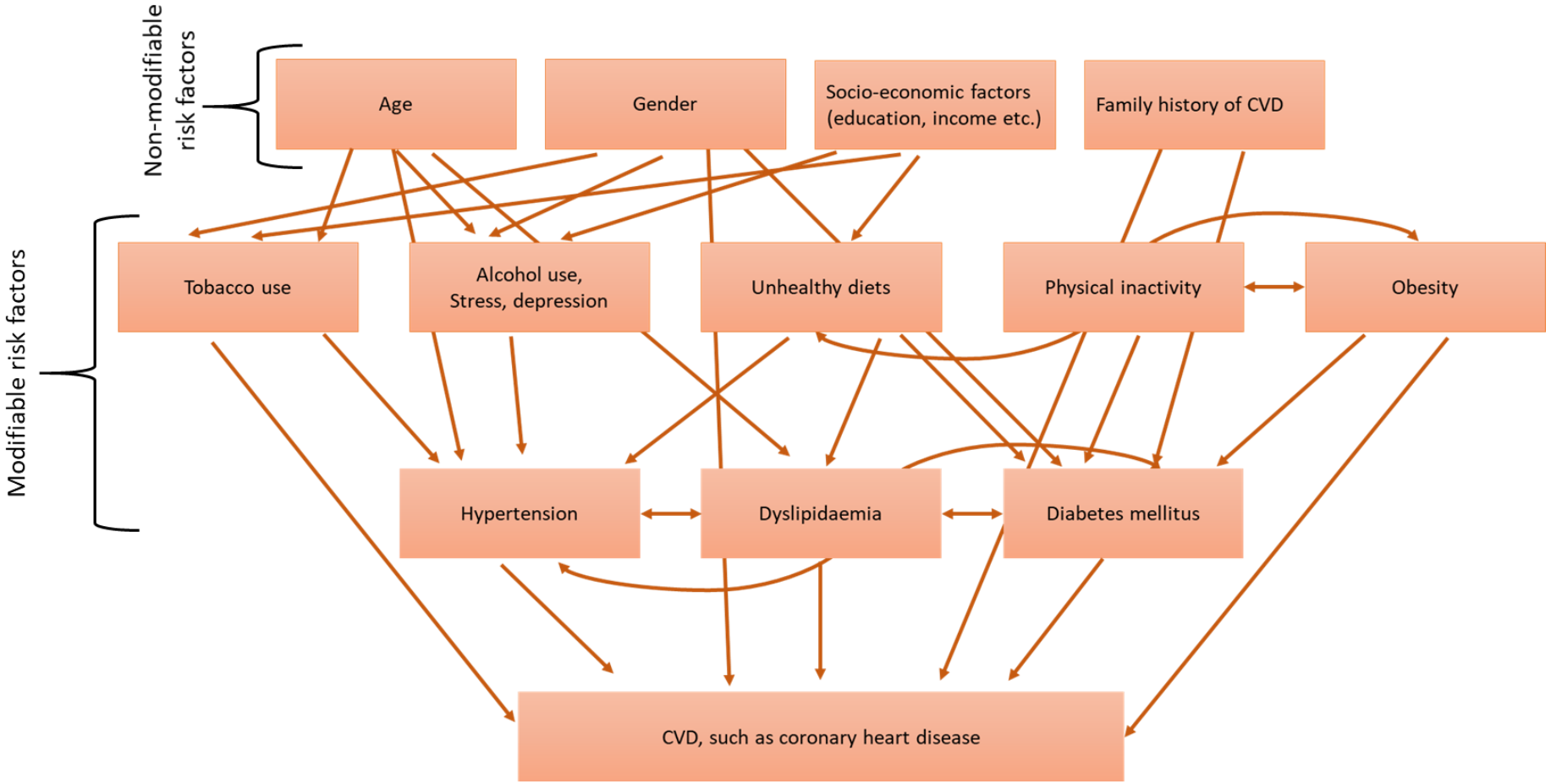


Figure 2.2: Interaction amongst CVD risk factors. Figure designed by the author of this dissertation.

2.2.3 Atherosclerosis

A major underlying pathophysiological mechanism of the two most common CVDs, viz. CHD and cerebrovascular disease (stroke), is atherosclerosis (Frostegård, 2013) . The major risk factors that play a role in the development of atherosclerosis are shown in **figure 2.3**. This chronic condition is characterized by plaque build-up, which ultimately cause a hardening of the arterial walls (Bergheanu, Bodde and Jukema, 2017).

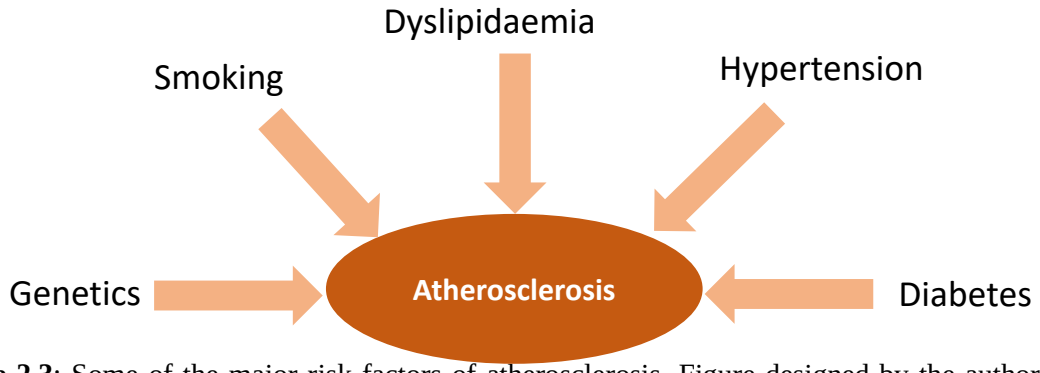


Figure 2.3: Some of the major risk factors of atherosclerosis. Figure designed by the author of this dissertation.

The pathophysiology of atherosclerosis is initiated by elevated levels of plasma cholesterol (hypercholesterolemia) that lead to alteration in the arterial endothelial permeability. The arterial endothelial permeability refers to structural and functional changes in the entire vessel wall which acts as the passage for macromolecules, fluids, and cells into the intima (Mundi *et al.*, 2018). This increase in endothelial permeability is primarily due to changes/ dysfunction in endothelial barrier function (main function is to regulate the exchange of biological molecules and maintain a low and selective permeability to fluid and solutes under normal physiological conditions) (Mundi *et al.*, 2018). Subsequent increase in endothelial permeability permits migration of cholesterol rich fractions of the blood (low density lipoproteins (LDL)) into the arterial wall (Sakakura, Nakano *et al.*, 2013, Naseem 2005). Endothelial cells that express adhesion molecules such as such as vascular adhesion molecule-1 (VCAM-1) and selectins, permit circulating monocytes to migrate via diapedesis in the subendothelial space. In this space, the monocytes acquire macrophage characteristics and change into foamy macrophages (Bergheanu, Bodde and Jukema, 2017). The accumulation, alteration and oxidation of LDL cholesterol in the subendothelial space convert them into strong chemo-attractants, which results in secretion of chemotactic protein 1 (MCP-1), selectins, intercellular adhesion molecules (ICAMs), vascular cell adhesion molecules (VCAMs), platelet and endothelial cell adhesion molecules (PECAMs) and junctional adhesion molecules (JAMs) in endothelial cells, all of which facilitate monocyte rolling and attachment of cholesterol to the endothelium (Braunersreuthe, Mach 2006, Montecucco, Mach 2009, Naseem 2005). All of this ultimately leads to a cascade of vascular modification, as shown in **figure 2.4**.

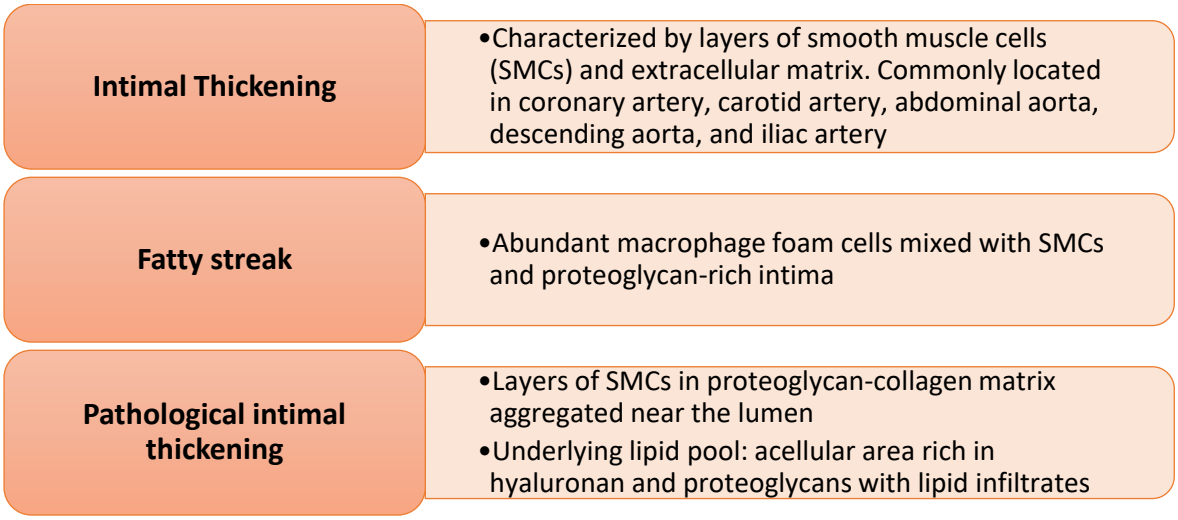


Figure 2.4: Vascular modification in atherosclerosis (*Adapted from* Bergheanu, Bodde and Jukema, 2017).

The surrounding inflammation leads to the release of soluble inflammatory mediators such as C-reactive protein (CRP), cytokines (interleukin 6 (IL-6) and tumour necrosis factor alpha (TNFα), interferon gamma and adipocytokines) endocannabinoids and hormones (Steffens, Montecucco and Mach, 2009; Hansson, 2017). All of these mediators further increase inflammation and activate immune and vascular cells. Smooth muscle cells then migrate to the intima and cause irreversible arterial remodelling which lead to arterial stenosis with calcium and collagen deposition. A rupture is possible when there is atherosclerotic plaque instability (which can be caused by toxic mediators from the vascular and immune cells). A rupture can essentially expose the blood flow to the intra-plaque pro-thombotic material which can result in the formation of thrombus. This can lead to a sudden occlusion of the artery, which can ultimately disrupt blood flow and result in ischaemia in the heart and brain (Montecucco, Mach 2009).

2.2.4 Endothelial Function & Dysfunction

2.2.4.1 The Vascular endothelium & its function

Atherosclerosis begins in the blood vessels, more specifically in the endothelium located in the tunica intima of blood vessel walls; hence, the vascular endothelium and its function have a crucial role in the development or prevention of atherosclerosis (Zhao, Vanhoutte and Leung, 2015). The blood vessels are composed of three layers (**refer to figure 2.5**): an intimal monolayer of endothelial cells (ECs), which forms the vascular endothelium, medial vascular smooth muscle and the adventitia or tunica

externa (Zhao, Vanhoutte and Leung, 2015). The ECs therefore represent the inner most interior surface of the blood vessels; and they cover the entire vascular tree from the heart to the capillaries.

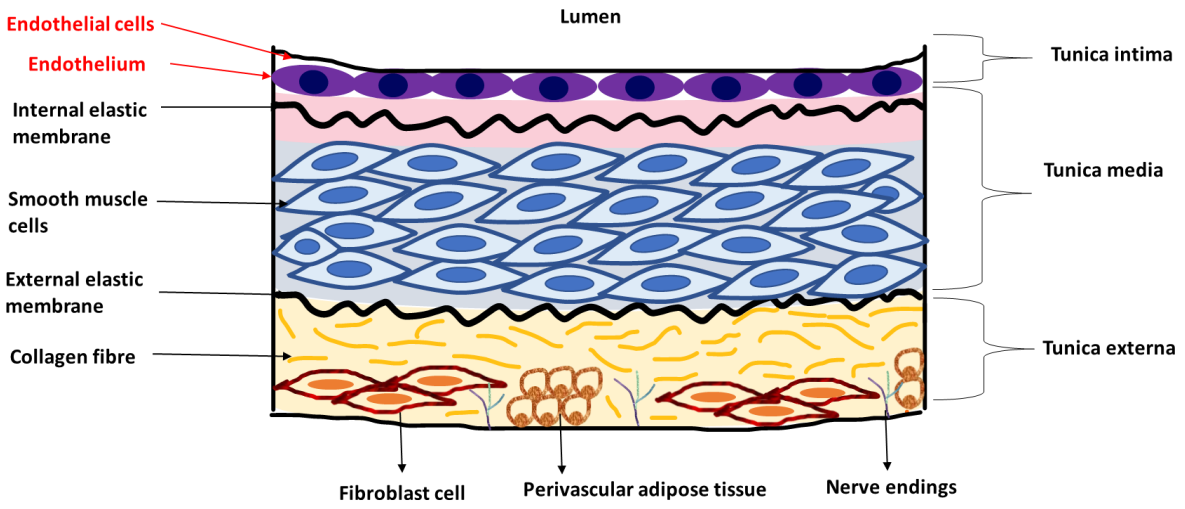


Figure 2.5: Structure of the arterial wall. Tunica intima is composed of ECs and internal elastic membrane; the smooth muscle cells (SMCs) and external elastic membrane form the tunica media; tunica externa is composed of adventitia. The inner layer of ECs forms the endothelium. Figure designed by the author of this dissertation based on content from (Zhao, Vanhoutte and Leung, 2015).

The endothelium has the most important role in regulating vascular homeostasis. It actively controls the vascular tone and permeability and regulates the exchange of molecules in response to extracellular and intracellular signals (Flammer and Luscher, 2011). One of the main outcomes of a healthy endothelium is its ability to maintain a balance between coagulation and fibrinolysis, inflammatory activity, and cell proliferation. In terms of atherogenesis, the endothelium prevents the adhesion of immune cells and infiltration of monocytes into the sub-endothelial space of lesion-prone areas, thus interfering and preventing the development of atherosclerotic plaques (Daiber *et al.*, 2017). Overall, a healthy endothelium is critical in preventing thrombotic events and further exerts anticoagulant, antiplatelet, and fibrinolytic properties. **Refer to figure 2.6** showing the effects of the healthy vascular endothelium.

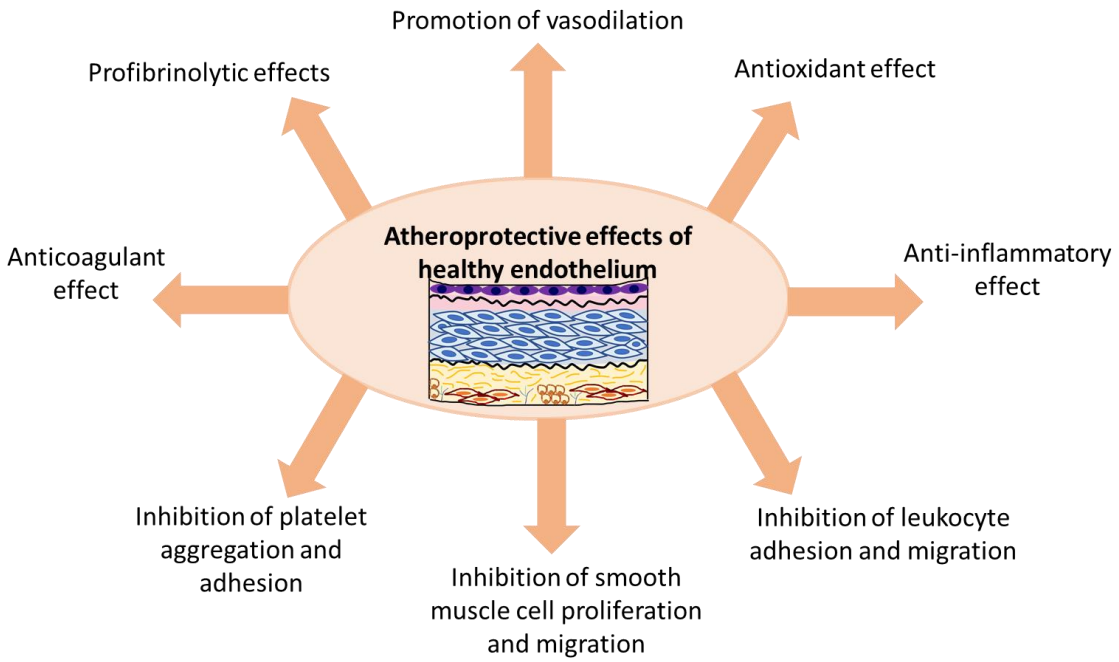


Figure 2.6: Atheroprotective effects of the endothelium. Figure designed by the author of this dissertation based on contents from (Favero *et al.*, 2014).

The ECs have the ability to sense mechanical stimuli such as pressure and shear stress through its mechanosensors. In addition, they have receptors for ligands such as hormones and other vasoactive substances, to which the endothelium responds to by releasing agents that regulate vasomotor function, trigger inflammatory processes, and affect homeostasis (Endemann and Schiffrin 2004, Limaye and Vadas 2007). The endothelium is able to achieve most of its functions by releasing endothelium-derived relaxing factors (EDRF) and endothelium-derived constricting factors (EDCF), **refer to table 2.3** for a summary of EDRFs and EDCFs. At optimal function, the endothelium maintains a balance between EDRFs and EDCFs.

Table 2.3: Summary of relevant endothelial derived factors and their function.

Endothelium derived relaxing factors		
	Description	**Function**
Nitric Oxide (NO)	The most important EDRF, produced by endothelial nitric oxide synthase (eNOS) in response to shear stress (Flammer and Lüscher, 2010). Described in detail under section 2.2.4.2	Key function is the induction of a vasodilatory effect on the vasculature (Davignon and Ganz, 2004). Other functions include inhibition of smooth muscle cell proliferation, prevention of platelet adhesion and aggregation, as well as leukocyte adhesion and migration into the arterial wall (Strijdom, Chamane and Lochner, 2009). Described further in section 2.2.4.2
Prostacyclin (PGI2)	Produced by cyclooxygenase-1 (COX-1) from arachidonic acid and increases cAMP in smooth muscle cells and in platelets. Released partly in response to shear stress and when there are disturbances in endothelial function (Flammer and Lüscher, 2010).	Main function is to limit vasoconstriction. Also known to aid in the release of NO by endothelial cells and vice versa, the action of PGI2 in the vascular smooth muscle is also potentiated by NO (Flammer and Lüscher, 2010).
Endothelium-derived hyperpolarisation factors (EDHF)	Hydrogen sulphide is known to be a major EDHF. Other EDHFs include K+, cytochrome P450 metabolites (epoxyeicosatrienoic acids- arachidonic acids) and hydrogen peroxide (Ozkor and Quyyumi, 2011)	EDHFs are responsible for vascular smooth muscle hyperpolarisation and eventually vasodilation (Ozkor and Quyyumi, 2011).
Endothelium derived constricting factors		

	Description	Function
Endothelin 1 (ET-1)	ET-1 is more dominant in the cardiovascular system and its production is stimulated by Interleukin (IL-1ß), tumor necrosis factor (TNFα), transforming growth factor (TGFß), platelet derived growth factor (PDGF), vasopressin, hypoxia, and shear stress (Flammer and Lüscher, 2010; Haque, Welch and Loizidou, 2013). ET-1 is one of a family of three endothelins (ET-1, ET-2, ET3) (Haque, Welch and Loizidou, 2013).	ET-1 elicits its function by binding to two G-protein coupled receptors, ET_A and ET_B. Main function is to exert opposite effects to NO and promote vasoconstriction. Binding to ET_Areceptors in the vascular smooth muscle cells lead to vasoconstriction, meanwhile, binding to ET_B. in the endothelium reduces ET-1 production by promoting NO and prostacyclin production (Bourque, Davidge and Adams, 2011).
Thromboxane A (TXA_2)	TXA_2 is converted from prostaglandin H2 through TXA_2 synthase in the cyclooxygenase (COX) pathway (Chen, 2018).	TXA_2 is a proatherogenic prostanoid that can induce vasoconstriction, platelet activation and adhesion. In the cardiovascular system, its main role is signalling endothelium dependent contractions in the arteries (Chen, 2018).
Angiotensin II (Ang II)	Ang II is a hormone, bioactive peptide of the renin-angiotensin system (RAS). The diverse actions of Ang II are mediated via AT1 and AT2 receptors, which couple to many signalling proteins, e.g. small G proteins, phospholipases, mitogen-activated protein (MAP) kinases, phosphatases, tyrosine kinases, NADPH oxidase, and transcription factors (Cat & Touyz 2011).	It is a potent vasoconstrictor that increases peripheral vascular resistance and elevates arterial blood pressure. It is known for its pathophysiological role in CVDs such as hypertension, heart failure and atherosclerosis (Cat and Touyz, 2011).

2.2.4.2 Nitric oxide (NO)- the most important vasodilating factor

NO is the most crucial EDRF in the vascular system. It is synthesized in the ECs by endothelial nitric oxide synthase (eNOS) during conversion from L-arginine to L-citruline (Sena, Pereira and Seiça, 2013). **Refer to figure 2.7** for NO production and release description. The production of NO by eNOS requires cofactors such as tetrahydrobiopterin (BH4) and nicotinamide adenine dinucleotide phosphate (NADPH). Shear stress and vasodilatory agonists such as acetylcholine, activate eNOS to produce NO by increasing intracellular calcium by acting on specific receptors (muscarinic receptors) (Davignon and Ganz, 2004; Zhao, Vanhoutte and Leung, 2015). This displaces caveolin and calcium then binds with calmodulin, activating eNOS to produce NO, hence the shear stress-induced synthesis of NO by eNOS is calcium-dependent. In contrast, NO can also be produced via a calcium independent pathway where eNOS is phosphorylated independent of calcium and ultimately leads to the production of NO (Zhao, Vanhoutte and Leung, 2015). Once NO is produced, it diffuses to the vascular smooth muscle cells (VSMCs) and activates soluble guanylate cyclase (sGC), elevating levels of cyclic guanosine-3,5-monophosphate (cGMP) and leading to relaxation of VSMCs (Davignon and Ganz, 2004; Zhao, Vanhoutte and Leung, 2015) (**refer to figure 2.7**).

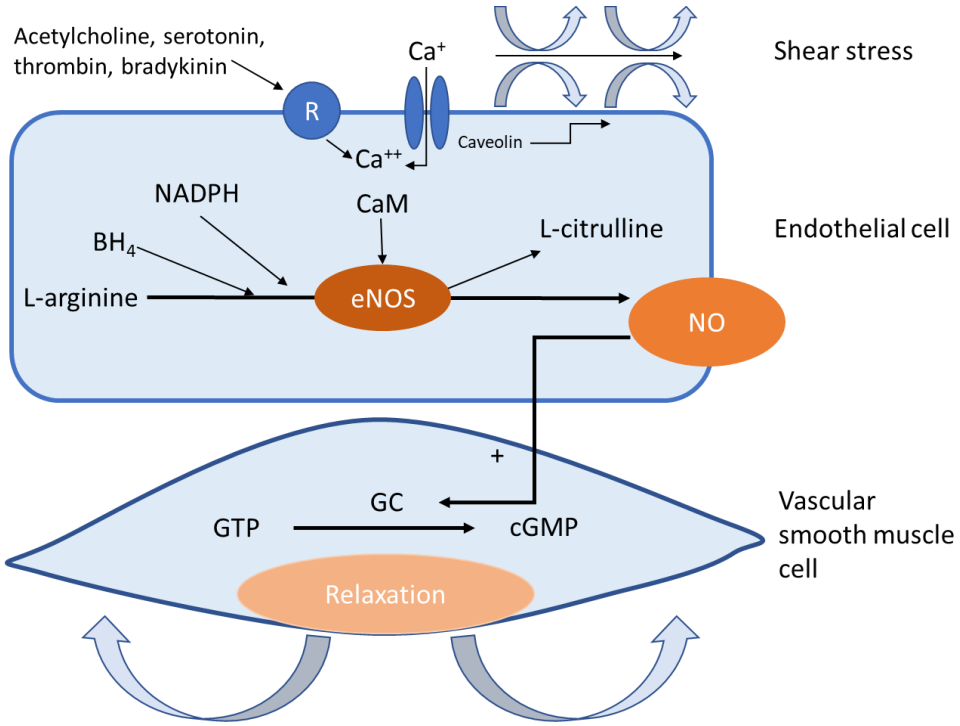


Figure 2.7: Production of nitric oxide (NO) by endothelial cells and relaxation of the vascular smooth muscle cells. Figure designed by the author of this dissertation based on content from (Davignon and Ganz, 2004; Toblli *et al.*, 2012). eNOS, endothelial nitric oxide synthase; NO, nitric oxide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; CaM, calmodulin; GTP, guanosine 5'-triphosphate; GC, guanylate cyclase; cGMP, cyclic guanosine monophosphate.

The most important function of NO is its vasodilatory effect on the vasculature. NO mediates this vasodilation by opposing the effects of EDCFs such as endothelin and angiotensin II (Davignon and Ganz, 2004). Apart

from vasodilation, NO can also exert anti-thrombotic and anti-inflammatory effects on the vasculature by inhibiting platelet adherence and aggregation, leukocyte adhesion/infiltration, and proliferation of vascular smooth muscle cells (Strijdom, Chamane and Lochner, 2009). Furthermore, NO is known to prevent oxidative modification of LDL- cholesterol which is found to be one of the crucial mechanisms in atherosclerosis (Davignon and Ganz, 2004). Apart from NO's direct role, it can also modulate the release of other endothelium derived factors, for example, in many large arteries, EDHF dependent vasodilation only becomes prominent when NO is inhibited, also demonstrating NO's gatekeeping abilities (Vanhoutte *et al.*, 2017). Hence, overall, NO is the most important vasodilating factor in maintaining vascular homeostasis and endothelial function.

2.2.4.3 Endothelial dysfunction

Pathophysiological alterations to the endothelium towards reduced vasodilation and a pro-inflammatory and prothrombotic state collectively result in endothelial dysfunction (Rajendran *et al.*, 2013; Daiber *et al.*, 2017). The hallmark of endothelial dysfunction is reduced NO bioavailability. Additionally, endothelial dysfunction is characterized by poor hemodynamic deregulation, impaired fibrinolytic ability, overproduction of growth factors, increased expression of adhesion molecules and inflammatory genes, excessive generation of ROS, increased oxidative stress, and enhanced permeability of the endothelium (Sena, Pereira and Seiça, 2013), collectively resulting in a loss of vascular homeostasis. **Refer to table 2.4** for a summary of the major modifications associated with endothelial dysfunction.

Table 2.4: Summary of characteristics associated with endothelial dysfunction (*Adapted from* Sena, Pereira and Seiça, 2013).

Major modifications associated with endothelial dysfunction
• ↓ vasodilation (↓NO, PGI2) • ↑oxidative stress & uric acid • Pro-coagulant abilities (von Willebrand Factor (vWF), plasminogen activator inhibitor (PAI-1), P selectin) • Pro-inflammatory state (↑ICAM, VCAM, E-selectin, CRP, TNF-α, IL-6, MCP-1) • ↑ damage of ECs & ↓ repair of endothelial progenitor cells.

The pathophysiology of endothelial dysfunction is characterized by impaired NO bioavailability which is largely in response to increased oxidative stress and inflammation (Simsek *et al.*, 2010). Oxidative stress can be due to either an increase in oxidant production, or a reduction in antioxidant protection or a failure in repairing oxidative damage (Simsek *et al.*, 2010). Hyperglycaemia, insulin resistance, dyslipidaemia, inflammation, and cigarette smoking are some of the many cardiovascular risk factors associated with oxidative stress. In some cases, oxidative stress can lead to uncoupling of eNOS which can produce more ROS

instead of NO (Endemann 2004, Van den Oever, Raterman *et al.*, 2010) . ROS can induce EC damage, and free radicals are known to disrupt the balance of NO, which leaves the endothelium overtly permeable, allowing toxins and acute phase and pro-inflammatory proteins such as CRP to pass through the blood vessels and into adjacent tissues (Rajendran *et al.*, 2013). Inflammation is additionally known to reduce NO bioavailability. When NO and its physiological actions are inhibited, endothelial signalling is impaired leading to endothelial dysfunction and, if not reversed, resulting in widespread pathophysiological consequences in the body. One of the major sequelae of endothelial dysfunction is atherogenesis and thrombosis, largely due to the loss of endothelium's protective abilities and for the induction of pro-atherothrombotic mechanisms (Sena, Pereira and Seiça, 2013). Additionally, apart from atherosclerosis, endothelial dysfunction is associated with the development of many other CVDs such as hypertension, and coronary artery disease, as well as metabolic conditions such as diabetes mellitus (Sena, Pereira and Seiça, 2013).

2.2.5 Biomarkers of Atherosclerosis (inflammation) & Endothelial dysfunction

A biomarker is defined as a measurable characteristic by which a physiological process or pathology can be identified (Group *et al.*, 2001; Stoner *et al.*, 2013). Generally, a biomarker should be quantifiable in samples such as blood, serum, urine or tissue and should be associated directly or indirectly with the represented disease (Stoner *et al.*, 2013). As discussed in earlier sections, CVD pathogenesis includes the complex mechanisms of atherosclerosis and ED and hence there is not only one biomarker present to predict CVD, but several which can be implicated with the disease. Additionally, it is imperative to note that not all biomarkers are equal with some similar in their function and offering a better prognostic information than others or some better suited to predict certain specific CVDs. For the purposes of this PhD dissertation, only known and major biomarkers of atherosclerosis and ED will be discussed. **Refer to table 2.5** for some of the major circulating biomarkers of ED and atherosclerosis with their functions.

Table 2.5: Summary of biomarkers of endothelial dysfunction and atherosclerosis with their relevant function.

Biomarker	Function
Vascular Cellular Adhesion Molecule-1 (VCAM-1)	VCAM-1 is expressed in blood vessels after the ECs are stimulated by cytokines. Its main function is to mediate rolling, adhesion and migration of leukocytes across the endothelial barrier (Stoner *et al.*, 2013). VCAM-1 is up-regulated by cytokines in response to TNF-α and IL-1 and through stabilization of mRNA through the action of IL-4. Furthermore, VCAM-1 is known to promote monocyte chemotaxis (Goncharov *et al.*, 2017).
Intercellular cell-adhesion molecule-1 (ICAM-1)	ICAM-1 is expressed on the surface of ECs, leukocytes and SMCs in response to shear stress, bacterial toxins, pro-inflammatory cytokines and oxidants. ICAM-1 is involved in mediating attachment of circulating leukocytes to the endothelium and

	their subsequent transmigration and accumulation in the arterial intima (Stoner *et al.*, 2013). ICAM-1 also participates in angiogenesis, and EC activation or damage (Goncharov *et al.*, 2017). All these processes play a vital role in the development and progression of atherosclerosis. Hence measurement of circulating soluble ICAM-1 released from endothelial cell membranes may predict on-going atherosclerosis.
Platelet/endothelial cell adhesion molecule 1 (PECAM-1)	PECAM-1 belongs to the immunoglobulin gene superfamily and is highly expressed (high density) in ECs and moderately (low density) on the surface of hematopoietic cells (Galkina and Ley, 2007; Stoner *et al.*, 2013). Functions of PECAM-1 include involvement in angiogenesis, integrin regulation, apoptosis and transendothelial migration of monocytes. Importantly, it plays a role in plaque formation and thrombosis. Its role in the pathogenesis of atherosclerosis can be attributed to its ability to mediate leukocyte infiltration (Stoner *et al.*, 2013; Goncharov *et al.*, 2017).
E-selectin	E-selectin is one of the 3 members of the selectin family which includes P-selectin and L-selectin (Goncharov *et al.*, 2017). E-selectin can serve as a surrogate marker of increased expression of cellular adhesion molecules such as VCAM and ICAM on vascular ECs, and reflect inflammation and activation of ECs (Stoner and Sabatier, 2010; Goncharov *et al.*, 2017).
P-selectin	P-selectin is a transmembrane adhesion protein that is upregulated in activated platelets and vascular ECs. P-selectin is known to mediate the initial interaction of circulating leukocytes with activated ECs which lead to a characteristic rolling of the leukocytes on the endothelium. Hence, it plays an important role at sites of inflammation and vascular injury by contributing to thrombus formation through leukocyte involvement (Goncharov *et al.*, 2017).
Interleukin- 6 (IL-6)	IL-6 is a pleiotropic cytokine, produced by a variety of cells, including ECs. The physiological effects of IL-6 are found in a variety of tissues and include cell growth, differentiation, and importantly angiogenesis, re-vascularization, and tissue healing (Goncharov *et al.*, 2017).
Tumour necrosis factor- alpha (TNF-α)	TNF-α is a cytokine which is produced by macrophages, ECs and SMCs of atherosclerotic arteries (Stoner *et al.*, 2013). It has a wide range of pro-inflammatory functions. It contributes to atherosclerotic processes by causing metabolic perturbations and increasing the expression of cellular adhesion molecules such as VCAM-1 and ICAM-1 (Meager, 1999). Furthermore, TNF-α is known to induce the expression of chemokines and enhance the production of other cytokines and growth factors (Stoner *et al.*, 2013). Additionally, TNF-α also stimulates new vessel formation and induces the process of atheroma development (Hotamisligil and Spiegelman, 1994).

C-reactive protein (CRP)	CRP is an acute phase protein. A growing body of literature points to CRP as a potential, crucial etiological factor in inflammation and atherosclerosis (Jialal, Devaraj and Venugopal, 2004). Liver and hepatocytes are the primary sites of CRP production. Production is usually in response to the stimulation by IL-6 and TNF-α from the inflammation site. CRP is also known to enhance the expression of adhesion molecules such as ICAM-1, VCAM-1, E-selectin, and P-selectin, as well as MCP-1 in ECs, further promoting endothelial activation dysfunction and atherosclerosis (Pasceri *et al.*, 2001; Stoner *et al.*, 2013).
Von Willebrand factor (vWF)	vWF is a glycoprotein which plays a critical role in in blood coagulation. Both the expression and function of vWF is carefully regulated by protease ADAMTS-13. Another important function of vWF is the regulation of angiogenesis and any changes in its function can lead to can lead to thrombotic thrombocytopenic purpura (Goncharov *et al.*, 2017).
Monocyte chemoattractant-1 (MCP-1)	MCP-1, a key chemokine, is produced either constitutively or after induction by oxidative stress, cytokines, or growth factors (Deshmane *et al.*, 2009). Its main functions include regulations of migration and infiltration of monocytes and macrophages, all of which have a crucial role in atherosclerosis (Stoner *et al.*, 2013).
Advanced glycation end products (AGEs)	AGEs are products of non-enzymatic glycation and, oxidation of proteins and lipids in response to exposure to sugar. They accumulate in the vessel wall following oxidative stress (Basta, Schmidt and De Caterina, 2004). AGEs exert their pathogenic functions through activating their receptor for advanced glycation end products (RAGE) and macrophages in an NF-κB-dependent fashion. All of this leads to the induction of platelet-derived growth factor (PDGF), insulin-like growth factor (IGF)-I, and pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and TNF-α. AGEs binding with RAGE produces ROS by reducing the cellular antioxidant defence mechanisms. Elevated ROS further activates ECs and promotes expression of procoagulant tissue factors and adhesion molecules such as E-selectin, VCAM-1 and ICAM-1 (Basta *et al.*, 2002; Stoner *et al.*, 2013).
Vascular endothelial growth factor (VEGF)	VEGF is a hypoxia inducible cell mitogen (Paulus, Jennewein and Zacharowski, 2011). It stimulates endothelial cell migration along with vessel permeability (Dvorak *et al.*, 1999) and promotes survival of the newly formed blood vessels. VEGF is crucial for endothelial cell survival (Gerber *et al.*, 1999).
Plasminogen activator inhibitor (PAI-1)	PAI-1 belongs to the family of serine protease inhibitors (SERPINs) (Paulus, Jennewein and Zacharowski, 2011). They are known to be the principal inhibitors of the tissue-type and the urinary-type plasminogen activator (Cesari, Pahor and Incalzi, 2010). Both plasminogen activators can activate plasminogen and fibrinolysis. PAI-1 is known to be involved in the inflammatory process and endothelial cell migration.

	Under physiological conditions, PAI-1 is released into the circulation and the extracellular space by liver cells, smooth muscle cells, adipocytes, and platelets (Paulus, Jennewein and Zacharowski, 2011).
Angiotensin II (ANG II)	ANG II is a key player in the renin–angiotensin–aldosterone system (RAAS). Its main function is to maintain blood pressure, salt and fluid volume homeostasis (Rautureau, Paradis and Schiffrin, 2011). It has vasoconstrictor and anti-natriuretic properties. ANG II can also induce production of ROS, and both production and expression of pro-inflammatory cytokines and adhesion molecules. Furthermore, it is known to promote the functional adhesion of monocytes to ECs and enhance expression of TNF-α, IL-6, and IL-1β as well as chemokines and chemokine receptors (Stoner *et al.*, 2013).
Anti-oxidant biomarkers	In literature many biomarkers have been identified that may represent the balance between free radical formation and protective antioxidants (Stafforini *et al.*, 1997). Oxidative stress biomarkers include F2 isoprostane, lipoprotein-associated phospholipase A2 (LpPLA1), nitrotyrosine, and oxidized LDL. Important antioxidant biomarkers include coQ10, GSH, and SOD. Most of these biomarkers are pro-atherogenic and play a role and correlate with atherosclerosis and CVD (Stoner *et al.*, 2013).

Endothelial dysfunction is accompanied by inflammation, hence fragments of activated endothelium, endothelial microparticles, and as well as whole ECs are released into the circulation. These can typically be measured in the blood and their circulating levels are found to elevated in CVD (Halcox, 2012). The proinflammatory and procoagulant state of the ECs lead to endothelial activation in the lesion-prone regions of the vessels. This is characterized by an increase in leukocytes and is associated with early stages of atherosclerosis (Coker, 2017). Inflammatory makers associated with endothelial activation are some of the major known biomarkers of both endothelial dysfunction and atherosclerosis, **refer to figure 2.8** for endothelial activation.

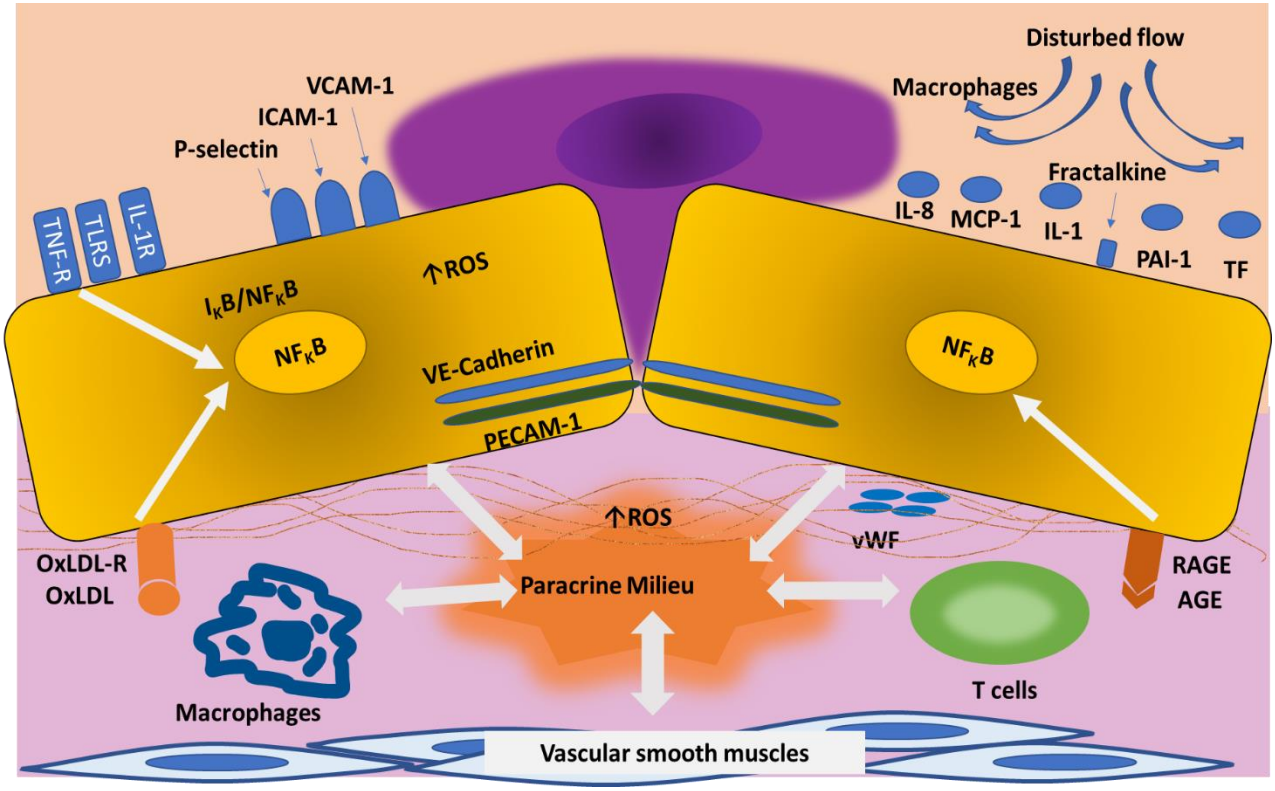


Figure 2.8: The role of the various inflammatory markers in endothelial activation. Figure designed by the author of this dissertation based on content from (Gimbrone and García-Cardeña, 2016). IL-1,8, interleukin 1, 8; IL-R, receptor for IL-1; TNF-R receptor for TNF; Ox-LDL-R, receptor for oxidized LDL; RAGE, receptor for AGE; TLRs, Toll-like receptors; VCAM-1, vascular cellular adhesion molecule-1; ICAM-1, intercellular cell adhesion molecule-1; ROS, reactive oxygen species; VWF, von Willebrand factor; MCP-1 monocyte chemoattractant-1; and PAI-1, Plasminogen activator inhibitor-1.

Endothelial activation begins in the lesion-prone regions of the vessels where actions of proinflammatory agonists such as IL-1, TNF, and endotoxin, oxidized lipoproteins and advanced glycation end products (AGE), as well as biomechanical stimulation by disturbed blood flow take place (**refer to figure 2.8**) (Gimbrone and García-Cardeña, 2016). These factors and disturbed blood flow act largely through the pleiotropic transcription factor, nuclear factor-κB (NF-κB), and lead to cellular expression of adhesion molecules such as ICAM-1 and VCAM-1, as well as secreted and membrane-associated chemokines such as MCP-1, and prothrombotic mediators such as tissue factor (TF), vWF and PAI-1 (Pober *et al.*, 1986; Gimbrone and García-Cardeña, 2016). This leads to selective monocyte and T lymphocytes recruitment which then relocate in the subendothelial space. The combined actions of activated ECs, SMCs, monocyte/macrophages and lymphocytes result in the production of a complex paracrine milieu/ environment of cytokines, growth factors, and ROS within the vessel wall, all of which promote chronic proinflammatory state and atherosclerotic progression (Gimbrone and García-Cardeña, 2016) (**refer to figure 2.8**).

In the activated state, endothelial cells have an increased expression of proinflammatory cytokines, enzymes and adhesion molecules (Baghai *et al.*, 2018). Thus, it is highly possible that endothelial activation plays a crucial role in development of endothelial dysfunction. In many ways endothelial activation is an early step in the development of endothelial dysfunction (Daiber *et al.*, 2017). Therefore, identification of endothelial cell-derived inflammatory factors may serve as biomarkers of endothelial dysfunction (Sun *et al.*, 2020). **Refer to figure 2.9** for the link and role of endothelial activation and dysfunction in vascular disease.

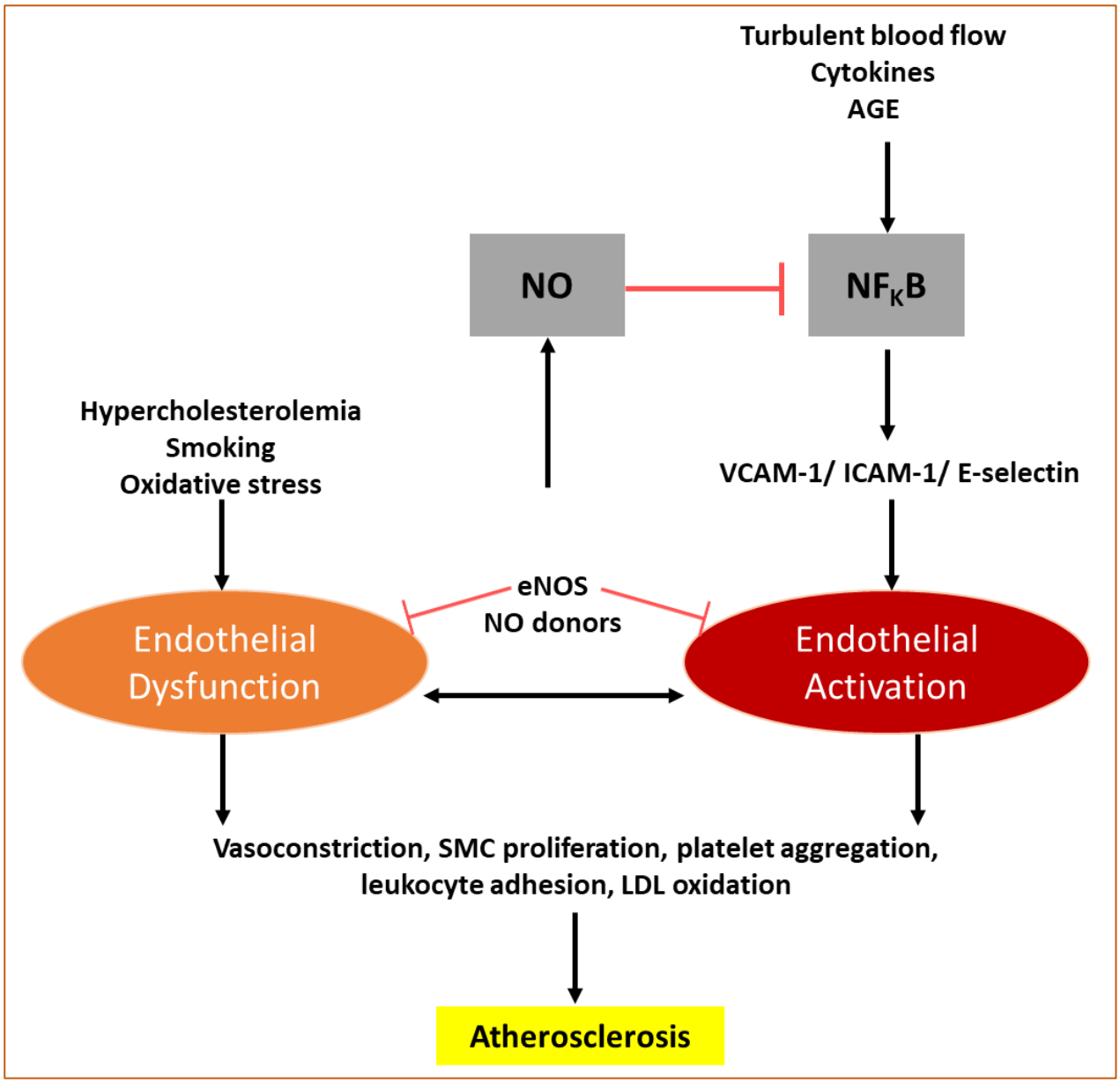


Figure 2.9: The role of endothelial activation and dysfunction in vascular disease. CVD risk factors such as hypercholesterolemia, smoking and oxidative stress, promote endothelial dysfunction; meanwhile proinflammatory cytokines, turbulent blood flow and advanced glycation end-products (AGEs) are important mediators of endothelial cell activation via the activation of the transcription factor, NF-κB. NO from eNOS or NO donors reduces endothelial cell activation through inhibition of NF-κB. Reduced or loss of NO leads to increased endothelial cell activation. Likewise, endothelial cell activation can cause endothelial dysfunction.

Both endothelial dysfunction and endothelial cell activation lead to atherosclerosis by increasing vasoconstriction, SMC proliferation, platelet aggregation, leukocyte adhesion, and LDL oxidation. Figure designed by the author of this dissertation based on contents from (Liao and Liao, 2013). NO, nitric oxide; LDL, low density lipoprotein; eNOS, endothelial nitric oxide synthase; SMC, smooth muscle cell; VCAM-1, vascular cellular adhesion molecule-1; ICAM-1, intercellular cell adhesion molecule-1; and AGE, advanced glycation end-products.

2.2.6 The role of CVD risk factors, endothelial dysfunction, atherosclerosis and biomarkers in CVD

Prolonged exposure to cardiovascular risk factors such as age, obesity, smoking, dyslipidaemia, hypertension and diabetes, among others, is known to impact the endothelium. Vascular endothelium can lose its defence mechanisms, resulting in endothelial activation and dysfunction (**refer to figure 2.10**). Endothelial dysfunction is known to be one of the major links between CVD risk factors and atherosclerosis (Mudau *et al.*, 2012). The progression to atherosclerosis from cardiovascular risk factors is mediated by a range of mechanisms and inflammatory markers (**refer to figure 2.10**), however, endothelial dysfunction is a reversible step in the process of CVD and hence clinical screening, or measurement thereof can prove to be useful. Furthermore, there are established circulating biomarkers associated with endothelial dysfunction and atherosclerosis, which too can be screened and measured to predict cardiovascular outcome (Park and Park, 2015).

Atherosclerosis exists along a continuum and begins with subclinical atherosclerosis which starts early with processes of ED and inflammation. The term “subclinical atherosclerosis” refers to early pro-atherosclerotic changes in blood vessels that are not detected by normal physical examination. It has no or minimal recognizable clinical findings and is often asymptomatic (Shiel 2018). Hence, subclinical atherosclerosis is defined as an early precursor of atherosclerosis. Subclinical atherosclerosis can remain clinically undetected throughout the life of the individual until an acute cardiovascular event transpires (**refer to figure 2.10**) (Singh *et al.*, 2018). Hence, measuring subclinical atherosclerosis can prove to be an early indicator of atherosclerosis and if detected, it can slow or prevent the progression to overt CVD (Singh *et al.*, 2018).

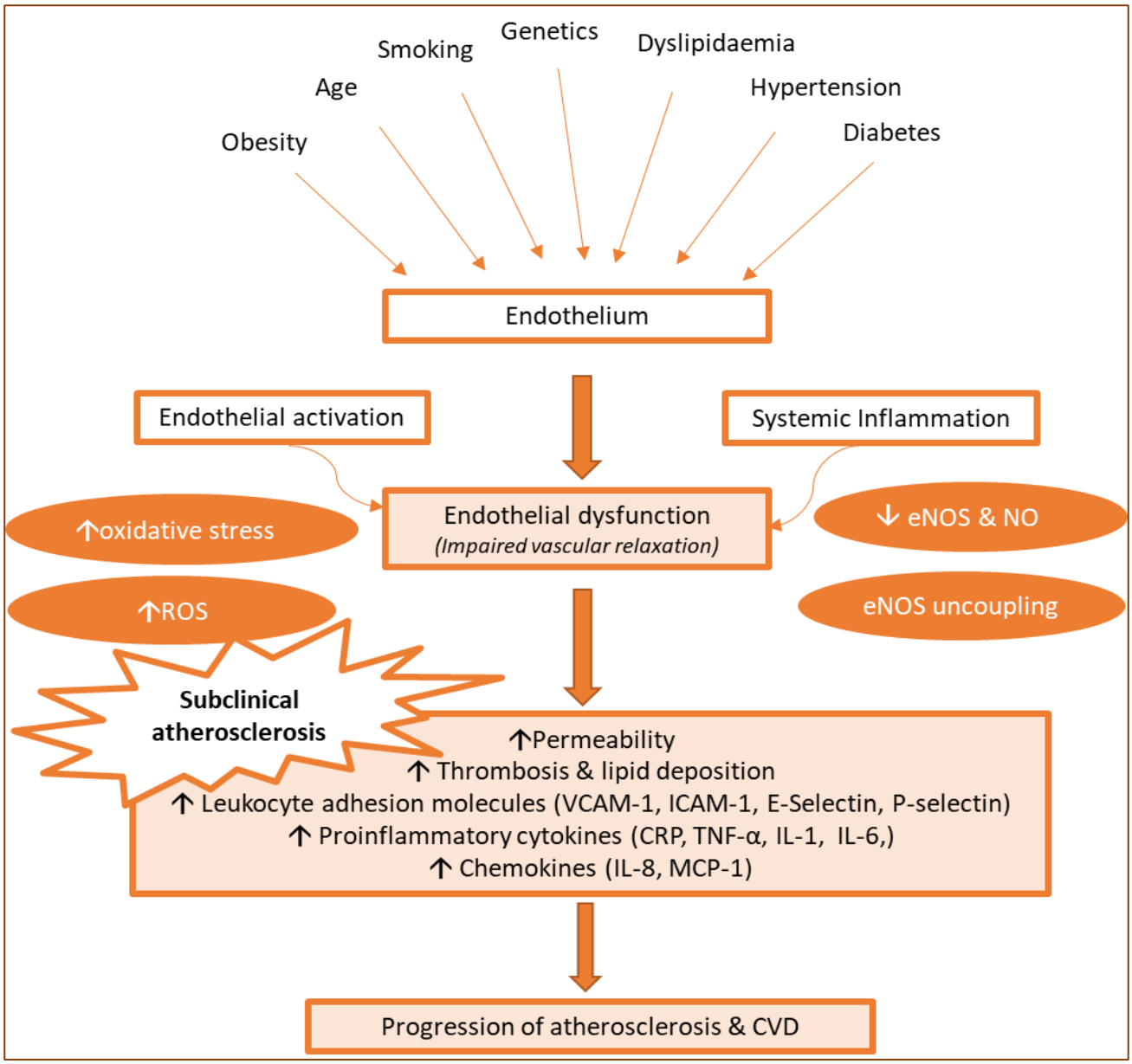


Figure 2.10: The progression from CVD risk factors to ED to atherosclerosis and ultimately CVD. Figure designed by the author of this dissertation.

2.2.7 Measurement of Atherosclerosis and Endothelial function

2.2.7.1 Measurement of Atherosclerosis

As discussed in earlier sections, atherosclerosis is a chronic, progressive inflammatory disease which typically has a long asymptomatic phase. Eventually, atherosclerosis progression can lead to an acute cardiovascular event (Toth, 2008). However, while the disease is still in its subclinical phase, it can remain undetected until a symptom or acute event occurs. Hence the subclinical phase can be useful for measurement, particularly from the clinical perspective as changes in the arterial wall precede clinical signs and symptoms (Holewijn *et al.*, 2010). Thus, measurement and quantification of subclinical atherosclerosis could serve as an early indicator of atherosclerotic burden and if detected in a timely fashion, it can slow or prevent the progression to CVD (Singh *et al.*, 2018). Atherosclerosis measurement further represents the overall risk exposure and genetic susceptibility which may be beneficial in refining CVD risk stratification and therapeutic strategies (Singh *et al.*, 2018).

There are several invasive and non-invasive techniques available to measure subclinical atherosclerosis. These techniques can identify and measure distributions and parameters in the vasculature, such as luminal diameter, vessel wall thickness, and plaque volume (Toth, 2008). Currently there are many invasive techniques available that visualize the arterial system and measure the extent of atherosclerosis. These techniques include intravascular ultrasound and angiography. Less invasive techniques include computed tomography and magnetic resonance imaging. However, it is understood that these invasive techniques are not ideal for screening in the general population (Holewijn *et al.*, 2010). Some of the techniques require injected detergents to optimize image quality and may also expose the individuals to radiation. Overall, these techniques are found to be expensive, and not widely available and applicable to everyone. Therefore, recently more non-invasive techniques have been developed that are both affordable and more widely available (**Refer to table 2.6**) (Holewijn *et al.*, 2010). One such successful technique, which is also relevant to the current dissertation, is the measurement of carotid intima medial thickness (IMT) (further explained under section 2.2.7.1.1).

Table 2.6: Various techniques for the measurement of subclinical atherosclerosis

Technique	Brief description
Coronary angiography	A highly invasive technique using a catheter and detergents to localize plaque and reveal the degree of coronary luminal stenosis. A downfall of this technique is that it cannot identify specific lesions that are prone to rupture and hence can lead to acute coronary syndromes (ACS) (Little *et al.*, 1988; Toth, 2008).
Intravascular ultrasound (IVUS)	IVUS is an invasive technique that relies on the miniaturisation of ultrasound transducers that are placed at the tip of a coronary catheter (Tabas, García-Cardeña and Owens, 2015). This technique can measure both the size and composition of the plaques along the vessel wall and provide information regarding the lesion location and plaque burden (Toth, 2008).
Intima media thickness (IMT) measurement	IMT is a validated successful non-invasive technique that measures the thickness of the inner most two layers (tunica intima and tunica media) of the arterial wall by means of ultrasound technology. Clinically, IMT is often conducted on the carotid artery of the individuals (Toth, 2008; Holewijn *et al.*, 2010; Nezu *et al.*, 2016).
Magnetic resonance imaging (MRI)	High resolution MRI can be used to measure of plaque burden and susceptibility to rupture. The technique generates images of the arteries to visualize stenosis, occlusions and aneurysms. MRI assesses plaque volume and composition, fibrous cap integrity, and lesion type (Chu *et al.*, 2004; Toth, 2008).
Electron beam computed tomography (EBCT)	EBCT is clinically used to measure coronary artery calcification by means of rapid imaging technology and use of electron beam (gun). Plaque burden can be reflected through coronary calcium deposits as they are related to the lipid and apoptotic remnants of the plaque. Some studies point to coronary artery calcification as an independent cardiovascular risk factor, however, although EBCT can identify and measure the severity of coronary plaques within the coronary tree, it cannot predict plaque rupture (Toth, 2008).
Pulse wave velocity (PWV)	Clinically, PWV is a gold standard non-invasive method to measure arterial stiffness. It measures the velocity at which the blood pressure pulse circulates through the arteries. The technique records pulse waveforms at two sites sequentially and is usually measured between

	the right carotid and the left femoral artery (Townsend *et al.*, 2015). As PWV increases as the arteries become stiffer with ageing and progression of atherosclerosis (Holewijn *et al.*, 2010).

2.2.7.1.1 Intima media thickness (IMT) measurement

IMT measurement by ultrasound is a well-known non-invasive, sensitive, and reproducible technique. In the literature, it is an established marker of subclinical atherosclerosis (Holewijn *et al.*, 2010; Kim and Youn, 2017). With aging and progression of atherosclerosis, the arterial wall thickens and the ultrasound IMT technique measures these structural changes in the arterial wall (Holewijn *et al.*, 2010). To be more specific, carotid IMT measurements reflect the distance between the luminal border of the intima and the outer border of the media of the carotid artery far wall, which is represented as a double-line pattern on a B-mode ultrasound image (**refer to figure 2.11**) (Stein *et al.*, 2008; Kim and Youn, 2017) . Reference values for IMT has been discussed in many studies, the most common guideline includes an IMT value of > 0.9 mm or the presence of plaque as a sign of damage (Naqvi, Lee and Iana, 2015). There are four segments in the carotid artery, common carotid artery (CCA), bifurcation (bulb), external carotid artery and internal carotid artery (ICA). Although IMT can be measured at the bulb and ICA, the most common site of measurement as reported in the literature is the distal common carotid artery. This is largely due to the difficulty in obtaining images for measuring ICA IMT, whilst the CCA far wall IMT has been considered to have the best reproducibility and validated to display the true thickness of the vessel wall (Roman *et al.*, 2006; Kim and Youn, 2017). .

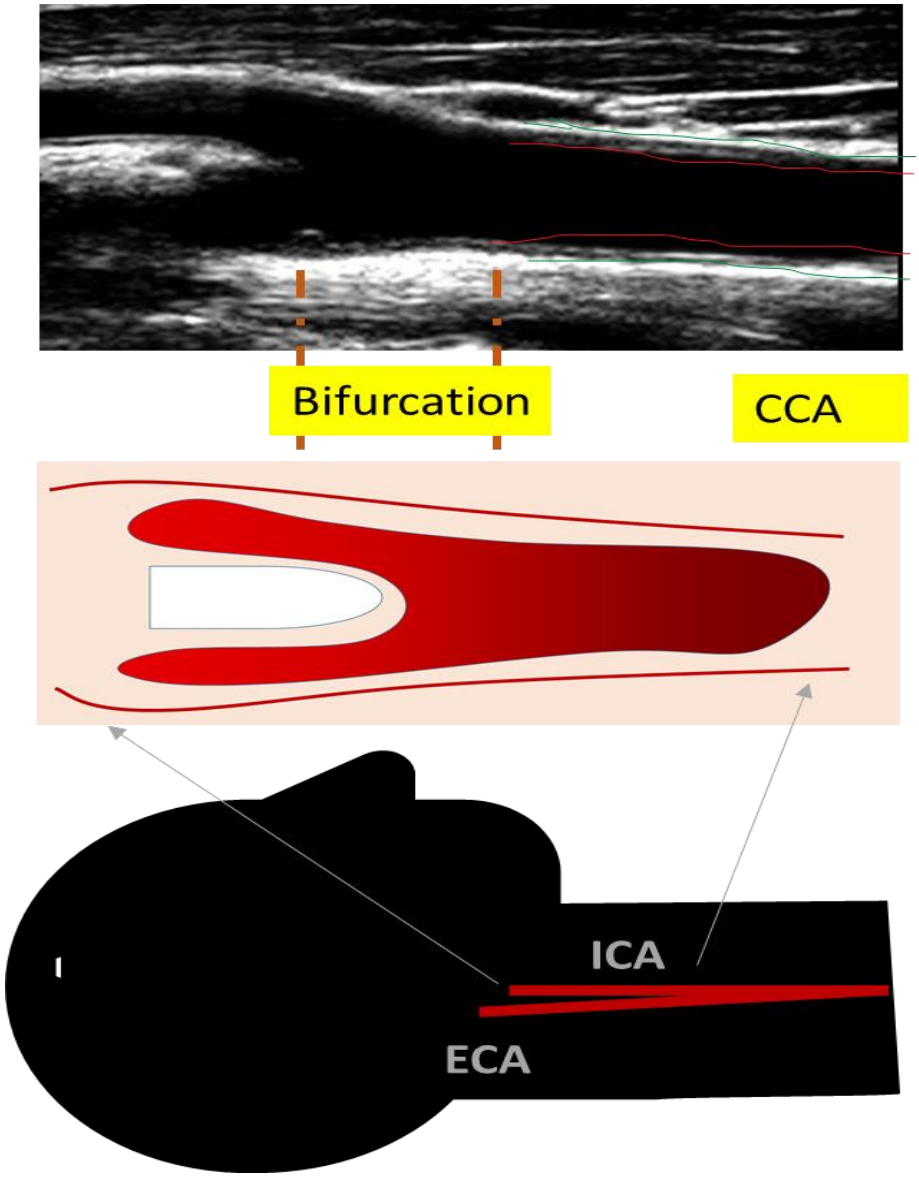


Figure 2.11: Measurement of carotid IMT. The most distal 10 mm of the common carotid artery is measured. Normal intima-media complex is depicted; ECA, external carotid artery; ICA, internal carotid artery; and CCA, common carotid artery. Figure designed by the author of this dissertation based on contents from (Holewijn *et al.*, 2010).

Although IMT data reported in the literature have been found to be generally consistent and reproducible, a measure of inter-study variability does exist, which could be ascribed to different protocols, ultrasound devices, sonography techniques, reading equipment, readers of the scans, and the thickness of the intima media complex (Holewijn *et al.*, 2010). However, since the development of automatic devices and edge detection software programs, the variability has decreased substantially (Kim and Youn, 2017).

2.2.7.2 Measurement of Endothelial function

Endothelial dysfunction is an early precursor of atherosclerosis and appears even before structural changes develop. If detected at an early stage, treatment and/ or lifestyle changes can potentially reverse the progression to atherosclerosis and CVD (Holewijn *et al.*, 2010). Clinically, endothelial dysfunction was first demonstrated in 1986 by intracoronary infusion of the NO-inducing agent, acetylcholine, and quantitative coronary angiography in atherosclerotic coronary arteries (Ludmer *et al.*, 1986). Since then, many less invasive and non-invasive techniques have been developed to measure endothelial function (**Refer to table 2.7** for techniques for the measurement of endothelial function). All these various techniques have their disadvantages and advantages; however, for clinical purposes, techniques that are non-invasive, reliable, reproducible and easily accessible are favoured as the ideal methods for the measurement of endothelial function (Deanfield, Halcox and Rabelink, 2007; Al-Qaisi *et al.*, 2008; Flammer and Lüscher, 2010). The current gold standard measurement of endothelial function in clinical research is the technique of flow-mediated dilatation (FMD) of the brachial artery (Flammer and Lüscher, 2010). This method was also used in the current PhD study and explained further below.

Table 2.7: Selected techniques for the measurement of endothelial function adapted from (Mudau *et al.*, 2012; Charania, 2017).

Method	Description
Intracoronary infusion of acetylcholine	An invasive technique which involves intracoronary infusion of the endothelium-dependent vasodilator, acetylcholine, and subsequent measurement of the vasomotor response due to relaxing factors
Flow mediated dilatation	A gold standard, non-invasive technique which relies on endothelium-dependent release of NO and other EDRF's in response to reactive hyperaemia. The method uses ultrasound technology to visualize a segment of the brachial artery
Forearm plethysmography	Involves intra-brachial infusion of endothelial-dependent vasodilators such as acetylcholine, metacholine, substance P and bradykinin, with subsequent measurement of changes in endothelial function of forearm arterioles
Finger plethysmography	A non-invasive technique that measures changes of the pulse-wave amplitude during reactive hyperaemia. Low pulse-wave amplitudes are associated with compromised endothelial function and are therefore good predictors of CVD

2.2.7.2.1 Flow mediated Dilatation (FMD)

Flow mediated dilatation (FMD) of the brachial artery was initially introduced in 1992. Since then it has become the gold standard non-invasive technique for the measurement of endothelial function (Flammer and Lüscher, 2010). FMD essentially measures transient changes in the brachial artery diameter in response to shear stress which is induced by reactive hyperaemia. To be more precise, an increase in blood flow in the brachial artery is achieved by rapid deflation of a pneumatic cuff which is placed on the forearm distal to the brachial artery (**refer to figure 2.12**) (Al-Qaisi *et al.*, 2008). This pneumatic cuff is first hyper-inflated up to 200 mmHg (or 50mmHg above the individual's supra systolic blood pressure) and deflated after 5 minutes. Upon deflation of the cuff, the blood flows back into the artery causing hyperaemia (increased flow), which induces shear stress and activation of eNOS. NO is released and diffuses to the smooth muscle cells causing them to relax (resulting in vasodilation) (Ghiadoni *et al.*, 2015). FMD is measured as the percentage change in the brachial artery diameter in response to increased flow (**refer to figure below 2.12**) (Al-Qaisi *et al.*, 2008). Although no standardized normal ranges have been determined yet, some studies in the literature have shown that normal FMD values in healthy populations range between 7-10%. Individuals with impaired cardiovascular function have lower or absent FMD value (Stout, 2009). The Framingham Heart Study further demonstrated mean FMD% values of ~3.3% in females and ~2.4% in males with ranges from 0 to 5% in individuals with known CVD (Benjamin *et al.*, 2004).

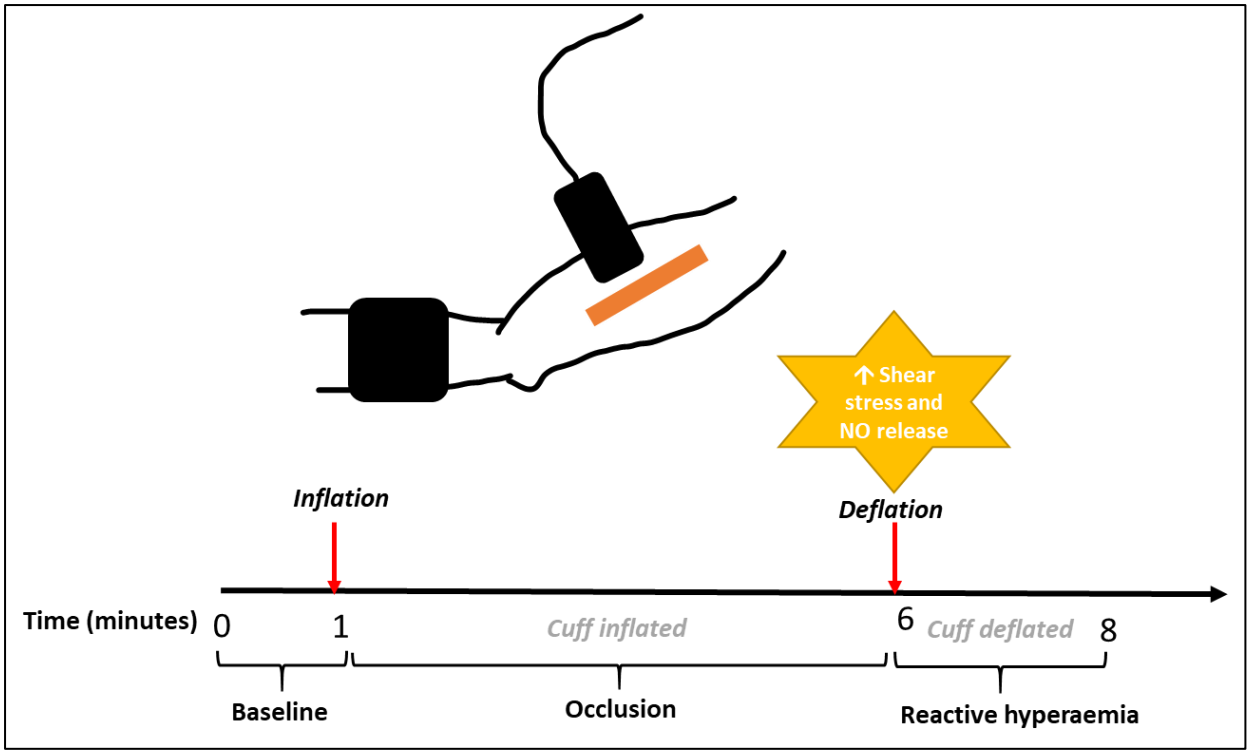


Figure 2.12: Schematic representation of the assessment of FMD of the brachial artery. The image displays the position of the cuff on the forearm as well as the ultrasound probe on the arm. The timeline shows the FMD technique, where baseline measurement is for 1 minute, after which the cuff is inflated causing an occlusion, followed by deflation after 5 min which leads to reactive hyperaemia which induces shear stress and NO release. Figure designed by the author of this dissertation based on contents from (Ghiadoni *et al.*, 2015; Jarrete, Zanesco and Delbin, 2016). NO, nitric oxide.

Flow mediated dilatation (FMD) is widely used in clinical research, however the application is found to be technically challenging and thus still requires standardization (Stout, 2009). When using FMD, the effect of the environment and physiological influences as well technical (equipment) and operator variability need to be considered (Stout, 2009; Ghiadoni *et al.*, 2015) .

Section B: Human Immunodeficiency virus and its underlying mechanisms

2.3 Human immunodeficiency virus

2.3.1 Introduction and Epidemiology

Acquired immune deficiency syndrome (AIDS) was first identified as a new disease in 1981 (CDC report, 1981). Two years later, a retrovirus, termed human immunodeficiency virus (HIV) was confirmed to be the aetiological agent that causes AIDS. Initially, HIV infection rates were mistakenly believed to be on a slow rise; however, the number of people living with HIV grew to approximately 8 million by 1990, reaching 30 million by the end of that decade, which demonstrated the devastating and widespread nature of the disease. The response to the HIV pandemic during the first two decades was poor and only began to improve in the mid-2000s largely due to the roll out of successful combination antiretroviral therapy (ART) (Delpech, 2013). Today, due to successful ART, the HIV epidemic has transformed from a rapidly terminal disease to a chronic and manageable condition with steady incremental improvements in life expectancy (Kaplan-lewis, Aberg and Lee, 2017).

Since the start of the HIV epidemic, approximately 75 million people globally have been diagnosed with HIV-infection of whom approximately 32 million have died of the disease (WHO, 2018). Furthermore, according to the Joint United Nations Programme on HIV/AIDS (UNAIDS), it is estimated that 37.9 million people were living with HIV by the end of 2018 (UNAIDS, 2019). According to the WHO, the burden of HIV/ AIDS varies considerably between countries and regions, with the sub-Saharan African region being affected most significantly, accounting for two-thirds of people living with HIV globally. It is estimated that 68% of the people living with HIV are from SSA with Eastern and Southern Africa accounting for 20.6 million HIV-infected people, and 800,000 new HIV infections in 2018 (UNAIDS, 2019). At present, South Africa has the highest number of people living with HIV, estimated to be 7.7 million at the end of 2018 (UNAIDS, 2019). In 2014, UNAIDS in partnership with their essential stakeholders, launched the so-called 90–90–90 targets with the aim to at least diagnose 90% of all HIV-positive individuals, provide ART for 90% of those diagnosed, and achieve viral suppression for 90% of those treated by 2020 (Bain, Nkoke and Noubiap, 2017). With regards to these goals, South Africa has made great progress in HIV testing rates and has subsequently achieved the first of the 90-90-90 target, with 90% of people living with HIV aware of their status in the country (UNAIDS AIDSinfo, 2019). Furthermore, till date, South Africa is home to the largest ART rollout program in the world (UNAIDS, 2019).

2.3.2 HIV pathophysiology

HIV is subdivided into two types, HIV-type 1 (HIV-1) and HIV-type 2 (HIV-2) (Eberle and Gürtler, 2012). HIV-1 is mainly responsible for AIDS and has been reported to have originated in the area around Kinshasa in Democratic Republic of Congo, from where it is believed to have spread through the transport networks to other areas in SSA, West Africa, Europe and the rest of the world (Bbosa, Kaleebu and Ssemwanga, 2019). HIV-2, on the other hand has been documented to be less infectious than HIV-1 (Gilbert *et al.*, 2003) and is largely reported to be endemic to Western Africa, however recently HIV-2 infections have also been described in in Europe (Portugal and France), India and the United States of America (Bbosa, Kaleebu and Ssemwanga, 2019). Genetically, HIV belongs to the Lentivirus genus of the Retroviridae family (Tavassoli, 2011). The HIV genome is characterized by retroviral genes such as gag, pol and env which is bordered by long terminal repeats (LTRs), consisting of the viral promoter (Kirchhoff, 2016).

The HI virus itself is spherical in structure with two identical copies of RNA strands at the core, tightly bound to nucleocapsid proteins (NC, also known as p7) **(Refer to figure 2.13)** (Zhu *et al.*, 2006; Tavassoli, 2011). The two RNA strands are further enclosed by a conical capsid which is composed of the p24 capsid protein (CA). This capsid, further consists of enzymes and proteins which are essential to the virus and not available in the host, this include copies of the viral protease and the accessory Vif, Vpr, and Nef proteins, as well as some cellular factors, such as tRNAlys3 which is used as a primer for reverse transcription (Tavassoli, 2011; Kirchhoff, 2016). The integrity of the virus is maintained by the matrix protein which surrounds the capsid. The envelope that surrounds this matrix is composed of a lipid membrane which is derived from the host cell and contains cellular proteins, as well as about 7–12 trimeric complexes of viral envelope Env protein. The Env protein consists of the external glycoprotein 120 (gp120) that mediates viral attachment and the transmembrane glycoprotein 41 (gp41) that is critical for viral fusion (Kirchhoff, 2016). All of these envelope proteins arrange into homotrimeric ‘‘spikes’’ that are essential for entry into the host cell (Zhu *et al.*, 2006; Tavassoli, 2011).

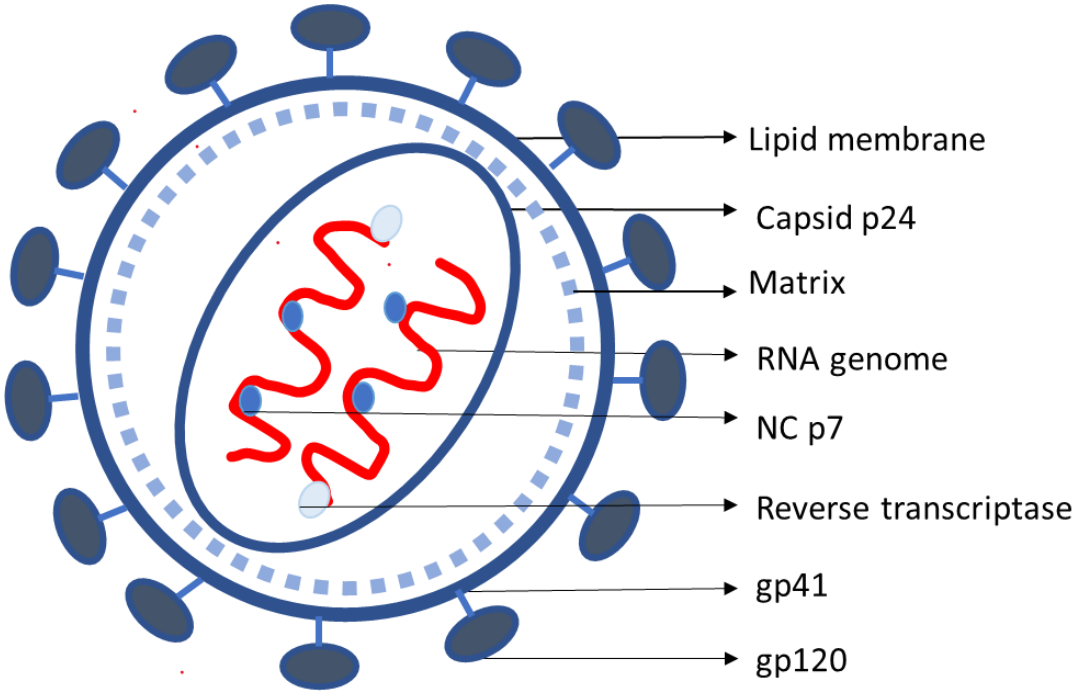


Figure 2.13: Structure of the HIV particle. Figure designed by the author of this dissertation based on content from (Fanales-Belasio, Raimondo *et al.* 2010; Tavassoli, 2011; Kirchhoff, 2016).

2.3.3 HIV life cycle

Refer to figure 2.14 for a schematic presentation of the HIV replication cycle. HIV is an obligate intracellular parasite and as with other viruses, it relies on host proteins to complete its life cycle (Ghimire, Rai and Gaur, 2018). The HIV life cycle is a highly complex and regulated process which recruits viral and cellular components to the plasma membrane for assembly into infectious particles (Mailler *et al.*, 2016). The replication cycle begins with the virus entry into the host. This multistep process includes envelope protein gp41 which is attached to and extends away from the viral membrane, with its extracellular domain non covalently binding to protein gp120 (**refer to figure 2.13** above) (Klimas, Koneru and Fletcher, 2008; Tavassoli, 2011). Both proteins associate into homotrimers that mediate binding and entry into the host cell (Tavassoli, 2011). Gp120 binds to the CD4 receptor which leads to a structural change in the virus envelope complex of gp120 and gp40 to expose a particular domain of gp120 which enables binding with a coreceptor CC chemokine receptor 5 (CCR5) or CX chemokine receptor 4 (CXCR4) that is located on the surface of the cell membrane (Kirchhoff, 2016; Ghimire, Rai and Gaur, 2018). Following the double binding to host (both to CD4 and chemokine receptor), gp120 further undergoes a conformational change that moves the gp41 hydrophobic region close to the host cell, leading to its insertion into the host cell's membrane (Fanales-Belasio *et al.*, 2010)(Fanales-Belasio, Raimondo *et al*. 2010; Tavassoli, 2011; Kirchhoff, 2016). This penetration into the cell membrane leads to a conformational rearrangement of the heptad repeat regions (HR1 and HR2) of gp41 which brings the transmembrane region of gp41 that is in the virus membrane in contact with

the gp41 hydrophobic fusion peptide which is inserted into the host's membrane. This leads to the formation of the fusion pore which permits the virus capsid to enter the cell (Tavassoli, 2011).

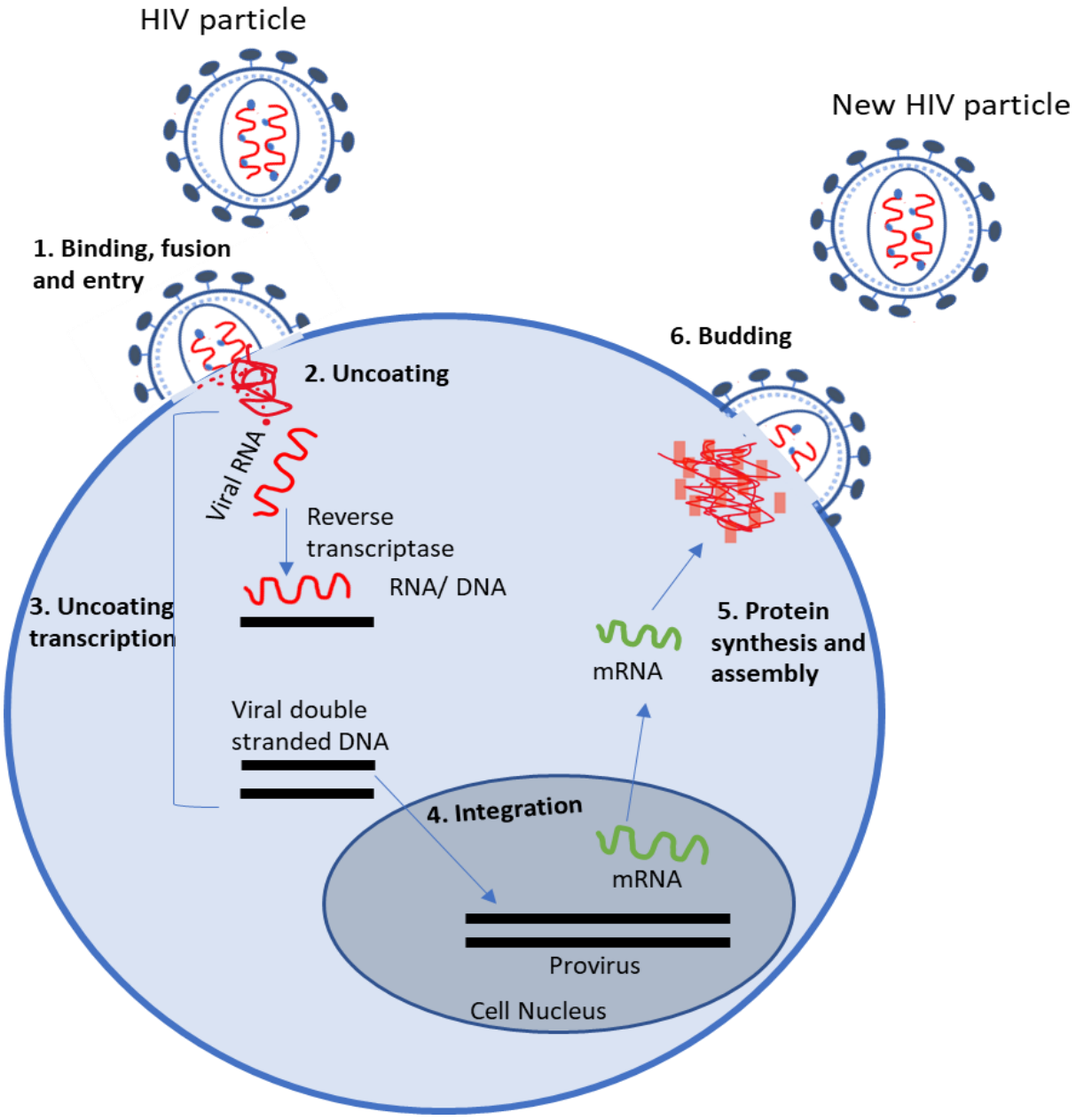


Figure 2.14: HIV life cycle. Figure designed by the author of this dissertation based on content from (Fanales-Belasio, Raimondo *et al.* 2010; Tavassoli, 2011; Kirchhoff, 2016).

Refer to figure 2.14. Following fusion, the HIV capsid accompanied by the genetic information of the virus is released into the host cell cytoplasm (Ghimire, Rai and Gaur, 2018). HIV contains its genetic information as two single stranded RNA, meanwhile the host cell uses DNA. Hence, the viral RNAs are transcribed into linear double- stranded DNAs through the process of reverse transcription which is mediated by the HIV reverse transcriptase enzyme (Tavassoli, 2011). This transcribed HIV DNA moves to the cell's nucleus where HIV integrase enzyme splices the viral DNA into the hosts DNA. This

integration process essentially completes the HIV infection with the viral DNA incorporated into the host's genome (Tavassoli, 2011). This integrated form of the virus, now termed 'provirus' can express viral proteins if the host's cell is in an activated state (Klimas, Koneru and Fletcher, 2008). Following cell activation, the provirus DNA is transcribed into a messenger RNA (mRNA) that initially yields synthesis of regulatory HIV proteins Tat and Rev. The viral protein Tat plays a crucial role in up regulating transcription. Initially only a small number of transcripts are produced, leading to a build-up pf Tat which subsequently triggers the phosphorylation of RNA polymerase II which greatly increases transcription of the viral genome (Tavassoli, 2011).

The complexity of the HIV genome leads to the possibility of more than 30 different viral mRNA species in the host cell (Tavassoli, 2011). The viral mRNA coding for long fragments migrates to the cytoplasm, this transport is promoted by the Rev protein (Kirchhoff, 2016). In the cytoplasm, the mRNA dominates the protein making apparatus of the cell and begins synthesizing structural proteins of the new virus through the process of translation (Klimas, Koneru and Fletcher, 2008). The sequence of the proteins of the mRNA molecules is translated into the RNA and proteins including the envelope and the core of the virus. The process of translation yields gene products which are much larger than those that are part of the final virus and thus they need to be spliced into smaller functional units (Klimas, Koneru and Fletcher, 2008). The viral protease, dominates the splicing process and cleaves large gp160 precursor molecules into gp120 and 41. The Gag and Pol proteins are also derived from a large 160 kD precursor molecule, from which the HIV protease cleaves the p24, p17, p9 and p7 Gag final products and the Pol proteins (Miceli and Parnes, 1993; Fanales-Belasio *et al.*, 2010). The cleavage of the precursor molecules by the HIV protease is necessary for the generation of infectious viral particles. In summary, the new viral particles are generated in a stepwise fashion. To begin with, the two viral RNA strands associate together with the replication enzymes while the core protein assemble over them to form the virus capsid (Fanales-Belasio, Raimondo *et al.*, 2010). Following this, the immature particle migrates towards the cell surface. This entire process is enabled by the Gag protein (Tavassoli, 2011). At this stage, the large precursor molecules are then cleaved by the viral protease, resulting in new infectious viral particles. Following this the virus then 'pinches' off the cells and 'buds' off through the host cell membrane while obtaining a new envelope (Fanales-Belasio, Raimondo *et al.*, 2010; Klimas, Koneru and Fletcher, 2008). The surface of this new HIV particle then acquires HIV glycoproteins that promote binding to CD4 cell co-receptors to infect other cells (Tavassoli, 2011). A single cell can generate thousands of infectious HIV particles either in a recurrent fashion, over a period of weeks or as a single burst leading to cell death (Klimas, Koneru and Fletcher, 2008).

2.3.4 Antiretroviral therapy (ART)

Initially, the clinical management of HIV largely consisted of prophylaxis against common opportunistic infections and managing AIDS related illnesses. The race for finding effective treatment was ongoing throughout the 1980s and 1990s with the first successful ART drugs rolled out in the mid-1990s (Ghimire, Rai and Gaur, 2018). Shortly after this, approval was given for monotherapy, and combination therapy based on highly active antiretroviral therapy (HAART), which transformed HIV from a progressive fatal illness to a chronic manageable disease (Arts and Hazuda, 2012). Although, HAART is not a cure for HIV, it substantially reduces viral load, maintains T cell counts and prevents opportunistic infections that may be fatal (Ghimire, Rai and Gaur, 2018). Overall, these successful advances in the ART space have led to significant reductions in morbidity and mortality in people living with HIV (Rana *et al.*, 2020).

ART predominantly acts on various stages of the HIV life cycle to inhibit key steps in viral replication (**Refer to figure 2.15** for a schematic representation (Arts and Hazuda, 2012). Currently the ART drugs are divided into seven different classes based on their target in the HIV life cycle. The first class approved by the Food and Drug Administration was the nucleoside reverse transcriptase inhibitors (NRTIs). Drugs from this class block the HIV reverse transcriptase and inhibit reverse transcription of viral RNA into double stranded viral DNA and thus prevents HIV from replicating (Arts and Hazuda, 2012; AIDSinfo, 2018). A few years after the discovery of NRTIs, non-nucleoside reverse transcriptase inhibitors (NNRTIs) were introduced. NNRTIs act by non-competitively inhibiting reverse transcriptase by binding to their allosteric site, thus altering their activity and blocking the development of double stranded viral DNA from the viral RNA (Arts and Hazuda, 2012; AIDSinfo, 2018). The third class of ARTs are the protease inhibitors (PIs) which act by blocking the viral protease enzymes responsible to produce mature virions after budding from the membrane (Arts and Hazuda, 2012). Drugs from this class specifically inhibit the cleavage of Gag and Gag-Pol precursor proteins (AIDSinfo, 2018).

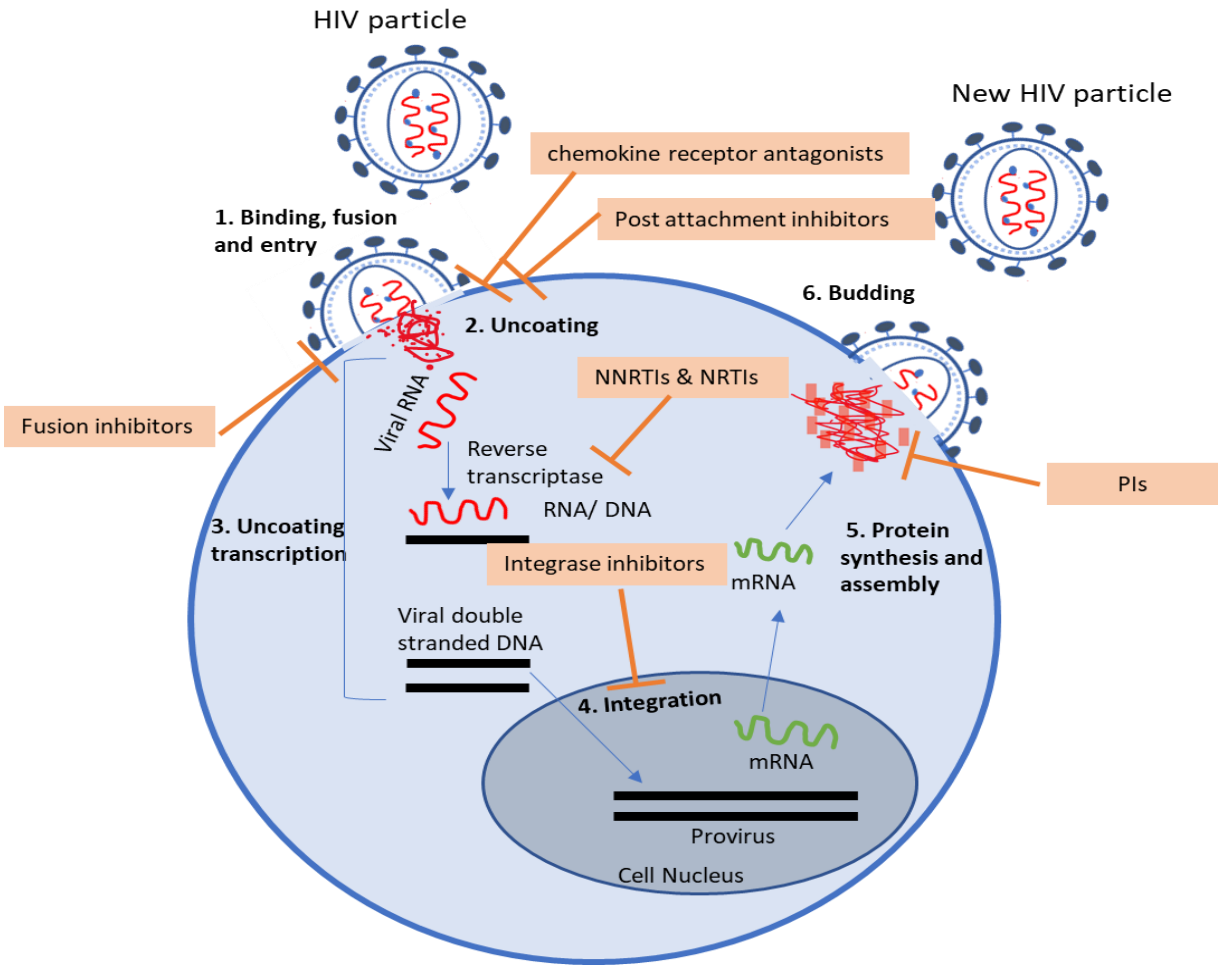


Figure 2.15: Schematic representation of the HIV life cycle and the sites of action of the various classes of ART. Figure designed by the author of this dissertation based on content from (Fanales-Belasio, Raimondo *et al.*, 2010; Tavassoli, 2011; Arts and Hazuda, 2012; Kirchhoff, 2016; AIDSinfo, 2018).

The integrase inhibitors (INIs), target and competitively inhibit the viral enzyme integrase which is responsible for integration of viral DNA into the DNA of the infected cell. More recently, entry inhibitors were introduced, including fusion inhibitors that block the HIV envelope from merging with the host CD4 cell membrane (fusion) (Arts and Hazuda, 2012; AIDSinfo, 2018), hence preventing the virus from entering the CD4 cell. Similar to this class, there are chemokine receptor antagonists which particularly target the CCR5 and inhibit fusion between the cell membranes (Arts and Hazuda, 2012; AIDSinfo, 2018). The last and most recent class of ART is the post-attachment inhibitors, which act by binding to the CD4 receptor on a host CD4 cell while subsequently inhibiting the virus attachment to CCR5 and CXCR4 coreceptors and entering the cell (AIDSinfo, 2018). **Refer to table 2.8 below** for a list of ART drugs approved in the most common classes.

Table 2.8: The most common ART classes with examples of ART drugs available in each class.

Nucleoside reverse transcriptase inhibitors	Non-nucleoside reverse transcriptase inhibitors	Protease inhibitors	Integrase Inhibitors	Fusion Inhibitors	CCR5 antagonists	Post-attachment inhibitors
Abacavir Didanosine Emtricitabine Lamivudine Stavudine Tenofovir disoproxil Zalcitabine Zidovudine	Delavirdine Efavirenz Nevirapine Etravirine Rilpivirin Doravirine	Atazanavir Darunavir Fosamprenavir Indinavir Lopinavir/ritonavir Nelfinavir Saquinavir Tipranavir	Elvitegravir Raltegravir Dolutegravir Bictegravir	Enfuvirtide	Maraviroc	Ibalizumab

Currently, international guidelines for clinical management of HIV recommend initiation of ART as soon as possible after diagnosis (Rana *et al.*, 2020). For many patients, this translates into taking a single well-oriented, fixed dose combination tablet once daily. However clinical management of patients on ART is riddled with adherence challenges with a sizeable proportion of patients not achieving viral suppression and subsequently resulting in poorer patient outcomes (Gardner *et al.*, 2011; Powers *et al.*, 2017; Rana *et al.*, 2020). It is noteworthy that despite chronic use of ART, there is no cure of the disease. HIV is coupled with persistent viral reservoir that harbours integrated, replication-competent provirus within host cellular DNA. This reservoir is resistant to ART and to actions by the immune system of the host. If treatment is stopped, these reservoirs reactivate and HIV can begin replicating again, and transmission can occur. ART does not eradicate viral reservoirs, hence one of the main challenges in the fight against HIV infection is to develop strategies that are able to eliminate these persistent viral reservoirs (Churchill *et al.,* 2016; National Institute of Allergy and infectious diseases, 2018). Other challenges include drug toxicity over a long treatment period, high viral mutation rates leading to ARV resistance (Ghimire, Rai and Gaur, 2018).

2.3.5 ART guidelines in South Africa

Initially ART commencement was based on the individual's CD4 cell count. Revised guidelines were issued in 2015 by WHO recommending the initiation of ART immediately after diagnosis (WHO, 2016). In accordance with the international guidelines, the South African ART guidelines recommend that all people living with HIV commence with ART regardless of age, CD4 cell count and clinical stage. According to the 2019 ART Clinical guidelines developed by the South African National Department of Health, all adult patients without any contraindications are recommended to begin ART within 7 days of diagnosis or on the same day if possible (South African National Department of Health, 2019).

Refer to table 2.9 for South African approved relevant ART drugs and their side effects. In South Africa, the first line treatment is largely available as a triple drug fixed dose combination (FDC) regimen (Meintjes, Moorhouse *et al.* 2017). The FDC regimen lowers the inconvenience of having to ingest multiple pills daily and largely simplifies prescription, dispensing and stock management of drugs. Furthermore, FDCs have shown to improve treatment compliance in individuals (Meintjes, Moorhouse *et al.,* 2017, Davies 2013). In line with international guidelines, South Africa recommends first line ART treatment containing two NRTIs and one NNRTI (tenofovir + emtricitabine (or lamivudine) + efavirenz (preferred). At the time of the PhD study and still largely today, the FDC ART largely comprises of tenofovir (T), emtricitabine (E) and efavirenz (E) (also known as TEE), which is available as Atripla™ or Odimune™ in South Africa (Deeks and Perry, 2010). In 2018, the WHO recommended that all countries using TEE or TLE (lamivudine in place of emtricitabine) should transition eligible

HIV patients to a different combination, which contains dolutegravir (D) in place of efavirenz, thus comprising the first line FDC to be TLD, instead of TEE or TLE (USAID, 2019; WHO, 2019). This further also included INIs in combination with NRTIs as first line treatment for HIV. In South Africa, the TLD transition has been on a slow rise and riddled with controversies around dolutegravir's adverse impact (increased risk of neural tube defects in the foetus) on women of child bearing age, however the WHO has supported the safety of the drug based on recent data from two clinical trials from Africa which demonstrate a significantly lower risk for these women (South African National Department of Health, 2019; WHO, 2019). More recently, dolutegravir has also been associated with abnormal weight gain, further questioning its adverse effects on these patients (Menard *et al.*, 2017; Bourgi *et al.*, 2020) Clinical guidelines in South Africa recommend that patients who are overweight should receive lifestyle interventions (such as prudent eating and moderate exercising plan) accompanying the dolutegravir based treatment while obese patients should be rather be considered for the TEE or TLE treatments (South African National Department of Health, 2019).

The second line treatment in South Africa includes a PI-containing combination (zidovudine + lamivudine + lopinavir booster with ritonavir (lopinavir/r)). Patients with anaemia and renal failure are recommended to switch to abacavir (an NRTI). TLD is also recommended for use as a second-line regimen for patients failing on efavirenz or nevirapine containing regimens or for those failing a non-dolutegravir-containing first-line regimen (WHO, 2019). Currently, however, the most common second-line ART contains PIs, lopinavir/ ritonavir which is available as Aluvia™ in South Africa (Chandwani and Shuter, 2008). HIV patients who demonstrate confirmed failure to both first and second line ART, a virologic failure and ART resistance are recommended to receive third line ART treatment, which is based on the individual's genotype resistance testing, managed by an expert panel (South African antiretroviral treatment guidelines, 2015; South African National Department of Health, 2019).

Table 2.9: Relevant South African approved ART drugs and their side effects. Content from (Charania, 2017; South African National Department of Health, 2019, AIDSinfo 2020).

Drugs	Side Effects
Emtricitabine	Common side effects include headache, diarrhoea, nausea, fatigue, dizziness, depression, insomnia, abnormal dreams, rash, abdominal pain, asthenia, increased cough, and rhinitis. In paediatric patients, skin hyperpigmentation is very common.
Tenofovir Disoproxial Fumarate	The most common side effects include nausea, vomiting, diarrhoea, and asthenia. Less frequent side effects include hepatotoxicity, abdominal pain, and flatulence. This drug has also been implicated in causing kidney toxicity, particularly at elevated concentrations.
Efavirenz	Common side effects include diarrhoea, dizziness, drowsiness, headache, increased sweating, poor concentration, trouble sleeping, depression and skin rash.
Lamivudine	Common side effects included headache, nausea, malaise, fatigue, nasal signs and symptoms, respiratory tract infections, throat and tonsils discomfort, abdominal discomfort and pain, vomiting, diarrhoea, and cough**.**
Abacavir	Common side effects include diarrhoea, dizziness, drowsiness, and headache.
Lopinavir	Common side effects include an increase in serum cholesterol and triglycerides.
Ritonavir	Common side effects include asthenia, malaise, diarrhoea, nausea and vomiting, abdominal pain, dizziness, insomnia, sweating, taste abnormality, metabolic hypercholesterolemia, hypertriglyceridemia, elevated transaminases and elevated creatine phosphokinase (CPK).
Dolutegravir	Usually mild and self-limiting. Side-effects include insomnia, headache, central nervous system (CNS) effects, gastrointestinal effects, and abnormal weight gain. Dolutegravir may increase the risk of neural tube defects. According to recent trials, the absolute risk is very low.

Section C: The link between CVD & HIV and its underlying mechanisms

2.4 Cardiovascular risk factors and disease in HIV infected individuals

2.4.1 Introduction

Advances in ART have been successful in transforming HIV/Aids from a life-threatening acute disease to a chronic disease condition, which can be managed with treatment (Estrada *et al.*, 2020). In fact, recently, some studies suggest that individuals on ART have a life expectancy approaching that of HIV-free individuals (Samji *et al.*, 2013; Kaplan, Hanna and Kizer, 2016). However, currently, CVD has become one of the main causes of morbidity in the HIV-infected population (Shah *et al.*, 2018; Estrada *et al.*, 2020). This could be partially attributed to the increased lifespan of the HIV-infected individuals as well as direct effects of the ART drugs (de Gaetano Donati, Cauda and Iacoviello, 2010). From the time effective ART was introduced, there have been mounting concerns and questions about the potential effect of ART on the development of CVD in the HIV population. These concerns continue to persist, even as HIV treatment is refined with fewer adverse effects, better adherence, more convenience, and earlier initiation of therapy (Kaplan, Hanna and Kizer, 2016). Furthermore, several studies have observed a high prevalence of traditional cardiovascular risk factors in HIV-infected individuals (Beltrán *et al.*, 2015). Within the HIV context, all factors such as the individual's genetics, traditional cardiovascular risk factors, adverse effects of ART as well as the inflammatory state of HIV may be considered as potential risk factors for CVD (de Gaetano Donati, Cauda and Iacoviello, 2010; Beltrán *et al.*, 2015) (**Refer to figure 2.16 below**).

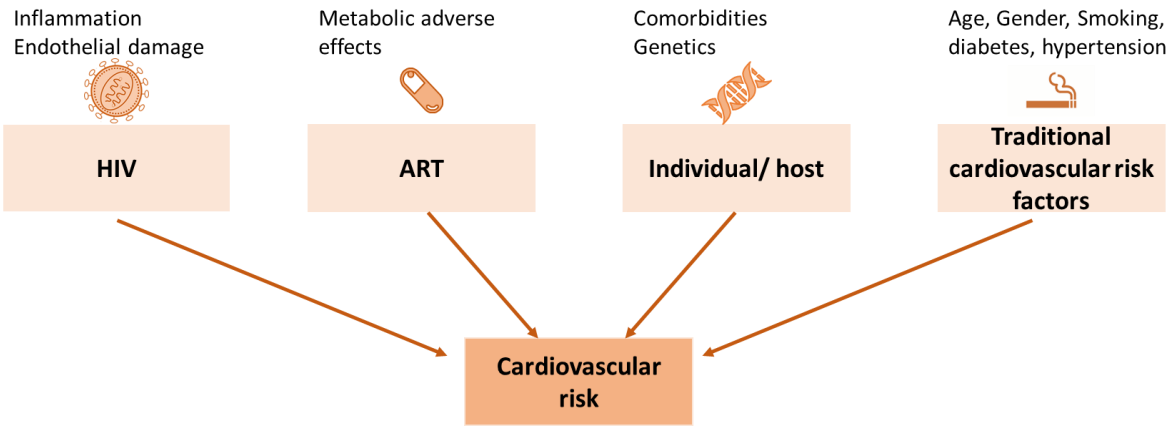


Figure 2.16: Risk factors of CVD in HIV. Cardiovascular risk in HIV-infected individuals can be attributed to the individual's genetics, traditional risk factors, adverse effects from antiretroviral therapy as well as the inflammatory state associated with HIV itself. Figure designed by the author of this dissertation based on content from (de Gaetano Donati, Cauda and Iacoviello, 2010).

2.4.2 Traditional cardiovascular risk factors and HIV

Many studies show that HIV infected individuals have a generally high prevalence of traditional cardiovascular risk factors, including smoking, dyslipidaemia, hypertension, and type 2 diabetes (Currier *et al.*, 2008; Beltrán *et al.*, 2015). Interestingly, studies that have controlled for traditional risk factors have largely demonstrated a significant effect of these factors on CVD events in the HIV population. Furthermore, many studies show that all these factors are strong predictors of CVD in the HIV population (Triant *et al.*, 2007; de Gaetano Donati, Cauda and Iacoviello, 2010). However, it is important to note that both ART and HIV infection may, additionally, influence the induction of particular risk factors such as dyslipidaemia and diabetes (Beltrán *et al.*, 2015).

Hypertension

Hypertension is found to be the leading risk factor for CVD in the HIV population, particularly in SSA (Okello *et al.*, 2020). Studies suggest that HIV-infected individuals in this region experience a high incidence of new-onset of hypertension, especially in the first two years after initiation of ART (Okello *et al.*, 2020). Additionally, data from around the world shows that 35% of all HIV-infected individuals on ART have hypertension in comparison to an estimated 30% of HIV-free individuals. Furthermore, 50% of HIV-infected individuals (over the age of 50 years) on ART have hypertension (Xu, Chen and Wang, 2017; Fahme, Bloomfield and Peck, 2018). In fact, there is evidence that HIV-infected individuals with hypertension have a higher risk of CVD and related mortality in comparison to HIV-free individuals with hypertension or HIV-infected individuals with normal blood pressure (Nüesch *et al.*, 2013; Fahme, Bloomfield and Peck, 2018). Although literature is clear on the growing concern of hypertension within the HIV context, the mechanism leading to hypertension in HIV remains unclear with many virus and treatment related factors implicated, including chronic inflammation, immune reconstitution, and lipodystrophy, all of which are suggested to uniquely influence common downstream pathways such as the sympathetic and renin–angiotensin–aldosterone systems (Maffongelli *et al.*, 2016; Fahme, Bloomfield and Peck, 2018). With regards to the type of treatment, PIs have particularly been implicated in the pathogenesis of hypertension due to their mechanistic effect on RAAS activation, endothelial dysfunction, arterial stiffness, and dyslipidaemia (Fahme, Bloomfield and Peck, 2018).

Smoking

In SSA, it has been found that HIV infected individuals are more likely to smoke in comparison to the HIV-free individuals (Okello *et al.*, 2020). A metanalysis has further demonstrated that there is a 47% higher risk of smoking amongst HIV-infected men and 87% higher risk of smoking in HIV-infected women (Mdege *et al.*, 2017). Reasons for smoking in this population have been speculated to include self-treatment of the comorbid anxiety and depression as well as self-management of HIV-associated symptoms (Humfleet *et al.*, 2009; Okello *et al.*, 2020). Although literature has often found smoking and

HIV infection to independently increase CVD risk, it remains unclear whether HIV-infected individuals have an increased susceptibility to smoking-associated CVD in comparison to HIV-free smokers (Okello *et al.*, 2020). In terms of the pathophysiological effect of smoking on CVD in the HIV context, the effect is similar to that of in the HIV-free context, however, it appears that smoking works synergistically with ART, especially with PIs, to promote CVD (Rahmanian *et al.*, 2011). Ultimately, smoking in this population provides unique and specific risks that promote more rapid development of cardiovascular and pulmonary disease (Cropsey, K *et al.*, 2017).

Obesity

A paradoxically high prevalence of obesity has been demonstrated in many HIV-infected populations, remarkably transforming the 'slim' and 'wasting' body image which was initially associated with the disease (Serwadda *et al.*, 1985; Okello *et al.*, 2020). Many studies in the SSA region have shown an increased weight gain following ART initiation (Koethe *et al.*, 2016). The PI class of ART has been proven to have adverse effects resulting in weight gain, visceral adiposity and eventually lipodystrophy that is further believed to drive subsequent dysglycemia (Price *et al.*, 2015; Okello *et al.*, 2020). Furthermore, the newer generation first-line ART drugs such as dolutegravir (integrase inhibitor) have also been demonstrated to significantly increase weight gain in comparison to the previously recommended first line ART (Bourgi *et al.*, 2020). Literature also shows that HIV-infected women have a higher prevalence of obesity and overweight in comparison to men, this is further in line with the trends in the HIV-free population (Malaza *et al.*, 2012).

Dyslipidaemia

A higher burden of dyslipidaemia has been found in the HIV infected population in comparison to the HIV-free population (Dillon *et al.*, 2013; Okello *et al.*, 2020). A metanalysis across Africa further demonstrated a 26.2% prevalence of elevated total cholesterol in HIV-infected populations in comparison to 25.5% prevalence in the HIV-free population. Furthermore, the analysis confirmed a more than two-fold higher prevalence for elevated LDL cholesterol in HIV-infected (45.6%) versus HIV-free (21.4%) study populations (Noubiap *et al.*, 2018). Additionally, studies have observed a decrease in total, HDL and LDL cholesterol, but an increase in triglyceride levels, in untreated HIV individuals (Riddler *et al.*, 2003; Hemkens and Bucher, 2014). The HI virus itself is implicated in inducing these effects through persistent inflammation and immune activation as well as through its increased thrombotic activity (Hemkens and Bucher, 2014; Beltrán *et al.*, 2015). It is now well known that several types of ART also increase total cholesterol, LDL-cholesterol, and triglycerides while keeping the HDL-cholesterol low (Hemkens and Bucher, 2014). The extent of lipid changes differs between antiretroviral drugs and drug classes. For instance, ART (e.g. indinavir, lopinavir vs. atazanavir, and darunavir) from the PI class are known to increase total cholesterol, LDL-cholesterol,

and triglycerides, whereas ART(e.g. efavirenz) from the NRTI class are found to increase total and LDL-cholesterol compared with newer PIs (Sax *et al.*, 2009; Hemkens and Bucher, 2014).

Diabetes

Impaired glucose regulation associated with HIV and ART can lead to a rise in type 2 diabetes (Okello *et al.*, 2020). Some studies suggest up to a four-fold higher risk of diabetes in HIV-infected individuals on ART in comparison to HIV-free individuals (Maganga *et al.*, 2015). Furthermore, studies from Western countries showed that the positive association between HIV and diabetes, in fact correlated with traditional risk factors such as age and obesity, suggesting that these factors, and not the ART, may play a role in the progression to diabetes in the HIV-infected population (Tripathi *et al.*, 2014). ART induced risk of diabetes has been associated with the use of drugs belonging to the PI class and the older thymidine analogue reverse transcriptase inhibitor family, which are implicated in mitochondrial toxicity (Hemkens and Bucher, 2014). However, today in many settings, these drugs have mostly been replaced (Hemkens and Bucher, 2014; Okello *et al.*, 2020). Mitochondrial toxicity has been shown to lead to impaired insulin sensitivity and *in vitro* models suggest that PIs block glucose transporter (GLUT4) and may also affect glucose-sensing-cells, both causing impaired glucose sensitivity (Koster *et al.*, 2003; Hemkens and Bucher, 2014). Furthermore, recently, the integrase strand inhibitor, dolutegravir, has been under speculation for its association with insulin resistance and diabetes (McLaughlin, Walsh and Galvin, 2018). It has been hypothesized that dolutegravir may have the ability to chemically bond with magnesium which in turn may affect the glucose transport via GLUT 4 receptor and gluconeogenesis leading to insulin resistance (Kamal and Sharma, 2019). Other factors that may be associated with an increased risk of diabetes within the HIV context are high-sensitivity C-reactive protein and tumour necrosis factors 1 and 2 (Brown *et al.*, 2010).

Age

Due to the success of ART, the proportion of HIV-infected individuals over the age of 50 has significantly increased since the early days of the epidemic (Kirk and Goetz, 2009; de Gaetano Donati, Cauda and Iacoviello, 2010). Furthermore, the incidence and prevalence of HIV is rising in older adults. It also seems that the older adults with HIV have an accelerated decline in immune function and are more likely to be diagnosed later in comparison to younger adults (Önen *et al.*, 2010). Although ART is likely to improve prognosis in older adults as well, a higher risk of co-morbidity remains (De *et al.*, 2013). Furthermore, it is speculated that the increased rate of CVD may also be due to the acceleration of biological aging which can be promoted by both the HI virus itself and/ or ART, suggesting that premature aging is in fact associated with HIV-infection. This in turn can influence development of other age-related risk factors and CVD (Deeks and Phillips, 2009; Pirrone *et al.*, 2013).

Sex

Literature from developed countries suggests that HIV-infected women have an approximately 2 to 4 times higher risk of myocardial infarction, stroke, and heart failure in comparison to HIV-free women (Chow *et al.*, 2012; Janjua *et al.*, 2017). The reasons for this could be elevated levels of systemic inflammation and interaction with reproductive hormones, as well as increased behavioural and psychosocial risk factors as compared to the general population (Zanni *et al.*, 2019; Okello *et al.*, 2020). Furthermore, generally many studies, especially from the SSA region, have observed a higher prevalence of increased risk factors such as obesity, dyslipidaemia and hypertension in both HIV-infected and HIV-free women in comparison to HIV-infected and HIV-free men, respectively (Gaziano *et al.*, 2017; Magodoro *et al.*, 2019). Additionally, several studies measuring IMT as a surrogate marker to assess the prevalence of CVD have demonstrated mixed results when comparing HIV-infected women to HIV-infected men (Schoffelen *et al.*, 2015; Okello *et al.*, 2020). Future large-scale studies are warranted to further understand the CVD risk between men and women and their mechanism associated with HIV and ART (Okello *et al.*, 2020).

2.4.3 HIV, ART, endothelial dysfunction, and biomarkers of endothelial injury

Endothelial dysfunction is the first step in atherogenesis (Kearns *et al.*, 2017). A large body of literature provides evidence that endothelial dysfunction can progress to atherosclerosis and predict future cardiovascular events in many populations. In the HIV context, both the virus and certain types of ART drugs are found to be associated with endothelial dysfunction (Torriani *et al.*, 2008; Wang, Yi, Green, *et al.*, 2015). **Refer to figure 2.17** for an overview of endothelial dysfunction in the HIV context. The ability of the HI virus to infect ECs can directly lead to endothelial dysfunction. HIV can enter ECs through chemokine receptors 3 and 4 (CCR-3 and CCR-4), cluster of differentiation 4 (CD4) or galactosyl-ceramide receptors (Skowyra *et al.*, 2012). Furthermore, HIV can penetrate ECs with the assistance of its viral proteins such as GP120, tat and Nef (Anand, Rachel and Parthasarathy, 2018).

Many studies have suggested that GP120 has a crucial role in the pathogenesis of endothelial dysfunction (Anand *et al.*, 2009; Anand, Rachel and Parthasarathy, 2018). GP120 has been associated with apoptosis, adhesion molecule expression, pro-inflammatory cytokine production, and endothelial cell permeability (Fiala *et al.*, 2004; Anand, Rachel and Parthasarathy, 2018). Additionally, GP120 is found to be responsible for the stimulation of macrophages to produce excessive amounts of NO, which can progress to direct endothelial damage (Wang, Yi *et al.*, 2015). Furthermore, studies confirm that GP120 is directly involved in the upregulation of pro-inflammatory cytokines such as IL-6 and IL-8 in primary ECs (Yang *et al.*, 2009; Anand, Rachel and Parthasarathy, 2018). In addition, the HIV TAT protein can activate inflammatory pathways via mononuclear cells that are known to produce TNF-α and the pro-inflammatory transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Willerson, Ridker 2004, Wang, Yi *et al.*, 2015). Tat protein also elicits the expression

of adhesion molecules E-selectin, VCAM-1 and ICAM-1, all of which alter endothelial permeability and ultimately impact negatively on endothelial function (Anand, Rachel and Parthasarathy, 2018). Like GP120 and Tat, Nef has also been associated with several aspects of HIV-induced endothelial dysfunction (Anand, Rachel and Parthasarathy, 2018). A study has shown that Nef is involved in upregulation of ERK kinase mediated ICAM-1 in vascular ECs (Fan *et al.*, 2010). Additionally, Nef can activate macrophages and produce foam cells, which is suggested to contribute to endothelial dysfunction and facilitate development of atherosclerosis (Anand, Rachel and Parthasarathy, 2018). Moreover, apart from these direct effects on endothelial function, HIV can also infect and decrease circulating endothelial progenitor cells especially the colony forming unit endothelial cells, which are essential for endothelial damage repair (Teofili, Iachininoto *et al.*, 2010, Wang, Yi *et al.*, 2015).

Numerous studies have investigated the relationship between HIV and endothelial function (measured by the non-invasive method of FMD) in clinical settings (Margaritis, 2019). HIV-infected individuals with an elevated viral load are found to have an inverse association with FMD (Blum *et al.*, 2005; Margaritis, 2019). Additionally, another study of 75 HIV-infected participants and 223 HIV-free participants showed that FMD was significantly lower in the HIV-infected group compared to HIV-free group. Furthermore, this study showed that viral load was an independent predictor of the degree of reduction in FMD (Solages *et al.*, 2006; Margaritis, 2019). In addition, another study has shown that endothelial dysfunction is more pronounced when HIV infection is severe and more advanced (Wang, Yi *et al.*, 2015, Subbarao, Lowe *et al.*, 2011).

In addition to the HI virus itself, ART can contribute to endothelial dysfunction in many ways (Skowyra *et al.*, 2012). **Refer to figure 2.17** for an overview of endothelial dysfunction in the context of HIV and ART**.** Particularly drugs from the PI class have been shown to directly induce endothelial dysfunction. They are thought to be responsible for mitochondrial DNA damage in ECs which can lead to destruction of the endothelium independent of the individual's lipid profile (Zhong, *et al.*, 2002, Skowyra *et al.*, 2012). Furthermore, it has been found that HIV-infected individuals receiving PIs have significantly lower endothelium-dependent vasodilatation measurements as assessed by the FMD protocol (Stein, Klein *et al.*, 2001, O'Leary, Polak *et al., 1999,* Ogata, Yasaka *et al.*, 2005, Bots, Hoes *et al.*, 1999). However, other studies demonstrate contradicting findings. For instance, significant endothelial impairment was found with the drug indinavir, whereas the newer generation PI drugs such as lopinavir and ritonavir have not been confirmed to induce endothelial dysfunction in HIV-infected individuals (Dube, Shen *et al.*, 2008, Grubb, Dejam *et al.*, 2006, Skowyra, Zdziechowicz *et al.*, 2012).

With regards to biomarker analysis, it has been established that HIV infected individuals have higher levels of biomarkers of endothelial dysfunction such as cellular adhesion molecules compared to HIV-free individuals (Francisci, Giannini *et al.*, 2009, Wang, Melancon *et al.*, 2013). HIV is also associated with increased levels of plasma proinflammatory cytokines such as TNF- α, IL-1, IL-6, IL-8, MCP-1.

Furthermore, all these inflammatory biomarkers have shown contradictory results in the presence of ART. Some studies have reported lower levels of biomarkers of inflammation and endothelial dysfunction in HIV-infected individuals on ART, whereas other studies demonstrate that these biomarkers remain elevated even in the presence of ART (Skowyra *et al.*, 2012; Beltrán *et al.*, 2015). For instance, a study has showed elevated P-selectin, tissue plasminogen activator and plasminogen activator inhibitor levels in treated HIV individuals when compared to the ART naive group (de Gaetano Donati, Rabagliati *et al.*, 2003, Skowyra, Zdziechowicz *et al.*, 2012).

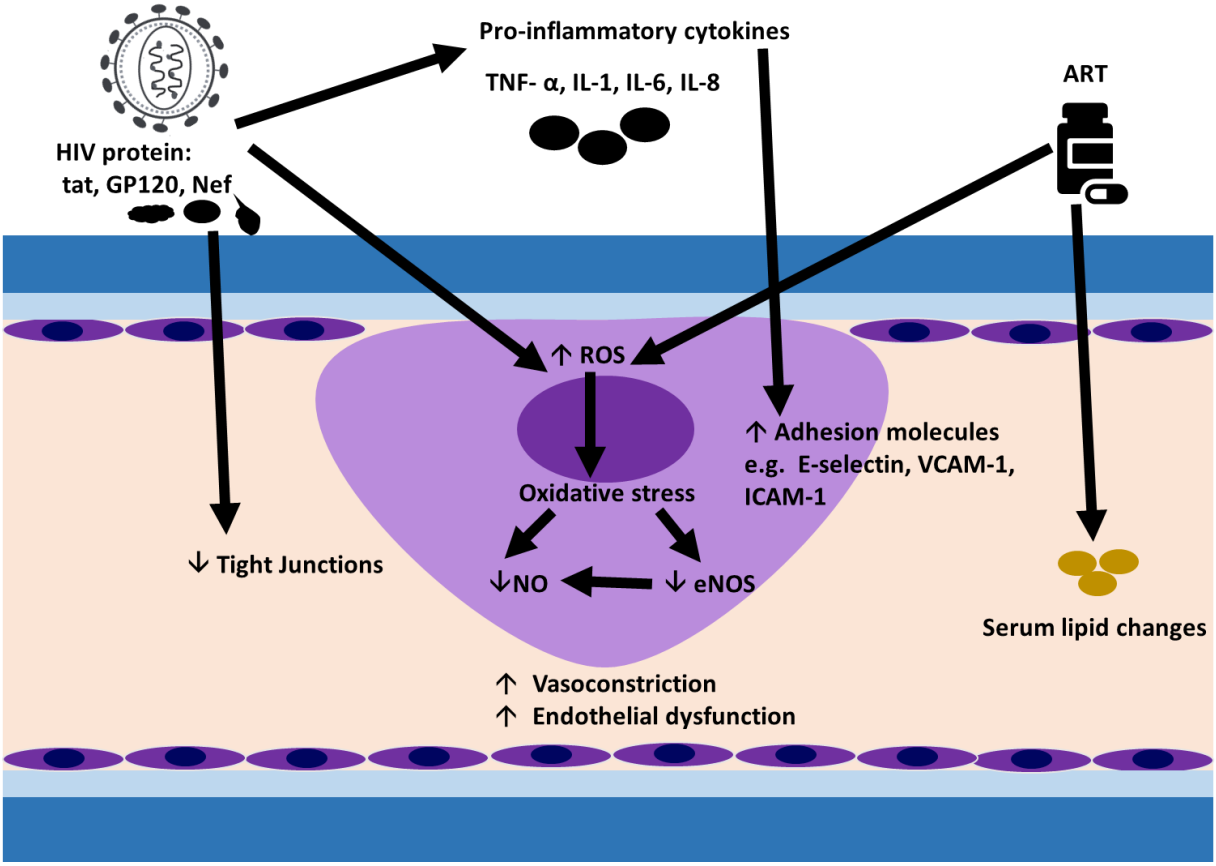


Figure 2.17: Overview of endothelial dysfunction in the context of HIV and ART. Figure designed by the author of this dissertation based on content from (Titanji *et al.*, 2020). Viral proteins, inflammatory cytokines, and ART all lead to increase in ROS. Oxidative stress induces eNOS uncoupling leading to decrease NO availability. These mechanisms trigger endothelial dysfunction, which is characterized by decreased endothelial tight junction proteins, increased vascular adhesion molecules and vasoconstriction. ART, antiretroviral therapy; ROS, reactive oxygen species; eNOS; endothelial nitric oxide synthase, NO, nitric oxide; VCAM-1, vascular cellular adhesion molecule-1; ICAM-1,

intercellular adhesion molecule 1; IL-1, IL6, Il-8, interleukins-1, 6, 8; TNF-α, tumour necrosis factor-alpha.

2.4.4 CVD risk, HIV, ART, and inflammatory biomarkers

Many studies have demonstrated that CVD risk is higher in the HIV infected population in comparison to HIV-free population, even after adjusting for traditional risk factors such as hypertension, hyperlipidaemia, smoking and diabetes (Yoshimura, 2017). In fact, data from the Veterans Aging Cohort Study, Virtual Cohort (longitudinal study of HIV-positive, vs. age, ethnicity, and clinical site matched uninfected veterans) further demonstrated that HIV infection is associated with a 50% higher risk of acute myocardial infarction (Freiberg *et al.*, 2013). Furthermore, there are many more studies that demonstrate increased risk of CVD, both in the absence and in the presence of ART (Grunfeld *et al.*, 2009; Wang, Yi, Ann, *et al.*, 2015). However, despite increased availability of data in this field, the higher risk of CVD in HIV infected individuals remains incompletely understood.

It is debatable as to which one of HIV-infection or ART poses a higher risk to CVD in HIV-infected individuals. Both the virus itself and ART have been implicated in the development of CVD. **Refer to table 2.10** for a list of mechanisms by which HIV may increase the risk of CVD.

Table 2.10: List of mechanisms by which HIV may increase the risk of CVD (Beltrán *et al.*, 2015).

Mechanisms
• **Persistent inflammation and immune activation** • **Endothelial damage** • **Increased thrombotic activity** • **Higher oxidative stress** • **Indirect metabolic disorders**

Refer to figure 2.18 for the role of HIV in the development of atherosclerosis. The HI virus is known to activate many inflammatory pathways which release cytokines and induce endothelial adhesion molecule expression. Both molecules facilitate leukocyte adhesion and transmigration and play a role in endothelial dysfunction which is discussed further in section 2.4.4 (Fisher, Miller and Lipshultz, 2006; Beltrán *et al.*, 2015). The persistent inflammation is accompanied by immune activation where several activation markers such as sCD163, sCD14, and CD14+/CD16+ monocyte expansion are elevated on monocytes/ macrophages (Beltrán *et al.*, 2015). These monocytes/ macrophages play a crucial role in the development of atherosclerosis wherein macrophages phagocytize modified lipoproteins, which promotes proinflammatory and chemotactic cytokine secretion, and mediate cholesterol efflux from the arterial wall. Furthermore, the HI virus increases the proportion of CD14+/CD16+ monocytes, as it exhibits an activated phenotype with increased secretion of

proinflammatory cytokines (Beltrán *et al.*, 2015). In addition, the virus suppresses the reverse cholesterol transport from arterial wall macrophages to HDL particles by blocking the adenosine triphosphate-binding cassette transporter A1 (ABCA-1) pathway (Mujawar *et al.*, 2006). This ultimately promotes the accumulation of foam macrophages within atherosclerotic plaques (Anand, Rachel and Parthasarathy, 2018). In addition, both HIV induced inflammation and immune activation have been found to increase thrombotic activity by increasing biomarkers such as von Willebrand factor (Funderburg, 2014). Furthermore, HIV is known to increase oxidative stress by promoting oxidative lesions and damaging DNA repair mechanisms (Aukrust *et al.*, 2005). Moreover, HIV promotes atherogenesis through its metabolic effects, mainly by decreasing HDL-cholesterol and apoA1, decreasing LDL particle clearance, as well as by increasing triglycerides and very low LDL cholesterol (Beltrán *et al.*, 2015). All these HIV mechanisms of inflammation, immune activation and a prothrombotic state participate in the development of atherosclerosis and ultimately CVD.

Refer to figure 2.18 for the role of ART in the development of atherosclerosis. In addition to the HI virus, a large body of evidence suggests that ART poses an increased CVD risk in HIV-infected individuals (Beltrán *et al.*, 2015; Wang, Yi, Ann, *et al.*, 2015). ART-induced hyperlipidaemia and hypercholesterolemia are known to promote the development and progression of atherosclerosis (Brown and Glesby, 2012). Furthermore, ART has been suggested to also promote the development of insulin resistance and type 2 diabetes (Colleen Hadigan *et al.*, 2001; Beltrán *et al.*, 2015). These alterations may occur either independently or as part of other disorders, such as metabolic syndrome or lipodystrophy (Beltrán *et al.*, 2015). With regards to the type of ART, drugs from the PI class have been largely reported to cause dyslipidaemia and contribute to the increased CVD risk in the HIV-infected population (Wang, Yi, Ann, *et al.*, 2015). In general, new generations of ART have substantially lowered adverse effects, however initiation of ART is usually associated with increased weight gain, triglycerides, total cholesterol and LDL cholesterol (Lazzaretti *et al.*, 2012; Beltrán *et al.*, 2015). Hence the increased CVD risk remains, even in the current treatment era (Wang, Yi, Ann, *et al.*, 2015).

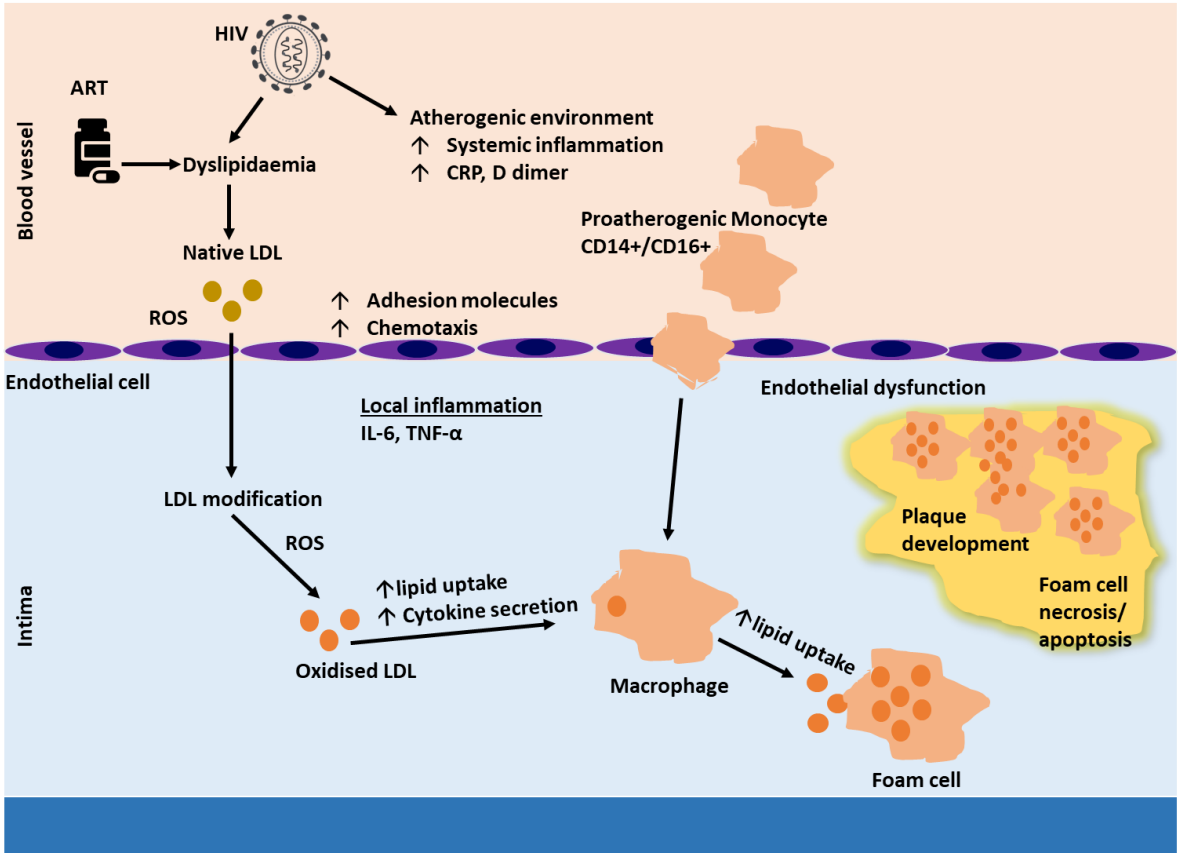


Figure 2.18: Role of HIV and ART in the pathogenesis of atherosclerosis. Figure designed by the author of this dissertation based on content from (Kearns *et al.*, 2017; Spadaro, Cecchetti and Fantuzzi, 2017). Both HIV and ART can induce dyslipidaemia and inflammation. This leads to accumulation and oxidation of LDL by ROS within the artery wall, which is followed by recruitment/infiltration of monocytes and their differentiation into macrophages, which become foam cells on uptake of oxidized LDL and then finally foam cell coalescence into a lipid necrotic core leading to plaque development. ART, antiretroviral therapy; LDL, low-density lipoprotein; ROS, reactive oxygen species; IL-6, interleukin-6; TNF-α, tumour necrosis factor- alpha; CRP, C reactive protein.

A growing body of evidence suggests that multiple biomarkers can be measured to assess the role of inflammation and immune activation in the development of CVD in the HIV-infected population (Kearns *et al.*, 2017). Biomarkers include inflammatory mediators and adhesion molecules that have been shown to be elevated in HIV –infection, such as high sensitivity (hs) CRP, IL-6, TNF-R, VCAM-1, ICAM-1, and asymmetric dimethylarginine (ADMA) (Kurz *et al.*, 2009; Neuhaus *et al.*, 2010; Hileman *et al.*, 2013). Particularly, increased hs CRP and IL-6 levels are found to be strong predictors of future cardiovascular events and overall mortality in both ART naïve and ART treated HIV infected patients, and are therefore regarded as useful biomarkers in this context (Kuller, Tracy *et al.*, 2008, Wang, Yi *et al.*, 2015). Furthermore, TNF-R has been associated with non-HIV related morbid events which includes CVD (Tenorio *et al.*, 2014). Additionally, numerous studies have demonstrated that the continuing HIV replication in CD4 t-helper cells, coupled with their depletion significantly contributes

to an increased prevalence of elevated biomarkers of inflammation, altered coagulation, and monocyte activation, independent of the effects of comorbid conditions (Sandler, Wand *et al.*, 2011, Wang, Yi *et al.*, 2015).

Levels of inflammatory and immune activation biomarkers in HIV-infected individuals can be affected by the type of ART being used (Laurence, Elhadad and Ahamed, 2018). Furthermore, the effect of ART on biomarker levels have been contradictory so far, with some studies suggesting that ART may impose protective effect on CVD in HIV-infected population (Kurz *et al.*, 2012; Beltrán *et al.*, 2015). For instance, it has been reported that ART is associated with lower levels of certain biomarkers of inflammation, particularly, reduced levels of VCAM-1, ICAM-1 and CRP were reported in a study of 115 HIV-infected individuals after 2 months and 14 months of ART (Duprez *et al.*, 2012). Furthermore, another study showed that TNFR-II, VCAM-1, ICAM-1, and IL-6 plasma levels were significantly decreased after 24 weeks and 96 weeks of ART (McComsey *et al.*, 2012).

However, it is noteworthy that none of the studies suggest that ART completely reverses inflammation and immune activation in HIV patients (Beltrán *et al.*, 2015). In fact, a few studies such as the Second Manifestations of ArTerial disease (SMART) study, Multi-Ethnic Study of Atherosclerosis (MESA) study, and Coronary Artery Development In young Adults (CARDIA) study, observed that virologically suppressed HIV-infected individuals on ART have elevated CRP and IL-6 levels amongst a few other biomarkers, in comparison to HIV-free individuals (Neuhaus *et al.*, 2010). Additionally, similar results were found in a case-control study where the presence of ART was found to have lowered VCAM-1, CRP and TNFR-II levels, however, the levels remained elevated in comparison to HIV-free individuals (Beltrán *et al.*, 2014). This further suggests that even in the presence of ART, HIV may induce many inflammatory pathways which release biomarkers that may contribute to CVD risk and development (Kearns *et al.*, 2017).

2.4.5 HIV, ART, subclinical atherosclerosis, and biomarkers of inflammation

Carotid artery intima-media thickness (IMT) is a measure of sub-clinical atherosclerosis that is known to predict future CVD events in many populations (Hanna *et al.*, 2016). Assessment of the carotid artery IMT via ultrasound, for evidence of subclinical atherosclerosis has previously been investigated in cross sectional and prospective studies as a marker of CVD risk in a HIV-infected population (Sarfo *et al.*, 2019). Many studies suggest that HIV-infected individuals have increased IMT in comparison to HIV-free individuals. For example, a study demonstrated that HIV infection was associated with premature atherosclerosis even with extreme low levels of viremia, overt immunodeficiency, and exposure to ART and this appeared to be independent of traditional cardiovascular risk factors (Hsue, Hunt *et al.*, 2009). This study further concluded that IMT and CRP were strongly associated with the presence of HIV

disease rather than viral load or CD4+ T cell count (Hsue, Hunt *et al.*, 2009). Another study compared carotid artery IMT in HIV-infected individuals with age and sex-matched controls with similar risk factors and patients with established coronary artery disease (CAD) and found that HIV-infected individuals had similar carotid artery IMT values in comparison with CAD patients. They further concluded that carotid artery IMT values in these groups were higher than those in the control group (Lekakis, Tsiodras *et al.*, 2008). Furthermore, a large case control study with 1500 participants showed that both HIV and ART are independent predictors of early carotid atherosclerosis as measured by IMT (Lorenz *et al.*, 2008; Margaritis, 2019).

It has also been found that IMT progression is more rapid in HIV-infected individuals. One study found faster progression among HIV-infected individuals on PI therapy compared to those on NNRTI-based regimens (Baker, Henry *et al.*, 2009). The same study showed that HIV infected individuals on PIs have a significantly higher IMT (Baker, Henry and Neaton, 2010). However, other studies investigating newer generation PI drugs such as lopinavir and ritonavir could not demonstrate changes in IMT (Dube, Shen *et al.*, 2008, Grubb, Dejam *et al.*, 2006, Skowyra, Zdziechowicz *et al.*, 2012). Interestingly, an increase in IMT was also found in HIV-infected individuals after initiation of ART, especially with tenofovir based therapy (Delaney, Scherzer *et al.*, 2010). Overall, there are many studies that conclude that HIV infection is independently associated with increased IMT (Ross, Armentrout *et al.*, 2008). In fact, a metanalysis of 20 studies, further confirms that both HIV and ART play a role in the progression of IMT (Msoka *et al.*, 2019).

From the literature, it is evident that inflammation, endothelial activation and endothelial dysfunction all have a major role in the development of plaque and atherosclerosis in the general population. In the non-HIV context, many studies have shown that increased levels of the proinflammatory cytokines, IL-6 and CRP, are associated with subclinical atherosclerosis (Amar, Fauvel *et al.*, 2006). In addition, CRP and IL-6 are independently predictive of future cardiovascular events (Amar, Fauvel *et al.*, 2006, Ross *et al.*, 2008). Furthermore, TNF-α has been implicated in myocardial dysfunction after acute coronary syndromes. Higher levels of TNF-α have been found in patients at baseline who experience recurrent myocardial infarctions or cardiac death. Moreover, it is interesting to note that there are not many studies that have observed the relationship between inflammatory markers, endothelial activation and subclinical atherosclerosis as measured by IMT in the non-HIV context. There is one study that found an association between IMT and inflammatory markers; however, the results are inconsistent with other studies (Thakore, Guo *et al.*, 2007).

The role of these biomarkers of inflammation and endothelial dysfunction in the HIV context has been explained previously under section 3.4.3 and 3.4.4. As discussed earlier, elevated levels of inflammatory markers, and endothelial dysfunction are evident in HIV infected individuals. This could further be correlated with increased risk of CVD (Ross, O'Riordan *et al.*, 2010, Ross, Rizk *et al.*, 2009b,

Tabib, Leroux *et al.*, 2000). However, it is interesting to note that there are not many studies that have observed the relationship between inflammatory markers, endothelial activation and subclinical atherosclerosis as measured by IMT in the HIV-infected population. A study from the USA that investigated the relationship between inflammatory markers, endothelial activation markers and IMT in an HIV-infected population, observed a significantly higher IMT, IL-6, VCAM, CRP and TNFα levels in the HIV-infected group compared to the HIV-free group. Overall, the study concluded that there was enhanced endothelial activation and inflammation in the HIV-infected individuals on ART with inflammatory markers being associated with endothelial activation, and both were associated with carotid artery IMT (Ross *et al.*, 2008). Another study, also from the USA, investigated the association of biomarkers, including biomarkers of inflammation (CRP, IL-6, TNF-R1 and 2) and endothelial dysfunction (ICAM-1) with subclinical carotid atherosclerosis amongst male HIV-infected and HIV-free study populations. This study suggested that levels of biomarkers of inflammation, and endothelial dysfunction were significantly elevated among HIV-infected men however no significant association was found between biomarkers of inflammation, and endothelial dysfunction and IMT in the HIV infected population (Subramanya *et al.*, 2019).

2.4.6 Rationale and problem statement

From the literature, it is evident that most studies investigating cardiovascular risk factors in HIV are performed in Western countries (Mashinya *et al.*, 2015a). Relatively few studies have investigated this topic in low-income countries (Mashinya *et al.*, 2015a). However, HIV-infected individuals in SSA are expected to be at a particular risk of CVD, especially as life expectancy rises with a widespread scale up of ART programs (Ssinabulya *et al.*, 2014; Schoffelen *et al.*, 2015). Furthermore, there is a paucity of studies on cardiovascular risk factors in the HIV context of South Africa (Malaza *et al.*, 2012; Fourie *et al.*, 2015; Mashinya *et al.*, 2015b; Roozen *et al.*, 2020). Additionally, lifestyle, socio-economic factors as well as healthcare services vary across different regions of South Africa with widespread rapid urbanization present countrywide. People have become less physically active, while dietary fat and sugar consumption has increased (Roozen *et al.*, 2020). Moreover, the burden of HIV remains high in the country.

There are very few studies evaluating cardiovascular risk in the HIV-infected population in South Africa. A study from the Mpumalanga province of South Africa suggested an association between ART and CVD risk in rural HIV-infected individuals, however the use of ART was self-reported in the study and according to the authors, the cardiovascular risk may have been underestimated due to the stigma associated with HIV (Clark, Gómez-Olivé *et al.*, 2015). Another study from the Limpopo province demonstrated that the CVD risk factors were common in the HIV-infected study group on ART, however, the risk score equation used was not developed particularly for the African context (Mashinya *et al.*, 2015b).

Moreover, very few studies have investigated the association between HIV, ART, and endothelial dysfunction in the South African context. The relationship between inflammatory pathways and the pathogenesis of HIV related endothelial activation is unclear. The interaction between factors linked with CVD and HIV, especially with ART addition is vastly complex and their overall impact on the vasculature warrants further investigation (Fourie, Schutte *et al.*, 2015). As discussed in the earlier sections, there are two studies from the USA that have investigated the association between HIV, ART, biomarkers and sub-clinical atherosclerosis which may be of relevance to this PhD study. The study by Ross et al., 2009b in the USA demonstrated increased inflammation, endothelial activation as well as increased IMT in their HIV-infected study population, and significant associations were also found between inflammatory markers and endothelial function, both of which were associated with IMT (Ross, Rizk *et al.*, 2009b). However, the study comprised of a relatively small sample size (n= 73 HIV-infected; n= 21 HIV-free participants), failed to control for important confounders such as smoking, blood pressure and clinical markers of overweight / obesity (e.g. waist hip ratio), all of which have been identified in literature to be linked with higher IMT (Ross, Rizk *et al.*, 2009b). The study by Subramanya *et al.*, 2019, demonstrated that levels of biomarkers of inflammation, and endothelial dysfunction were significantly elevated among HIV-infected men; however, no significant association was found between biomarkers of inflammation and endothelial dysfunction, and IMT in the HIV infected population (Subramanya *et al.*, 2019). However, this study comprised of only male participants, which may preclude the generalizability of their findings to females. Additionally, the authors suggest that they may have over adjusted their statistical models, which may have weakened the observed biomarker associations with their endpoints. Furthermore, the study evaluated the associations between multiple biomarkers and markers of subclinical carotid atherosclerosis, and it is possible some findings were significant solely due to chance (Subramanya *et al.*, 2019).

Moreover, it is noteworthy that HIV-infected populations in European and North American countries differ vastly from their counterparts in African countries, in terms of their genetic makeup, socio-economic and demographic profile and prevalence of traditional risk factors (Schoffelen *et al.*, 2015). Furthermore, the predominant HIV-1 subtype in North America (subtype B) differs from that found in South Africa (subtype C), and the ART treatment guidelines are also different.

Within the South African context, very few studies were found in the literature that may be of relevance to the current PhD study. A 2015 study from Limpopo showed an association between IMT and CVD risk factors and not HIV related factors; however, their study lacked an HIV negative control group which makes it impossible to conclude the true effect of HIV on IMT (Schoffelen *et al.*, 2015). Furthermore, no investigations on serum biomarkers were performed, and secondary clinical markers of vascular function such as FMD or pulse wave velocity were not included (Schoffelen *et al.*, 2015). A study by Fourie *et al.*, 2015 from the Northwest Province confirmed a link between endothelial activation and HIV-infection in their cohort, however an association with subclinical atherosclerosis (as

measured by carotid IMT) could not be demonstrated (Fourie *et al.*, 2015). A study from the Eastern Cape province, by Awotedu et al., 2015 demonstrated that arterial stiffness, cardiometabolic risk, age, and low CD4 count are associated with HIV-infected individuals in South Africa (Awotedu *et al.*, 2015). However, no measurements of IMT and FMD were conduced to measure endothelial function or subclinical atherosclerosis. The most recent study by Roozen *et al.*, 2020 assessed IMT and its determinants in two groups of HIV-infected individuals from different settings in South Africa. This study observed that CVD risk factors were the main drivers of IMT and the association of these traditional risk factors was increased with age (Roozen *et al.*, 2020). However, it is important to note that this study did not compare the HIV-infected group with a control, HIV-free group. Hence the true effect of HIV on IMT cannot be concluded. Furthermore, apart from the use of the IMT technique in the HIV population, no investigations on biomarkers and endothelial function were conducted.

Overall, there is a paucity of data from South Africa on a putative link between biochemical and clinical markers of early vascular injury in the context of treated and untreated HIV-infected individuals. While we wait for data from the ongoing EndoAfrica parent study, to date, no South African studies have incorporated the gold standard of endothelial function measurement in clinical research, namely FMD. And finally, no studies have incorporated both the measures of endothelial function (FMD) and sub clinical atherosclerosis (with IMT) in the South African HIV context. Furthermore, none of the studies discussed above assessed the HIV population of Western Cape, which is where the current study was conducted. Additionally, it is important to note that most studies found in literature were of the cross-sectional nature and no studies have evaluated the role of these biomarkers of inflammation and endothelial function in a longitudinal fashion to validate their results from the cross-sectional study. Especially considering the participants are recruited in the same manner as the cross-sectional study and belong to the same setting. Furthermore, no studies have evaluated the association between biomarkers, IMT and FMD even in the non-HIV context of South Africa. This PhD study aims to address these knowledge gaps in both cardiovascular and HIV research in South Africa.

2.4.7 Aims and objectives

The overarching aim of the PhD study is to investigate the relationship between subclinical atherosclerosis and serum biomarkers of endothelial dysfunction and vascular inflammation in HIV-infected (with or without ART) and HIV-free participants living in the Western Cape Province of South Africa. The PhD study incorporated two different epidemiological study designs with the main study comprising of a cross-sectional design and an exploratory sub-study being longitudinal in nature.

2.4.7.1 Objectives:

Specific objectives for the main cross-sectional study

- To identify and assess the cardiovascular risk profile (dyslipidaemia, hypertension, obesity, diabetes mellitus and smoking) in HIV-infected and HIV-free participants, using data generated from comprehensive health questionnaires, anthropometric measurements, and biochemical blood analyses.
- To measure the carotid IMT in the HIV-infected (with and without ART) and HIV-free groups.
- To investigate possible associations between HIV-status, cardiovascular risk factors and carotid IMT measurements.
- To measure and analyse known serum biomarkers of vascular endothelial dysfunction and vascular inflammation such as adhesion molecules (VCAM-1; ICAM-1); e-selectin; p-selectin; TNF-α, high sensitivity CRP and PAI-1 in all the study groups.
- To investigate possible associations between HIV-status, serum biomarkers and carotid IMT measurements.
- To measure vascular endothelial function in all the study groups by non-invasive flow-mediated dilation (FMD) of the brachial artery and investigate whether there is an association with carotid IMT and serum biomarkers.
- To investigate possible associations between endothelial dysfunction (FMD), subclinical atherosclerosis (IMT), serum biomarkers, and the duration of HIV infection and ART, viral load, and CD4 count in the HIV-infected study group.

Specific objectives for the exploratory longitudinal sub-study in HIV-free participants:

- To measure and analyse temporal progression over 12 months of IMT.
- To measure and analyse differences in IMT, FMD, serum biomarkers, and CVD risk factors at baseline and at 12 months.

Chapter 3: Methodology

3.1 Introduction

This chapter describes the experimental design, methods and techniques used for this PhD study. The study consists of a cross-sectional main study and a longitudinal sub-study, refer to **figure 3.1** for an overview of the two types of studies. The methods and techniques used are largely shared amongst the two studies. Where relevant, specific approach and design pertaining to the studies will be described separately in this chapter.

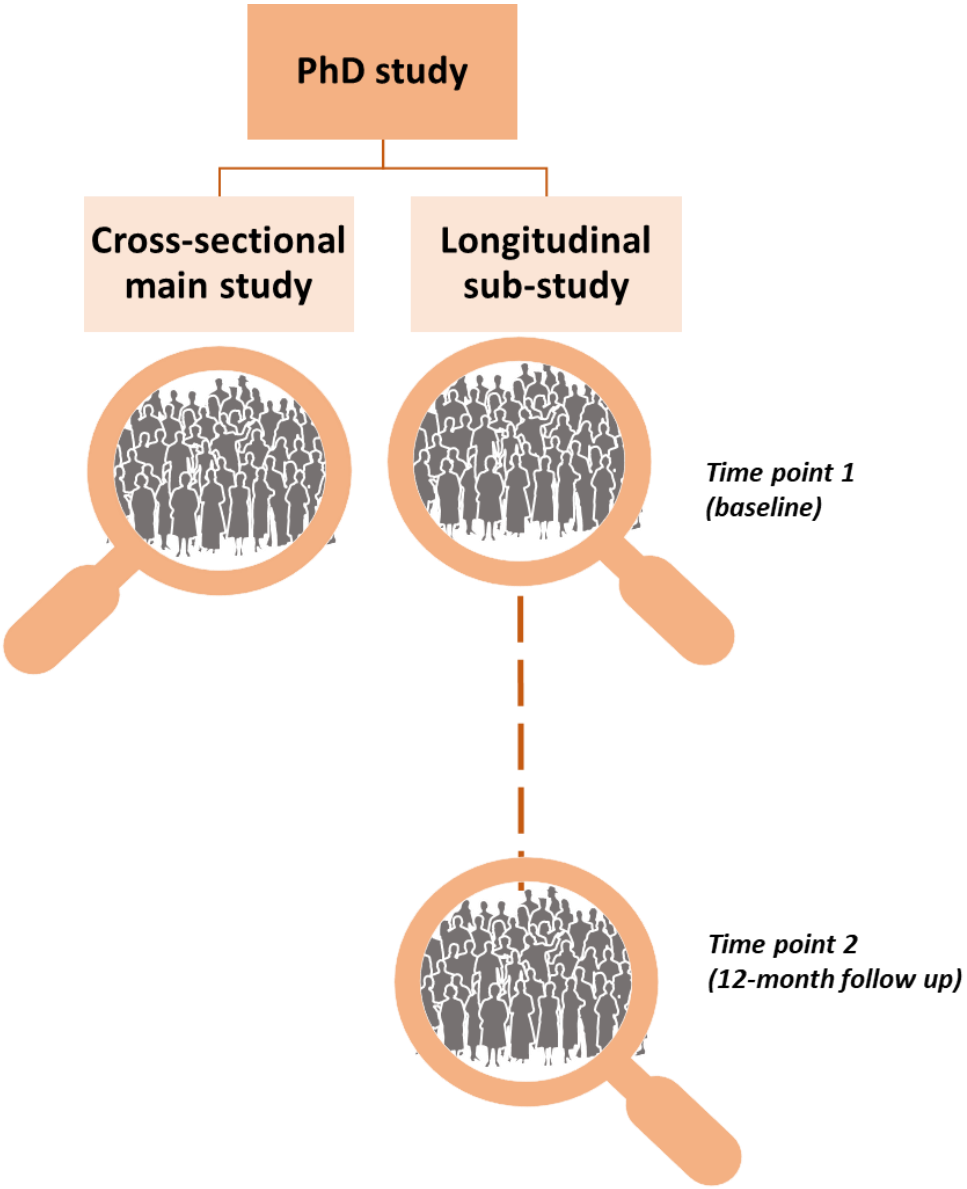


Figure 3.1: Division of the PhD study in a cross-sectional main study and the longitudinal sub-study.

3.2 Ethics and ethical considerations

The study received ethical clearance from the Health Research Ethics Committee of Stellenbosch University (HREC; Ethics Reference Number: N13/05/064B; N12/12/086) and conforms to the principles of the Declaration of Helsinki. Additionally, ethical clearance was renewed annually in line with HREC protocols. South African Good Clinical practice (GCP) guidelines were adhered to throughout the study. As part of the recruitment protocol, all eligible participants were briefed verbally as well as requested to read and sign the informed consent document prior to participating in the study. This process was conducted with utmost confidentiality and in the participant's home language or language of choice. Additionally, participants were encouraged to ask any questions to clarify any uncertainties that they had about the study. Furthermore, the participants were informed clearly that their enrolment in the study is strictly voluntary and they could withdraw from the study at any point if they wished to do so.

To maintain anonymity, a unique participant identification code (e.g. EA142) was assigned to each participant upon recruitment, and the code was used for all future study purposes. All data collected from the study was stored on a secure online database, Research Electronic Data Capture application (REDCap^{TM}), with password protection. Any data in hard copies / paper were stored in a lockable cupboard and office with strictly controlled access in the Division of Medical Physiology, Stellenbosch University. The methodology used in this study was largely non-invasive, except for blood collection. Blood test results were accessible to participants upon requests. Furthermore, any health concerns observed during the study assessments, were immediately escalated to the research nurse, who informed and referred the participants for treatment. HIV testing for participants with unknown status was managed with utmost confidentiality and pre and post-test counselling.

3.3 Study design and setting

The PhD study consists of a main study and an exploratory sub-study, both imbedded within an overarching parent study (EndoAfrica) (Strijdom *et al.*, 2017). The main study is cross-sectional in design and the exploratory sub-study is longitudinal in nature where the participants were assessed at baseline time point and again at 12-month period (**refer to figure 3.1**). Both studies are observational in design and were conducted in the Western Cape Province of South Africa. Participants for both studies were recruited at primary healthcare clinics in the northern suburbs of Cape Town, i.e. Bishop Lavis, Elsies river, Fisantekraal and Ravensmead, as well as in Worcester, a town approximately 120 km north east of Cape Town. Once recruited, the participants were transported to the Tygerberg campus, Fisan building, Division of Medical physiology, where the assessments were conducted. The participants from Worcester were assessed at the Worcester Community Health Centre.

3.4 Study inclusion, exclusion criteria and study groups

The main cross-sectional study had two groups of participants, HIV-infected (with or without ART) and HIV-free individuals that met the inclusion criteria, **refer to figure 3.2**. The longitudinal sub-study included a group of HIV-free participants who met the study inclusion criteria as described in **figure 3.2.** The sub-study participants were assessed at baseline and after 12-month time period.

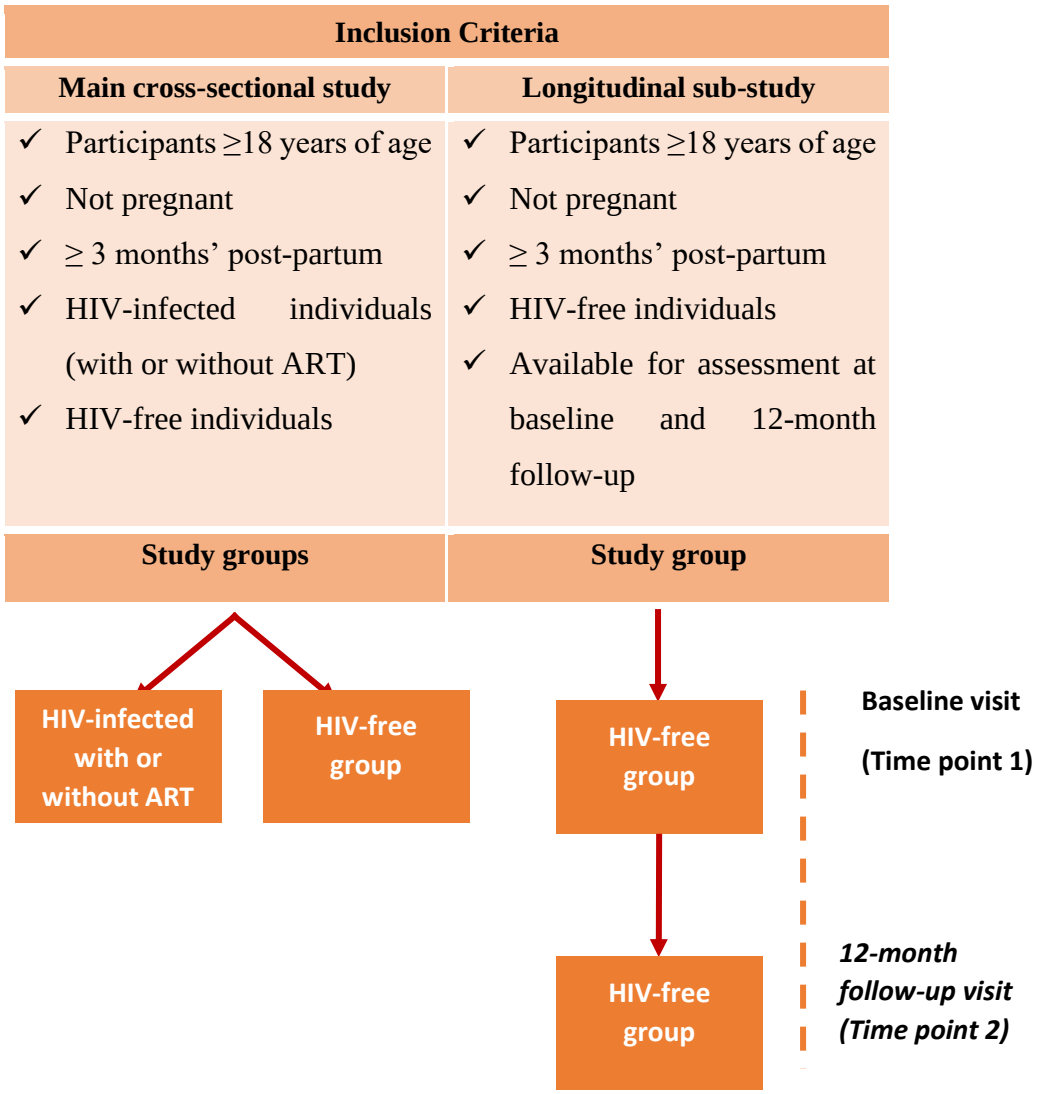


Figure 3.2: Inclusion criteria and study groups for both the main cross-sectional study and longitudinal sub-study. Figure designed by the author of this dissertation.

For both the studies, individuals below the age of 18, those terminally ill, with active tuberculosis as well as females who were pregnant (confirmed with pregnancy test) and less than three months post-partum were excluded from the study. Furthermore, for the longitudinal sub-study, HIV-infected individuals were excluded from the study.

3.5 Participant recruitment

Participants were recruited for both studies by experienced South African Nurses council (SANC) registered research nurses based on the inclusion criteria (**refer figure 3.2**). Volunteering participants with unknown HIV status were counselled and screened for HIV with a rapid HIV test (SD Bioline HIV 1/2 3.0 immunochromatographic test kit; Standard Diagnostics, Republic of Korea) by the qualified research nurses. The participants were also pre and post counselled by the research nurse who had training in HIV counselling. Female participants underwent a pregnancy test and were requested to report on their last normal menstruation date. For the main study, HIV-free participants were recruited in the same manner as the HIV-infected participants, and care was taken to ensure that all participants were from the same geographical areas and of similar socio-economic and demographic backgrounds as far as possible. The HIV-free participants were generally healthy individuals which the research nurse had access to either due those individuals residing in the same community/ geographical location or through them attending the clinic for other acute treatments for themselves and/or their family members.

The longitudinal sub-study comprised of a separate group of HIV-free participants who underwent baseline and 12-month follow-up assessments in a different arm of the parent EndoAfrica study cohort. No additional recruitment strategies were applied in the selection of these participants, except the sub-study inclusion/ exclusion criteria as shown in **figure 3.2**. Furthermore, it should be noted that although no participants were shared amongst the main PhD study and the PhD sub-study, both cohorts shared similar demographic and socio-economic profiles.

Following recruitment, verbal and written informed consent were obtained from all volunteering participants in the language of their preference. After which the participants were officially enrolled in the study and assigned a unique participant identification code. All medical samples and data received from the participants were labelled with bar codes linked to the participant's identification code. Appointments for clinical investigations were made in advance and participants were requested to fast for at least 10 hours prior to the appointment. They were further requested to refrain from doing physical exercises, smoking, and drinking coffee for 4- 6 hours prior to the assessments. For the longitudinal sub-study, participant assessments were performed at baseline and after 12-month follow up period.

3.6 General demographic, medical background, and lifestyle questionnaire

Demographic, medical history and lifestyle related data were collected from all participants by means of an interview and questionnaire on the assessment day (including at both times points for the longitudinal sub-study). **Refer to appendix A** for the questionnaires used in this study. General demographic data included age, gender, and ethnicity (self-reported). Medical background data included presence and history of heart disease, tuberculosis (TB) and any other long-lasting health

problems. HIV status, type of ART and duration of disease and ART was also recorded. This information was further validated by the patient's clinic file, which was accessible to the investigator and/ or the research nurse. Family planning and menstrual date information was also collected from female participants. Additionally, any medication use by all participants was also recorded. Lifestyle data included smoking and alcohol consumption status as well as the frequency of smoking and alcohol consumption. Furthermore, socio-economic data such as employment status and monthly income were also recorded. For data collection, the participants were asked the questions objectively in the language of their preference.

3.7 Anthropometric measurements

All anthropometric measurements were completed in accordance with international guidelines (Marfell-Jones, Stewart and De Ridder, 2012). Anthropometric measurements were conducted on the day of the assessment (including at both times points for the longitudinal sub-study). Measurements included body mass index (BMI), waist and hip circumference and wait-hip ratio. For the BMI calculation, body mass was recorded in kilograms (kg) using a digital electronic scale, height was measured using a stadiometer in centimetres (cm) to the nearest 0.1cm. BMI was calculated by division of body mass by the height in meters (m) squared. BMI was further categorised as per the guidelines shown in **table 3.1.** Waist and hip circumference were measured using a measuring tape in cm to the nearest 0.1cm. Waist-hip ratio was calculated by dividing waist circumference by hip circumference. Waist circumference and waist hip ratio was further categorized according to the guidelines shown in **table 3.2.**

Table 3.1: BMI categories according to the WHO guidelines**.**

Categories	BMI (kg/m^2)
Underweight	< 18.5
Normal weight	18.5–24.9
Overweight	25.0–29.9
Obese	> 30.0

Table 3.2: Waist hip ratio categories according to the WHO guidelines and waist circumference threshold according to Alberti *et. al.*, 2009.

Waist-hip ratio		
Categories	**Females**	**Males**
Low	≥ 0.80	≥ 0.95
Moderate	0.81–0.85	0.96–1.0

High	> 0.86	> 1.0
Waist circumference (cm)		
Categories	**Females**	**Males**
High	≥ 80	≥ 94

3.8 Blood pressure and heart rate measurements

Systolic blood pressure, diastolic blood pressure (SBP, SDP) and heart rate were measured using an Omron M6 automatic digital blood pressure monitor (Omron Healthcare, Kyoto, Japan). The blood pressure measurements were expressed in mmHg, were taken on the left arm in triplicate at 5-minute intervals and expressed as the mean value of the three measurements. The heart rate was measured in beats per minute (bpm). Blood pressure was further categorised according to guidelines shown in **table 3.3** below.

Table 3.3: Blood pressure categories according to Seedat *et. al.*, 2011.

Blood pressure categories	Systolic pressure (mmHg)	Diastolic pressure (mmHg)
Low	< 120	< 80
normal	120- 129	80- 84
elevated	130- 139	85- 89
High	≥ 140	≥ 90

3.9 Biochemical analyses

3.9.1 Introduction

Study participants were requested to fast from 22h00 the night before the assessment day. Qualified research nurses collected the fasting whole blood samples from the left arm of the participants in blood collection tubes (SGVac, The Scientific Group (Pty) Ltd.; Milnerton, Western Cape, SA) (**Refer to figure 3.3**). Mid-stream urine samples were also collected in urine containers (**Refer to figure 3.3**).

All blood and urine samples were sent to the National Health Laboratory Service (NHLS), which is a South African National Accreditation System (SANAS) accredited laboratory, located at Tygerberg Hospital in Cape Town. The samples were prepared according to standardized methods and transported as soon as possible, following SANS 10231 regulations. The following parameters were analysed by the NHLS: Serum C-reactive protein (CRP), triglycerides, high density lipoprotein (HDL), low density lipoproteins (LDL), and total cholesterol; whole blood fasting glucose, glycated haemoglobin (HbA1c), creatinine, GGT; CD4 count and HIV viral load in HIV-infected participants; urine creatinine, urine

microalbuminuria and urine albumin-creatinine ratio. **Refer to figure 3.3 G** for a photograph of the NHLS packet and tubes.

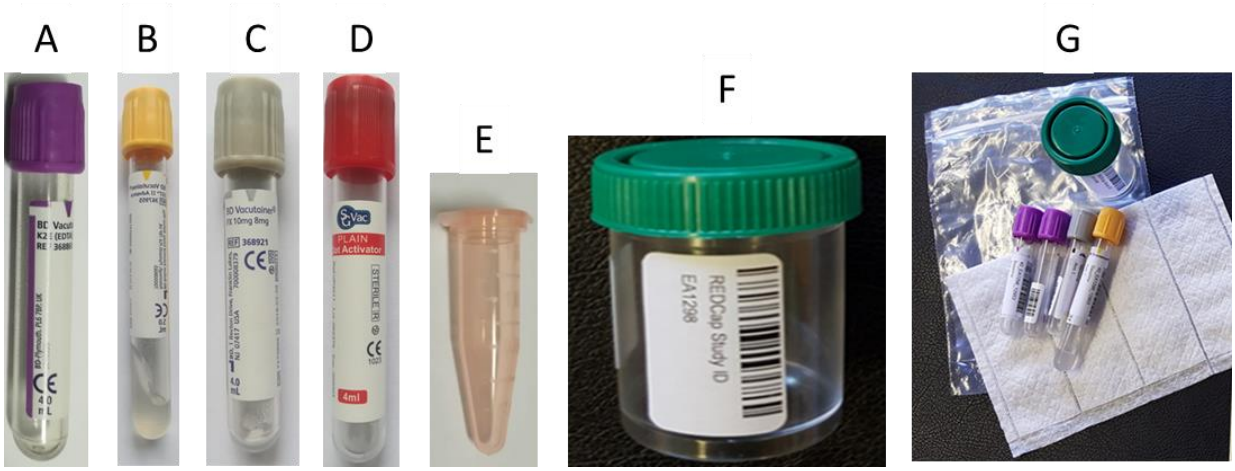


Figure 3.3: Blood and urine sample collection tubes used for the study. Samples collected in each tube for various analysis A) Purple top (3 x 5 ml) tube which contains ethylenediamine tetra-acetic acid (EDTA; strong anticoagulant), used for haemoglobin (Hb), haemoglobin A1c (HbA1c), HIV viral load and CD4 count analysis; B) Yellow top (1 x 7 ml) tube which is serum separator tube which contains a clot activator (acid citrate dextrose solution), used for total cholesterol, HDL, LDL, triglyceride, blood creatinine and GGT analysis; C) Grey top (1 x 5 ml) tube which contains a potassium oxalate (anticoagulant) and sodium fluoride (preservative), used for fasting glucose analysis; D) Red top (1 x 3 ml) serum blood collection tube which contains a lithium heparin (clot activator), used for serum preparation; E) Pink Eppendorf microcentrifuge tube (2 x 1.5 ml) used to aliquot 250 µl of serum from the red top tube for hsCRP analysis and vascular endothelial dysfunction biomarker analysis; F) (1 X 5ml) urine container, used creatinine and microalbuminuria levels analysis; G) Photograph of packet and tubes sent to NHLS for analysis. Photographs taken by researchers of the EndoAfrica team. Consent received for inclusion in this dissertation. Figure designed by the author of this dissertation.

Fasting serum samples were prepared by centrifuging the red top tube for 10 minutes at 2000 g or 43 revolutions per minutes (rpm) using a DM0412 clinical centrifuge from DLAD Scientific, Beijing, China. After centrifugation, 250 µl serum was extracted and aliquoted in 1.5 ml Eppendorf microcentrifuge tubes and stored at -80 °C. Serum biomarker analysis was conducted in collaboration with the Division of Molecular Biology and Human Genetics, University of Stellenbosch. Samples were selected by randomization and sent for evaluation for the following markers: TNF-α, adhesion molecules (VCAM-1 and ICAM-1), PAI-1, e-selectin, and p-selectin. Detailed biomarker analysis methodology is described later in section 3.9.3.

3.9.2 Blood chemistry analyses

The selection of the blood chemistry parameters was based on comprehensive literature analysis and relevance to the current PhD study. **Refer to table 3.4** for a summary of these parameters and reasons motivating their selection.

Table 3.4: Summary of the blood chemistry parameters measured in this PhD study and motivation behind their selection.

Parameter Measured	Motivation
Total Cholesterol, HDL, LDL and triglycerides	Dyslipidaemia is characterized by abnormal blood concentrations of one or more of the following: total cholesterol, LDL, triglyceride levels and / or low HDL levels (Arboix, 2015). In literature dyslipidaemia is a well-known cardiovascular risk factor in the general population (Noubiap, Bigna, Nansseu, Nyaga, Balti, Justin B Echouffo-Tcheugui, *et al.*, 2018) . Furthermore, both HIV and ART have been found to associate with dyslipidaemia in the literature (Okello *et al.*, 2020). Hence, these parameters were particularly selected to measure the lipid profile of the study participants and observe the prevalence of dyslipidaemia as well as its associations with the study's end points. For this PhD study, the criteria for dyslipidaemia include total cholesterol level of more than 5.1 mmol/l, or LDL level of more than 3 mmol/l, or triglyceride level of more than or equal to 1.7 mmol/l, or HDL level less than 1 mmol/l for males and less than 1.2 mmol/l for females (Seedat and Rayner, 2011; Alberti et al 2009).
High-sensitivity C-Reactive Protein (hsCRP)	Literature suggests that hsCRP is a marker of systemic inflammation and a predictor of cardiovascular risk in the general population. Within the HIV context, increased hsCRP is found to be a strong predictor of future cardiovascular events and overall mortality in both ART naïve and ART treated HIV-infected patients (Kurz *et al.*, 2009). Thus, it can be regarded as a useful biomarker of measurement in the HIV context (Wang, Yi, Green, *et al.*, 2015). For this PhD study, elevated hsCRP is defined as levels of more than 3 mg/L in the HIV-free population based on previous studies (Ridker *et al.*, 2000) ; and more than 3.3. mg/L in the HIV-infected population as suggested by literature (De *et al.*, 2013; Vishwanath, Quaiser and Khan, 2016).

Fasting glucose levels and HbA1c	Fasting glucose levels and HbA1c are known markers of cardiovascular risk in both the HIV-free and HIV-infected populations (Okello *et al.*, 2020; World Heart Federation, 2017). For the purposes of this study, elevated fasting glucose is considered as 5.6 mmol/L or more (Heart and Stroke foundation, South Africa, 2020). Elevated HbA1c levels is considered when above 5.9% and 6.5 % HbA1c or above suitable for the diagnosis of Diabetes (Heart and Stroke foundation, South Africa, 2020).
Haemoglobin levels (Hb)	Low Hb level has been associated with increased cardiovascular risk in the HIV-free population (Kim *et al.*, 2013). In the HIV context, persistent anaemia in HIV-infected individuals is associated with decreased survival and can be used as a measurement/ predictive indicator of the progression of HIV/AIDS (Obirikorang and Yeboah, 2009). For the purposes of this PhD study, anaemia is defined as less than or equal to 12 g/dl for females and 13 g/dl for males (WHO, 2019)
Albuminuria, serum and urine creatinine, urine albumin-to-creatinine ratio (ACR) and eGFR	Microalbuminuria is a well-known recognized early marker of renal dysfunction which is expressed by urine albumin to creatinine ratio (Glassock, 2010) . Elevated ACR has been associated with increased CVD risk in both HIV-free and HIV-infected populations (Pirro *et al.*, 2016). Decreased estimated glomerular filtration rates (eGFR) has been associated with CVD and HIV. For the purposes of this study, elevated ACR is considered to be 3 mg/mmol or higher and decreased eGFR was defined less than 60 mL/minute/1.73 m3 (Du Plessis, 2013).
γ-Glutamyl transferase (GGT)	Elevated GGT levels have been associated with increased cardiovascular risk (Ndrepepa and Kastrati, 2016). Furthermore, both HIV and ART have been associated with increased GGT levels (Osakunor *et al.*, 2015). Additionally, elevated GGT levels have also been demonstrated in individuals with high alcohol consumption. For this PhD, criteria for elevated GGT level is 60 U/L or more for males and 40 U/L or more for females (NHLS, South Africa).
CD4 count and viral load	CD4 count and viral load are well known markers of HIV/ AIDS progression. For the purposes of this PhD study, immunological failure was defined as CD4 count of lower than 250 cells/mm^3 and

	unsuccessful viral suppression at viral load above 1000 copies mRNA/ml according to the WHO guidelines (WHO, 2019)

All blood chemistry analyses were completed by the NHLS following standardized methodology and standard operating procedures. Details behind specific instruments used and the principles behind the techniques are described below.

Total cholesterol, HDL, LDL, and triglycerides

All cholesterol measurements (total cholesterol, HDL, LDL and triglycerides) were completed by the NHLS using an enzymatic, colorimetric method and expressed in millimoles per litre (mmol/L). A Roche/Hitachi cobas® c system was used. The analysis for total cholesterol and triglycerides is based on cleavage of the cholesterol esters by the action of cholesterol esterase which produces cholesterol and fatty acids (Roche, Total Cholesterol, accessed September 2020). Thereafter the cholesterol oxidase catalyses the oxidation of cholesterol to cholest-4-en-3-one and hydrogen peroxide. When in presence of peroxidase, the hydrogen peroxide mediates the effects of oxidative coupling of phenol and 4-aminophenazone which subsequently forms a red quinone-imine dye. The colour intensity of the dye formed is directly proportional to the cholesterol concentration (Roche, Total Cholesterol, accessed September 2020).

The analysis for HDL and LDL levels is based on the homogeneous enzymatic colorimetric test principle. In the presence of magnesium ions, dextran sulphate selectively forms water-soluble complexes with LDL which are resistant to polyethylene glycol-modified enzymes. HDL analysis is based on the formation of polyethylene glycol coupled amino groups by cholesterol esterase and cholesterol oxidase reaction (Roche, HLD, LDL, accessed September 2020). Similar to the cholesterol and triglyceride analysis, the colour intensities yielded during these reactions are directly proportional to the respective concentrations. This can be quantified photometrically using the instrument mentioned above (Roche/Hitachi cobas® c system).

High-sensitivity C-Reactive Protein (hsCRP)

The instrument used by the NHLS to quantify hsCRP was a Beckman Coulter IMMAGE® with Immunochemistry Systems and Calibrator 5 Plus assay. The analysis is based on near infrared particle immunoassay rate methodology. Presence of anti-CRP antibody-coated particles mediate the binding of CRP in the serum samples. This results in a formation of insoluble aggregate in the samples. The rate of the aggregation formation was measured which is directly proportional to hsCRP concentration. hsCRP was measured in milligrams per decilitre (mg/dL).

Fasting glucose levels and HbA1c

The instrument used by the NHLS to measure glucose and HbA1c levels was a cobas® 311/501 analyser; Roche/Hitachi cobas® c systems with Hemolysate application. An enzymatic reference method was used based on the UV test principle which relies on hexokinase catalysed phosphorylation reaction of glucose in the presence of adenosine triphosphate which ultimately produces glucose-6-phosphate. Following this, glucose-6-phosphate is oxidised in the presence of nicotinamide adenine dinucleotide phosphate (NADP) by glucose-6-phosphate dehydrogenase which produces gluconate-6-phosphate and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH). The rate of NADPH formation can be measured photometrically as it is directly proportional to the glucose concentration (Roche, GlUC3, accessed September 2020). Glucose levels are expressed in mmol/l.

The HbA1c measurement is based on using haemolysed whole blood in a turbidimetric inhibition immunoassay. The method relies on the usage of a haemolysing reagent, namely tetradecyltrimethylammonium bromide. Glycohemoglobin (HbA1c) in the sample reacts with anti-HbA1c antibody to produce soluble antigen-antibody complexes. Since the specific HbA1c antibody site is present only once on the HbA1c molecule, formation of insoluble complexes does not take place. The presence of polyhaptens react with excess anti-HbA1c antibodies to form an insoluble antibody polyhapten complex which can be determined turbidimetrically. HbA1c is expressed as mmol/mol HbA1c or % HbA1c (calculated as a % of HbA1c/Hb ratio) (Roche, Tina-quant Haemoglobin A1c Gen.3 - Hemolysate and Whole Blood Application, accessed September 2020).

Haemoglobin levels (Hb)

Measurements of Hb levels by NHLS was determined using whole blood application on a cobas® 311/501 analyser; Roche/Hitachi cobas® c systems. The analysis is based on chemiluminescence methodology where haemolysed blood cells have a specific absorbance spectrum. This can be analysed bichromatically during the pre-incubation phase of HbA1c immunoassay (Haemoglobin methods, NHLS: SOP), accessed September 2020). Hb levels are expressed in mmol/L.

Albuminuria, serum and urine creatinine, urine albumin-to-creatinine ratio and eGFR

A cobas® 501/502 analyser, Roche/Hitachi cobas® c system was used by the NHLS to determine albuminuria, expressed as mg/L. The technique is based on an enzymatic reaction method (immunoturbidimetric assay), where antigen in the sample reacts with anti-albumin antibodies and produces antigen/antibody complexes known as agglutinates. This can be measured turbidimetrically Roche, Albumin plus ver.2, accessed September 2020.

To determine serum and urine creatinine levels, a cobas® 311/501 analyser (Roche/Hitachi cobas® c system was used by the NHLS. The principle is based on the enzymatic method which relies on the formation of glycine, formaldehyde, and hydrogen peroxide from creatinine in the presence of creatininase, creatinase, and sarcosine oxidase. Catalysed by peroxidase the hydrogen peroxide reacts

with 4-aminophenazone to form a quinone imine chromogen. The colour intensity of the quinone imine chromogen formed is directly proportional to the creatinine concentration in the reaction mixture (Roche, Creatinine plus ver.2, accessed September 2020). Creatinine levels were measured in µmol/L.

Albumin-to-creatinine ratio was calculated and expressed as mg/mmol. Estimated glomerular filtration rates (eGFR) eGRF was calculated according the CKD-EPI formula (mL/minute/1.73 m3) as previously described (Levey *et al.*, 2009).

γ-Glutamyl transferase (GGT)

A cobas® 311/501 analyser Roche/Hitachi cobas® c system, was used by the NHLS to measure GGT levels. The principle behind the analysis is based on an enzymatic colorimetric assay, which relies on γ-glutamyl transferase to transfer the γ-glutamyl group of L-γ-glutamyl-3-carboxy-4-nitroanilide to produce glycylglycine. The amount of 5-amino-2-nitrobenzoate produced during this reaction is proportional to the level GGT (Roche, GGT2, accessed September 2020). Thus, GGT levels can be determined photometrically measuring the increase in this absorbance and is expressed in units per litre (U/L).

CD4 count and viral load

CD4 count was determined by NHLS using a flow cytometer (FC 500 MPL) with MXP software (Backman Coulter). The methodology is based on labelling cells with a surface marker which is then counted by the instrument. CD4 count was expressed as cells per microliter (cells / uL). For viral load analysis, a COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2 was used by the NHLS. The measurement is based on the HIV-1 nucleic acid amplification (PCR) test which relies on reverse transcription of the target messenger RNA (mRNA) to produce complementary DNA (cDNA). A cleaved dual-labelled oligonucleotide detection probe which is specific to its target is used to detect viral cDNA which is then quantified and expressed in mRNA/ml (Roche, HIV-1 Test, version 2.0, accessed September 2020).

3.9.3 Biomarker analysis

Serum biomarkers of vascular and endothelial dysfunction and inflammation were analysed using Luminex technology in collaboration with the Division of Molecular Biology and Human Genetics, University of Stellenbosch. The following biomarkers were determined: TNF-α, VEGF and adhesion molecules (VCAM-1 and ICAM-1, PAI-1 and e-selectin, and p-selectin). All of these parameters were selected based on literature analysis where they have been suggested as biomarkers that can be measured to assess the role of inflammation, endothelial activation / dysfunction and immune activation in the development of CVD in both the HIV-free and HIV-infected populations (Stoner *et al.*, 2013; Kearns *et al.*, 2017).

A Luminex Bio-Plex 200 system- Bio-Rad® BioPlex® 200 analyser was used, as described previously by Strijdom et al., 2017, for the analysis by a trained and highly experienced scientist. The Luminex 200 system is a versatile analyser and is based on the principles of flow cytometry. The system permits multiplex (multiple measurements simultaneously) of up to 100 different analytes in each of the 96-well filter microplates. Furthermore, the system can analyse relatively small sample volumes. Moreover, the Luminex cytokine/ chemokine/ protein assays are similar to sandwich ELISA assays, which enables multiplex measurements.

The biomarker analysis protocol was based on R&D Systems® Luminex® Immunoassay Principles (R&D System, 2014). (**Refer to figure 3.4)** Magnetic microparticles (colour coded magnetic beads) embedded with fluorophores were precoated with analyte specific antibodies. These fluorophores were set at protein specific ratios. Serum samples were pipetted into the plate wells where the specific antibodies bound to the proteins/ analytes of interest. Following this, excess unbound antibodies are washed off and a biotinylated antibody cocktail specific to the analytes of interest was added to each well. Excess unbound biotinylated antibody was washed off and a streptavidin-phycoerythrin conjugate (Streptavidin-PE) was added to each well. Streptavidin-PE then binds to the biotinylate antibody and excess unbound Streptavidin- PE is also washed off. Thereafter, the proteins of interest are resuspended in buffer and read using the Bio-Rad® BioPlex® analyser. (**Refer to figure 3.4**) The analyser can evaluate the sample based on dual-laser flow-based detection technique. One laser is used to excite the dyes inside each protein to identify the microparticle regions. The second laser is used to excite the Streptavidin -PE to measure the amount of analyte bound to the proteins. All fluorescence emissions from each protein as it passes through the flow cell is then analysed to differentiate emission levels using a photomultiplier tube and an avalanche photodiode.

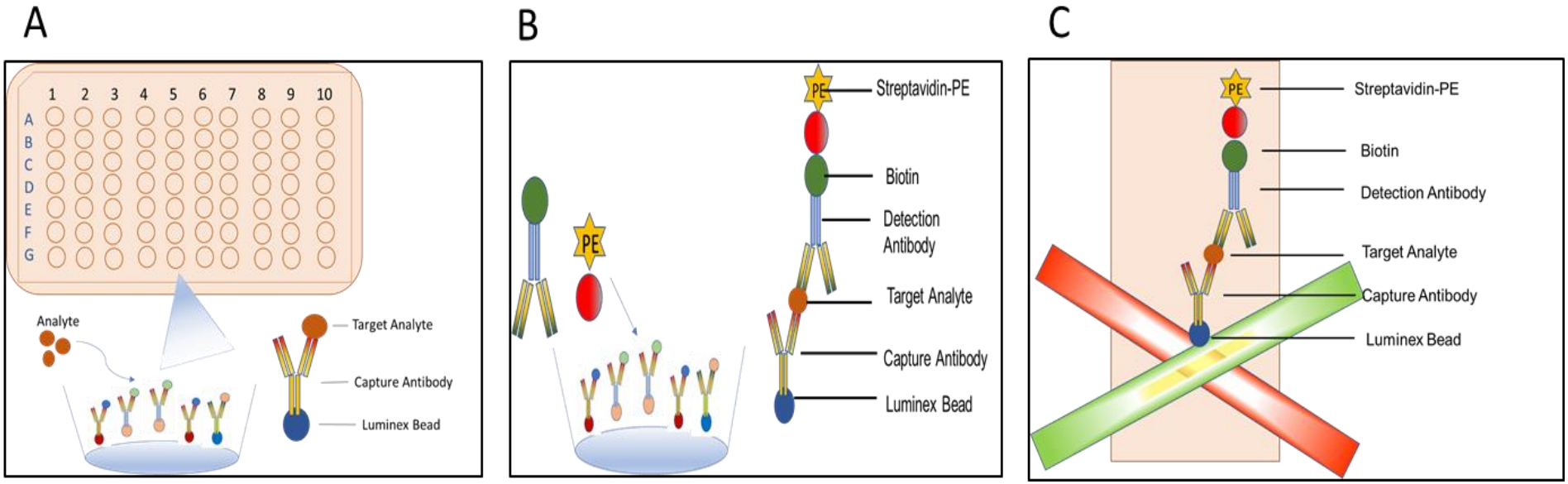


Figure 3.4: Biomarker analysis protocol. A) The sample is added to a mixture of color-coded beads which are pre-coated with analyte-specific capture antibodies. The antibodies bind to the analytes of interest; B) Biotinylated detection antibodies specific to the analytes of interest are added and sandwich the analyte between the capture and detection antibodies. Phycoerythrin (PE)-conjugated streptavidin is added which binds to the biotinylated detection antibodies; C) One laser classifies the bead and identifies the analyte being detected. The second laser determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound. Figure designed by the author of this dissertation based on content from R&D System, 2014.

3.10 IMT measurements

Carotid IMT measurements for this study were based on previously described guidelines and protocols. Little to no preparation is required by the participant for this assessment. For standardization purposes, the assessments were completed in the mornings and participants were requested to remove any jewellery or clothing that interfered in the neck area. To limit operator variability, only a few specific (highly trained and experienced) researchers (which included the author of this dissertation) were allocated to measure IMT. Furthermore, for quality control purposes, IMT was measured more than once on randomly selected participants to evaluate reproducibility.

IMT was measured using an Esaote MyLab™ Five portable ultrasound device (Genoa, Italy) with an Esaote Doppler probe (LA523, 12 MHz) and QIMT™ software which automatically and accurately measures all the parameters needed for the carotid IMT measurements (**Refer to figure 3.5 A**). For the IMT protocol, participants were requested to be in supine position with their head resting comfortably and their neck slightly hyperextended and tilted 45° to the opposite side of the carotid artery that was being assessed (**Refer to figure 3.5 B**). The operator (author of this dissertation) used the index and middle fingers to locate the pulsating carotid artery to guide the position of the probe on the participant's neck. The position of the probe is further adjusted until the image is clear and stable on the ultrasound. The lateral angle of the image was assessed as far as possible. In the participants where a lateral view was not optimal (clear), an anterior or posterior angle was used. Once the image was clear and stable, the region of interest was manually placed 5 mm proximal to the dilatation of the carotid bulb. Thereafter, QIMT™ software automatically detected and analysed the vascular boundaries in radio frequency (RF)-mode (**Refer to figure 3.5 C**). The software further calculated the diameter and the thickness of the intima media layer as well as the median and standard deviation IMT values, using high spatial resolution. At this stage, the IMT measurements are also calculated and expressed in micrometres (µm) with the standard deviation changing. As soon as the standard deviation is below 25 µm (the value also turns green), the IMT image is frozen and saved (**Refer to figure 3.6**). This automatic and accurate measurements are largely independent of the investigator and the device settings. For this study both the left and right common carotid arteries were assessed, and values from both sides were averaged and used in the data analysis. Furthermore, for the purposes of the current study, sub-clinical atherosclerosis was defined as an average IMT of > 900 µm based on guidelines by European Society of Cardiology and European Society of Hypertension (Mancia *et al.*, 2013). A plaque was defined as an average IMT of more than 1000 µm as described in a previous study from South Africa (Roozen *et al.*, 2020).

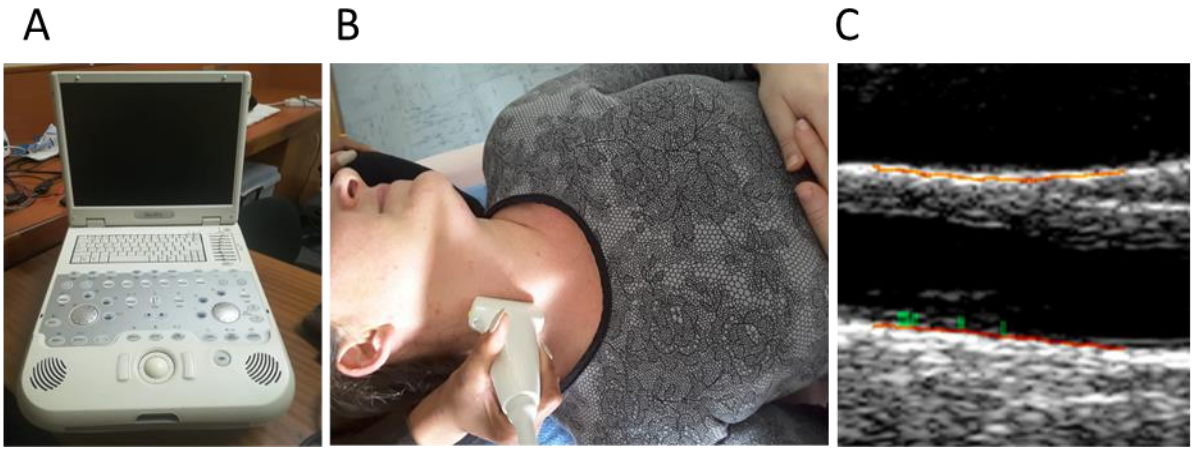


Figure 3.5: Instruments and technique used for the IMT measurement. A) Esaote MyLabTM Five portable ultrasound device (Genoa, Italy); B) Participant and Esaote Doppler probe (LA523, 12 MHz) position for the lateral angle of the image; C) QIMT™ software automatically detecting the vascular boundaries of the intima media layer in radio frequency (RF)-mode. Photographs were taken by the author of this dissertation. Consent was obtained by the individuals in the photograph.

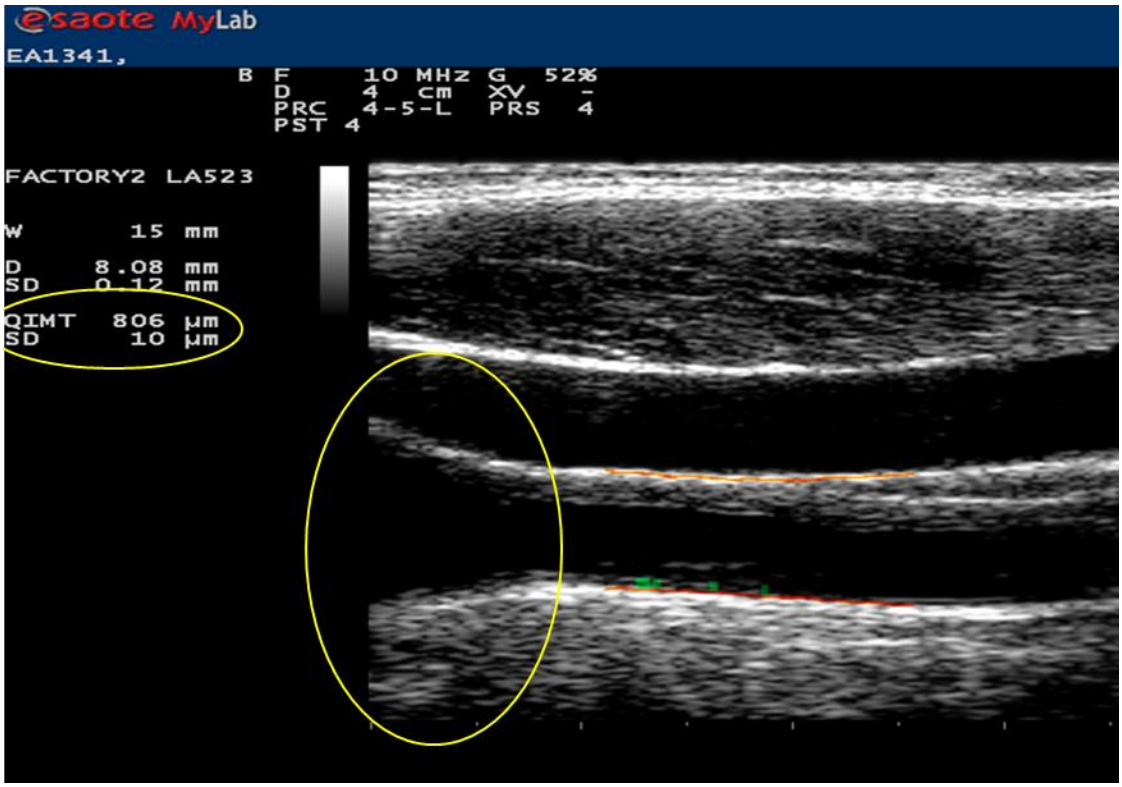


Figure 3.6: Longitudinal scan of 1 cm segment of the common carotid artery IMT measurement using the QIMT™ software. The IMT value and standard deviation (SD) is expressed in µm and circled in yellow. The Carotid bulb is also circled in yellow. Image was captured during an IMT assessment and figure designed by the author of his dissertation.

3.11 FMD measurements

The FMD procedure for this study was based on previously described guidelines and protocols (Corretti *et al.*, 2002; Charakida *et al.*, 2010; Thijssen *et al.*, 2011). Based on these recommendations, participants were requested to fast for at least 8-10 hours prior to the assessment. Apart from dietary intake, FMD measurement can also be influenced by recent exercise, caffeine, intake, alcohol and tobacco use as well as some medications. For females, hormonal changes due to the menstrual cycle may also affect FMD (Corretti *et al.*, 2002; Charakida *et al.*, 2010). Keeping this in mind, the participants were requested to also refrain from smoking, exercise, caffeine and medication for 4 to 6 hours prior to the assessments. Additionally, information regarding the menstrual cycle was also recorded from female participants.

Ambient temperature and acute sympathetic nervous system activation can also influence FMD (Thijssen *et al.*, 2011). Hence, for standardization, the FMD procedure was performed in the mornings, in a quiet, temperature-controlled room at a constant 22°C. Furthermore, time (more than 10 minutes) was accommodated for participants to acclimatise and get comfortable in the environment before assessments began. To mitigate the influence of operator variability on the FMD measurements, only a selected few highly trained and experienced researchers (which included the author of this dissertation) were allocated to measure FMD. Additionally, each operator was assigned specific tasks in the assessment which remained constant throughout the study. Furthermore, for quality control purposes, FMD results were randomly selected and assessed by blinded experts.

For the FMD protocol, participants were in the supine position with their right arm comfortably positioned (extended) at an 80° angle from their body (**Refer to figure 3.7A**). A blood pressure cuff was wrapped below the antecubital fossa (**Refer to figure 3.7B**). The FMD was measured using an Esaote MyLab™ Five portable ultrasound device (Genoa, Italy) with an Esaote Doppler probe (LA523, 12 MHz) connected to an Apple MacBook Pro laptop (2010 edition) with software supported by edge detection technology (Quipu Cardiovascular Suite™; Pisa, Italy) (**Refer to figure 3.8 A & B**.). CLINICA ultrasound gel was applied to the probe which was securely fixed on a precision probe holder from SMT Medical (Wuerzburg, Germany) which allows for micro-metric adjustments (**Refer to figure 3.8C).** The probe (attached to the holder) was placed approximately 3 to 4 cm proximal to the elbow and the brachial artery was localised by adjusting (manoeuvring) the probe's angle and position until a clear and stable image was visualized (**Refer to figure 3.8C**). To visualize and locate the brachial artery on the ultrasound, doppler mode was used, which displayed the real-time image in colours (arteries appeared in red and veins in blue) (**Refer to figure 3.8D**). This further verified that the image located was indeed of the brachial artery and not of a vein. Pulse repetition frequency which is determined by the velocity of blood flow rate detected in the blood vessel, was set at 6.7 Hz. The frequency of the ultrasound was set to 6.6 Hz and the depth was increased to 3 cm before switching to pulse wave mode. In the pulse wave mode, the steer was angled at 60° to match the angle of the vessel.

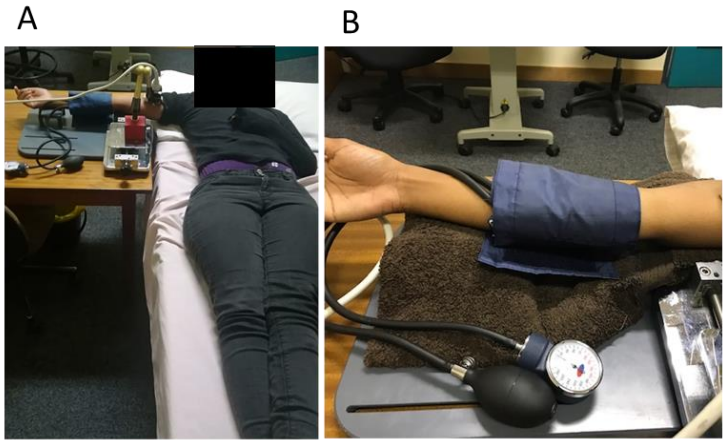


Figure 3.7: Participant position for the FMD assessment. A) Participant in supine position with the right arm extended at an 80° angle from their body; B) A blood pressure cuff wrapped around the right arm, below the antecubital fossa, of the participant. Photograph A was taken by an EndoAfrica team member, photograph B was taken by the author of this dissertation. Consent was obtained by the individuals in the photograph.

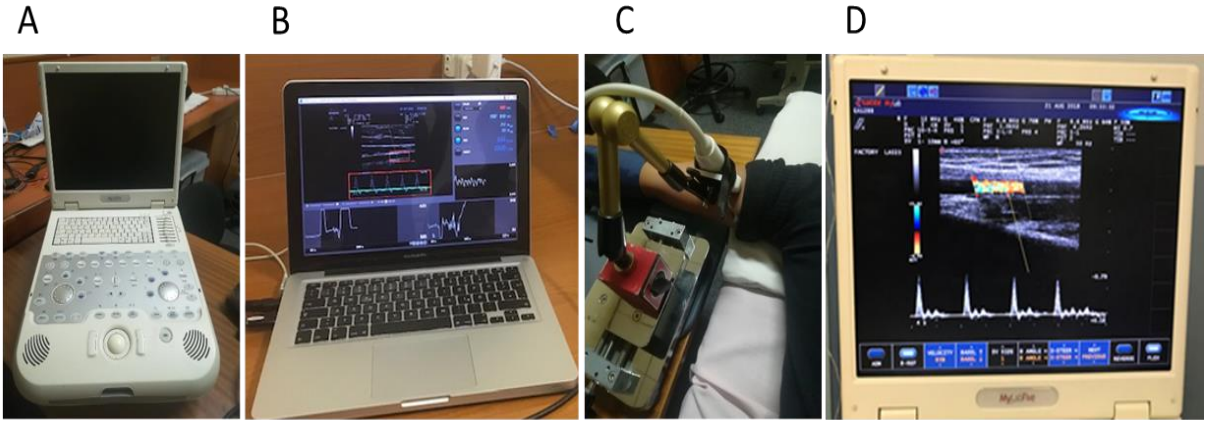


Figure 3.8: Photographs of the instruments used for FMD measurements. A) Esaote MyLabTM Five portable ultrasound device (Genoa, Italy); B) Apple MacBook Pro laptop (2010 edition) with software with edge detection technology (Quipu Cardiovascular Suite™; Pisa, Italy); C) Probe holder from SMT Medical (Wuerzburg, Germany) with an Esaote Doppler probe (LA523, 12 MHz) attached. D) Doppler mode on the ultrasound, displaying image in colour. Photographs were taken by the author of this dissertation.

Following this, Cardiovascular SuiteTM UE 2.8.1. (build 120) computerised software was used for edge detection and calculations. The software settings were selected for vascular measurements and the recording times were set to 60 seconds for the baseline diameter assessment, 300 seconds for the period

of ischaemia and 120 seconds for the period of reactive hyperaemia. The region of interest on the vessel image was selected manually by a highly trained operator and once the edges of the vessel were clearly detected, the FMD measurements and recording began (**Refer to figure 3.9A**). After the baseline recording phase, occlusion of the brachial artery was achieved via inflation of the blood pressure cuff to a supra- systolic pressure of 200 mmHg (or 50 mmHg above the prior systolic pressure measurement) for 300 seconds (**Refer to figure 3.9B**). Thereafter, the cuff was deflated, which triggered the reactive hyperaemia and the maximum brachial artery diameter, and this post occlusion period was recorded for 120s (**Refer to figure 3.9C**). The FMD studio and Cardiovascular Suite QUIPU software calculated FMD as a percentage change in the brachial artery diameter (maximum diameter minus baseline diameter %) in response to the increased shear stress induced by reactive hyperaemia as blood flows back into the artery. The basic FMD formula used by the software was $\left(\frac{\text{Maximum diameter} - \text{Mean Baseline Diameter}}{\text{Mean Baseline diameter}} \text{ X } 100\right)$.

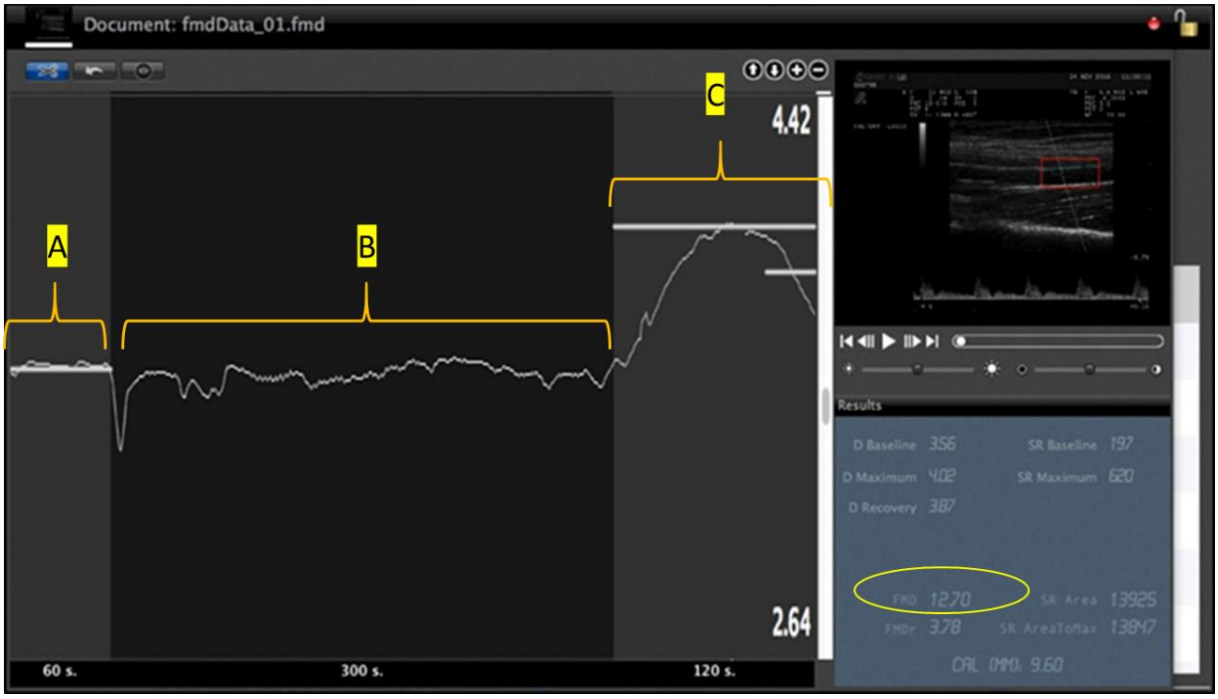


Figure 3.9: FMD measurement recording by Cardiovascular SuiteTM UE 2.8.1. (build 120) computerised software. The line graph indicates the brachial artery diameter A) Mean Baseline artery diameter is measured for a duration of 60 seconds. The recording is relatively stable indicating no or minimal changes in the artery diameter. B) Inflation of the blood pressure cuff (occlusion) for a total duration of 300 seconds which leads to ischaemia C. Deflation of blood pressure cuff leading to hyperaemia and subsequent shear stress-induced arterial dilation with maximum diameter recording followed by recovery diameter recording (total duration 120 seconds). FMD is then calculated as a percentage change in the arterial diameter from the baseline to maximum diameter. The FMD % is

circled in yellow on the image for this assessment. Images were captured during an assessment and figure designed by the author of his dissertation.

3.12 Statistical Analysis

All statistical analyses were performed with IBM® SPSS® software (version 26; USA). At the start of the PhD study, a biostatistician from the Biostatistics Unit (Faculty of Medicine and Health Sciences, Stellenbosch University) was consulted to determine whether the proposed sample sizes for the main PhD study (proposed HIV-free sample size: n=150 and proposed HIV-infected sample size: n=150) would be sufficiently powered. In this regard, a literature search was undertaken. A previous study by Ross et al. (2009), demonstrated that a sample size of n= 73 HIV-infected and n= 21 HIV-free participants was sufficient in elucidating associations of IMT with serum biomarkers as well as finding significant differences in IMT between the two groups in their study. Another study by Donald *et al.* (2008) demonstrated that a case sample size of between n= 100-400 was sufficiently powerful to secure endothelial function measurement reproducibility and statistical significance (1-2% effect size; 80% statistical power and 5% significance). Additionally, in the preliminary studies of the EndoAfrica parent study, significant differences were found in ICAM-1 and VCAM-2 levels between the two groups at a sample size of n= 84 (HIV-infected group) and n= 54 HIV-free group. Therefore, after consultation with the biostatistician and evaluation of relevant literature, it was concluded that the proposed sample sizes were adequate and sufficiently powerful to demonstrate clinical reproducibility and statistical significance in the main cross-sectional study.

The data distribution (normal distribution or non-normal distribution) was identified for each continuous variable using Shapiro- Wilk tests, data histograms, and Q-Q plots. For continuous data description: Normally distributed results were expressed as mean ± standard deviation (SD) and non-normally distributed results was expressed as median with interquartile range. Categorical variable results were expressed as the number of participants and % of the group sample size (n, %).

For the main study, comparison between the HIV-infected and HIV-free groups, independent sample t-tests were conducted for normally distributed data, and for non-normally distributed data, non-parametric Mann Whitney tests were conducted. For the independent sample t-tests, equal variances were assumed unless Levene's test indicated otherwise, for which correction for unequal variances was used. For the longitudinal sub-study, baseline and 12-month follow-up were considered as two dependent samples, and hence a paired analysis was completed. For normally distributed data, a paired samples t-test was used, and Wilcoxon signed ranked test (non-parametric analysis) was completed for the non-normally distributed data. For comparison between categorical data between the two groups for the cross-sectional main study, Pearson's chi square test was conducted, while for the longitudinal sub-

study, cross tabulation between results at two time points was conducted using the paired McNemar test or McNemar-Bowker test.

For the main cross-sectional study, Pearson's correlation test was used to evaluate the relationship between continuous variables. For categorical variables and their relationship with the outcome variables (IMT & FMD %), one-way ANOVA was performed with post hoc test for four categories and t-test was performed for those variables with two categories. Variables that were significantly correlated with the study's endpoints were shortlisted for regression analysis. Generalized linear regression model was utilized for all regression analysis due to its flexible nature of including both continuous and categorical variables in the model. Regression analysis was completed for the total cohort as well as for each study group. Mean IMT and FMD % were used separately as dependent variables for the regression analyses. Confounding variables such as gender and age were forced to remain in all models. For regression analysis in the total cohort, HIV status was forced to remain in the model and in the HIV-infected group, HIV duration, ART status, viral load and CD4 count were forced to remain in the model. Specific independent variables adjusted for in each regression model will be further discussed in the results chapter. The significance threshold for all statistical analysis was set at $p < 0.05$. The PhD candidate performed all statistical analyses, which were verified and validated by a professional biostatistician from the Biostatistics Unit, Faculty of Medicine and Health Sciences of Stellenbosch University.

3.13 Clinical study protocol

In summary, all assessments related to this study were performed in a stepwise manner. Refer to **figure 3.10** for summary flow diagram depicting the methodology and protocol used in this PhD study.

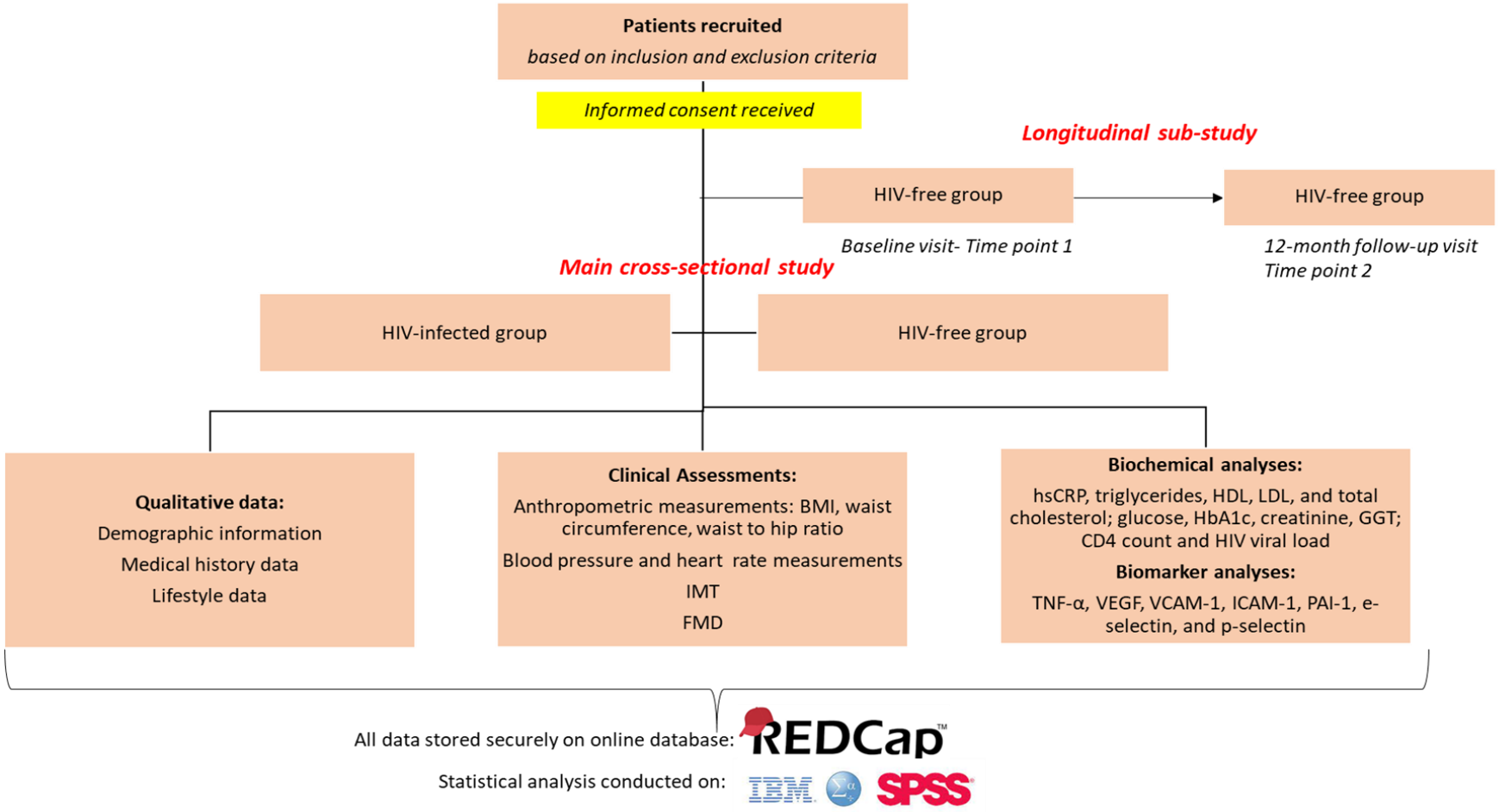


Figure 3.10: Clinical protocol of the study. Figure designed by the author of this dissertation.

Chapter 4: Results

4.1 Introduction

This chapter describes the results obtained from this PhD study in a logical, interpretable format, according to the aims and objectives discussed in Chapter 2. The PhD study obtained results from the cross-sectional main study, which is described under section A, and a longitudinal sub-study which is described under section B of this chapter (**refer to figure 4.1** for the breakdown of this chapter).

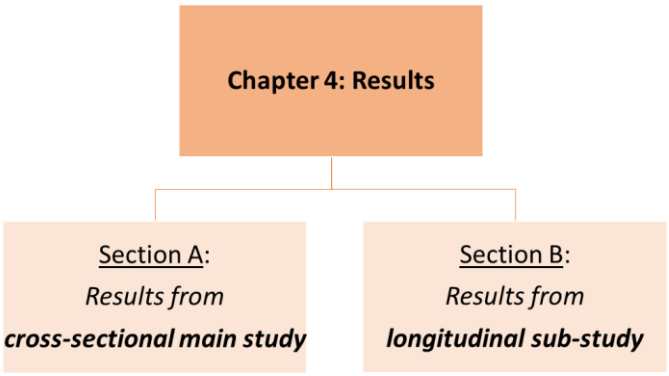


Figure 4.1: Division of results chapter into two sections.

Section A: Results from the cross-sectional main study

4.2 Descriptive profile of the study population

4.2.1 Demographic profile, medical background, lifestyle, and socioeconomic characteristics

A total of 347 volunteers participated in the cross-sectional main study. The mean age was 40.99 years, and 23.6% of the total cohort was more than or equal to 50 years of age. The total cohort was mostly represented by females (n= 251, 72.3%) and individuals who self-identified as being of mixed ancestry (n= 288, 83.0%). The HIV-free group consisted of n= 143 participants and the HIV-infected group comprised of n= 204 participants. There were no significant differences in age and ethnicity between the HIV-free and HIV-infected groups (**refer to table 4.1).** Although no significant differences were found in gender between the two study groups, additional gender subgroup analyses for all study parameters were conducted (**refer to appendix B**).

Table 4.1: Demographic profile of the total, HIV-free and HIV-infected study groups.

Variable	Total cohort	HIV-free	HIV-infected	P value
Sample size, n (%)	347 (100%)	143 (41.2%)	204 (58.8%)	-
Age (years) (Mean ± SD)	40.99 ± 10.41	41.36 ± 11.83	40.73 ± 9.31	0.56
Age Categories				0.28
Age < 50, n (%)	265 (76.4%)	105 (73.4%)	160 (78.4)	
Age ≥ 50, n (%)	82 (23.6%)	38 (26.6%)	44 (21.6%)	
Gender				0.07
Female, n (%)	251 (72.3%)	111 (77.6%)	140 (68.6%)	
Male, n (%)	96 (27.7%)	32 (22.4%)	64 (31.4 %)	
Ethnicity				0.24
Mixed ancestry, n (%)	288 (83.0%)	137 (95.8%)	151 (74.0%)	
African, n (%)	52 (15.0%)	5 (3.5%)	47 (23.0%)	

Data presented as mean ± SD or n (% of group). n, number of participants; SD, standard deviation.

Previous history of TB was the most prevalent form of comorbidity in the total cohort and the HIV-infected group. Additionally, HIV-infected group presented with a significantly higher previous history of TB prevalence in comparison to HIV-free group ($p < 0.001$). Previous history of hypertension was significantly lower in the HIV-infected group in comparison to HIV-free ($p= 0.03$). Furthermore, previous hypertension was the most prevalent form of comorbidity in the HIV-free group. With regards to lifestyle characteristics, more than half of the total cohort, and HIV-free and HIV-infected study groups (58.8%, 62.9%, 55,9%, respectively) reported to be current smokers, and to have consumed alcohol in the past 12 months (**Refer to table 4.2**).

Table 4.2: Medical background and lifestyle characteristics of the total, HIV-free and HIV-infected study groups.

Variable	Total cohort	HIV-free	HIV-infected	P value
History of comorbidities				
Previous TB, n (%)	109 (31.4%)	22 (15.4%) [a]	87 (42.6%) [a]	<0.001
Previous stroke, n (%)	13 (3.7%)	4 (2.8%)	9 (4.4%)	0.43
Diabetes mellitus, n (%)	11 (3.2%)	5 (3.5%)	6 (2.9%)	0.78
Previous hypertension, n (%)	61 (17.6%)	33 (23.1%) [b]	28 (13.7%) [b]	0.03
Previous dyslipidaemia, n (%)	15 (4.3%)	10 (7.0%)	5 (2.5%)	0.11
Previous heart disease, n (%)	3 (0.9%)	1 (0.7%)	2 (1%)	0.85
Smoking				
Current smoker n (%)	204 (58.8%)	90 (62.9%)	114 (55.9%)	0.37
History of smoking				0.06
History of smoking n (%)	31 (8.9%)	10 (7.0%)	21 (10.3%)	
No history of smoking n (%)	111 (32.0%)	43 (30.1 %)	68 (33.3%)	
Alcohol consumption				
Alcohol consumption in the past 12 months, n (%)	192 (55.3%)	80 (55.9%)	112 (54.9%)	0.85
Frequency of alcohol consumption				0.22
< 8 days per month, n (%)	129 (83.2%)	53 (37.1%)	91 (44.6%)	

Data presented as n (% of group). n, number of participants; SD, standard deviation; TB, tuberculosis. Means with same superscript letter: differ significantly (p <0.05).

There were no significant differences in socioeconomic characteristics such as employment status and monthly income between the HIV-free and HIV-infected groups (**Refer to table 4.3**)

Table 4.3: Socioeconomic characteristics of the total, HIV-free and HIV-infected study groups.

Variable	Total cohort	HIV-free	HIV-infected	P value
Employment status				0.71
Unemployed, n (%)	179 (51.6%)	79 (55.2%)	100 (49.0%)	
Employed full-time, n (%)	102 (29.4%)	39 (27.3%)	63 (30.9%)	
Employed part-time, n (%)	57 (16.4%)	22 (15.4%)	35 (17.2%)	

Monthly income				0.06
> R1,000, n (%)	100 (28.0%)	45 (31.5%)	55 (27.0%)	
R1,000 - R4,999, n (%)	191 (55.0%)	74 (51.7%)	117 (57 %)	
R5,000 - R9,999, n (%)	30 (8.6%)	10 (7.0%)	20 (9.8%)	
R10,000 - R20,000, n (%)	13 (3.7%)	6 (4.2%)	7 (3.4%)	

Data presented as n (% of group). n, number of participants.

4.2.2 HIV-related and ART characteristics

The majority of the HIV-infected participants were receiving ART, with 81.9% on NRTI / NNRTI-containing first line ART. Viral load was significantly lower ($p < 0.001$) and CD4 count was significantly higher ($p= 0.011$) in the HIV-infected on ART group in comparison to the HIV-infected/no ART group. Additionally, there was a significantly higher prevalence of participants with high viral load measurements (> 1000 mRNA/ml) in the HIV-infected/ no ART group compared to the HIV-infected on ART group ($p < 0.001$) (**Refer to table 4.4**).

Table 4.4: HIV related and ART characteristics of the total HIV-infected, HIV-infected/ no ART and HIV-infected + ART study groups.

Variable	Total HIV-infected cohort	HIV-infected/ no ART	HIV-infected + ART	P value
Sample size, n (%)	204 (100%)	16 (7.8%)	188 (92.2%)	-
HIV duration, (> 5 years), n (%)	87 (46.8%)	6 (42.9%)	81 (47.1%)	0.82
Viral Load (copies mRNA/ml)	50.00 (10.00-495.50)	11786.50 (2861.00-80110.25) [a]	50.00 (10- 161.50) [a]	< 0.001
Viral load category [b] High (> 1000 mRNA/ml), n (%)	44 (21.6%)	13 (81.3%) [c]	31 (16.5%) [c]	< 0.001
CD4 count (cells/mm^3)	509.03 ± 239.220	353.21 ± 206.30 [d]	520.99 ± 238.52 [d]	0.01
CD4 category [b] Low (< 250 cells/mm^3), n (%)	28 (13.7%)	4 (25.0%)	24 (12.8%)	0.17
ART Duration (Weeks)	-	-	175.00 (96.00-361.00)	-
First line ART, n (%)	-	-	167 (81.9%)	-

Data presented as mean ± SD or n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. Means with same superscript letter: differ significantly ($p <0.05$). [b] Viral load and CD4 categories based on guidelines by WHO.

4.2.3 Anthropometric characteristics

The mean BMI in the total cohort and the HIV-infected group fell within the normal range according to WHO guidelines. However, the mean BMI in the HIV-free group was in the overweight range according to the same guidelines by WHO (**Refer to table 4.5**). Furthermore, the HIV-infected group had a significantly lower BMI ($p < 0.001$) and waist circumference ($p= 0.001$) in comparison to HIV-free group. Additionally, there were significant differences between the HIV-infected and HIV-free group with regards to BMI categories ($p= 0.001$) and high waist circumference categories ($p= 0.003$). No differences were seen in waist to hip ratio between any of the groups (**Refer to table 4.5**). For gender differences in anthropometric measurements, **refer to appendix B.**

Table 4.5: Anthropometric characteristics of the total, HIV-free and HIV-infected study groups.

Variable	Total cohort	HIV-free	HIV-infected	P value
BMI (kg/m^2)	23.30 (19.30-29.30)	25.50 (20.30-33.10) [a]	22.26 (18.86-27.63) [a]	< 0.001
BMI categories [b]				0.001
Underweight (BMI < 18.5 kg/m^2), n (%)	59 (17.0%)	15 (10.5%)	44 (21.6%)	
Normal weight (BMI 18.5 to < 25 kg/m^2), n (%)	141 (40.6%)	53 (37.1%)	88 (43.1%)	
Overweight (BMI 25 to < 30 kg/m^2), n (%)	66 (19.0%)	27 (18.9%)	39 (19.1%)	
Obese (BMI > 30 kg/m^2), n (%)	81 (23.3%)	48 (33.6%)	33 (16.2%)	
Waist circumference (cm)	86.00 (75.00-98.00)	92.00 (76.00-104.00) [c]	83.00 (73.25-92.75) [c]	0.001
Waist circumference category [d] High (≥ 80/ ≥ 94 females/ males), n (%)	193 (55.6%)	93 (65.0%)	100 (49.0%)	0.003
Waist to hip ratio	0.89 (0.83- 0.95)	0.89 (0.84- 0.95)	0.89 (0.83 - 0.96)	0.96
Waist to hip ratio categories [d]				0.24
Low (≥ 0.80/ ≥ 0.95 females/ males), n (%)	105 (30.3%)	40 (28.0%)	65 (31.9%)	
Moderate (0.81–0.85/0.96–1.0 females/ males), n (%)	71 (20.5%)	25 (17.5%)	46 (22.5%)	
High (> 0.86/ > 1.0 females/ males), n (%)	171 (49.3%)	78 (54.5%)	93 (45.6%)	

Data presented as n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; BMI, body mass index. Means with same superscript letter: differ significantly ($p < 0.05$). [b] BMI category based on WHO. [d] Waist circumference and waist to hip ratio category based on guidelines by Alberti *et al*., 2009.

In HIV sub-group analyses, the HIV-infected group on ART demonstrated a significantly higher BMI (p= 0.02) and waist circumference (p= 0.01) in comparison to HIV-free group (**Refer to table 4.6**).

Table 4.6: Anthropometric characteristics of the HIV/ no ART and HIV on ART study groups.

Variable	HIV-infected/ no ART	HIV-infected + ART	P value
BMI (kg/m^2)	19.00 (17.62- 21.30) [a]	22.60 (18.91- 27.66) [a]	0.02
BMI categories [b]			0.32
Underweight (BMI < 18.5 kg/m^2), n (%)	6 (37.5%)	38 (20.2%)	
Normal weight (BMI 18.5 to < 25 kg/m^2), n (%)	7 (43.8%)	81 (43.1%)	
Overweight (BMI 25 to < 30 kg/m^2), n (%)	2 (12.5%)	37 (19.7%)	
Obese (BMI > 30 kg/m^2), n (%)	1 (6.3%)	32 (17.0%)	
Waist circumference (cm)	71.00 (69.00- 81.00) [c]	84.00 (75.00- 93.00) [c]	0.01
Waist circumference category [d] High (≥ 80/ ≥ 94 females/ males), n (%)	5 (31.3%)	95 (50.5%)	0.14
Waist to hip ratio	0.86 (0.80- 0.93)	0.89 (0.83- 0.96)	0.17
Waist to hip ratio categories [d]			0.91
Low (≥ 0.80/ ≥ 0.95 females/ males), n (%)	5 (31.3%)	60 (31.9%)	
Moderate (0.81–0.85/0.96–1.0 females/ males), n (%)	3 (18.8%)	43 (22.9%)	
High (> 0.86/ > 1.0 females/ males), n (%)	8 (50.0%)	85 (45.2%)	

Data presented as n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation; BMI, body mass index. [b] BMI category based on WHO. Means with same superscript letter: differ significantly (p < 0.05) [d] Waist circumference and waist to hip ratio category based on guidelines by Alberti et al., 2009.

4.2.4 Blood pressure and heart rate measurements

The median SBP and DBP in the total cohort fell within the normal clinical ranges according to Seedat and Rayner, 2013. The median SBP (p= 0.02) and median DBP (p= 0.03) values were significantly higher in the HIV free group compared to the HIV infected group. Additionally, there was a significant difference in DBP categories between HIV-infected and HIV-free group (p= 0.02). Furthermore, there was a higher prevalence of participants with hypertension in the HIV free group compared to HIV infected (p= 0.002). The median heart rate was also significantly different between HIV-infected and HIV-free group (p= 0.03) (**refer to table 4.7)**.

Table 4.7: Blood pressure and heart rate measurements of the total, HIV-free and HIV-infected study groups.

Variable	Total cohort	HIV-free	HIV-infected	P value
Systolic Blood Pressure (mmHg)	120 (110.00-136.00)	125.00 (112.00-138.00) [a]	119.00 (109.00-132.00) [a]	0.02
Systolic blood pressure categories [b]				0.10
Low (< 120 mmHg), n (%)	162 (46.7%)	57 (39.9%)	105 (51.5%)	
Normal (120- 129 mmHg), n (%)	69 (19.9%)	28 (19.6%)	41 (20.1%)	
Elevated (130- 139 mmHg), n (%)	46 (13.3%)	24 (16.8%)	22 (10.8%)	
High (≥ 140 mmHg), n (%)	70 (20.2%)	34 (23.8%)	36 (17.6%)	
Diastolic Blood pressure (mmHg)	84.00 (76.00-94.00)	86.00 (76.00-96.00) [c]	82.50 (75.25-91.00) [c]	0.03
Diastolic blood pressure categories [b]				0.02
Low (< 80 mmHg), n (%)	123 (35.4%)	44 (30.8%)	79 (38.7%)	
Normal (80- 84 mmHg), n (%)	51 (14.7%)	20 (14.0%)	31 (15.2%)	
Elevated (85- 89 mmHg), n (%)	54 (15.6%)	17 (11.9%)	37 (18.1%)	
High (≥ 90 mmHg), n (%)	119 (34.3%)	62 (43.4%)	57 (27.9%)	
Hypertension [b] (SBP > 140 mmHg or DBP >90 mmHg), n (%)	125 (36.0%)	65 (45.5%) [d]	60 (29.4%) [d]	0.002
Heart Rate (bpm) (median (IQR))	70.00 (63.00-79.00)	69.00 (60.00-77.00) [d]	71.00 (65.00-80.00) [d]	0.03

Data presented as n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation; SBP, systolic blood pressure; DBP, diastolic blood pressure. Means with same superscript letter: differ significantly ($p < 0.05$) [b] Systolic, diastolic blood pressure category, as well as hypertension cut off based on guidelines by Seedat and Rayner, 2013

In HIV subgroup analyses, there were no significant differences in blood pressure and heart rate measurements between the HIV on ART group and HIV/no ART group (**refer to table 4.8**)

Table 4.8: Blood pressure and heart rate measurements of the HIV/ no ART and HIV on ART study groups.

Variable	HIV-infected/ no ART	HIV-infected + ART	P value
Systolic Blood Pressure (mmHg)	117.00 (109.00- 132.00)	119 (109.00- 133.50)	0.88
Systolic blood pressure categories [a]			0.55
Low (< 120 mmHg), n (%)	10 (62.5%)	95 (50.5%)	
Normal (120- 129 mmHg), n (%)	1 (6.3%)	40 (21.3%)	
Elevated (130- 139 mmHg), n (%)	2 (12.5%)	20 (10.6%)	
High (≥ 140 mmHg), n (%)	3 (18.8%)	33 (17.6%)	
Diastolic Blood pressure (mmHg)	86.00 (75.00- 93.00)	82.00 (75.25- 91.00)	0.60
Diastolic blood pressure categories [a]			0.44
Low (< 80 mmHg), n (%)	4 (25.0%)	75 (39.9%)	
Normal (80- 84 mmHg), n (%)	3 (18.8%)	28.14.9%)	
Elevated (85- 89 mmHg), n (%)	5 (31.3%)	32 (17.0%)	
High (≥ 90 mmHg), n (%)	4 (25.0%)	53 (28.2%)	
Hypertension [a] (SBP > 140 mmHg or DBP > 90 mmHg), n (%)	4 (25.0%)	56 (29.8%)	0.69
Heart Rate (bpm)	75.00 (70.00- 81.00)	70.00 (64.00- 80.00)	0.10

Data presented as n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation; SBP, systolic blood pressure; DBP, diastolic blood pressure. Means with same superscript letter: differ significantly ($p < 0.05$). [a] Systolic, diastolic blood pressure category, as well as hypertension cut off based on guidelines by Seedat and Rayner, 2013.

4.2.5 Fasting lipid, glucose and HBA1c measurements

There were no significant differences in the lipid profile between the HIV-infected and HIV-free groups (**refer to table 4.9**). Median fasting glucose was found to be significantly higher in the HIV-infected group in comparison to HIV-free group (p= 0.02). Furthermore, HbA1c% was found to be significantly lower in the HIV-infected group in comparison to HIV-free (p= 0.01).

Table 4.9: Fasting lipid, glucose and HBA1c measurements of the total, HIV-free and HIV-infected study groups.

Variable	Total cohort	HIV-free	HIV-infected	P value
Lipid profile				
Total cholesterol (mmol/L)	4.31 (3.82- 4.96)	4.30 (3.80- 4.90)	4.40 (3.86- 5.07)	0.51
Total cholesterol categories [a] Elevated (≥ 5 mmol/L), n (%)	76 (21.9%)	28 (19.6%)	48 (23.5%)	0.38
High-Density Lipoprotein Cholesterol (HDL) (mmol/L)	1.35 (1.10- 1.71)	1.32 (1.10- 1.63)	1.43 (1.08- 1.83)	0.21
HDL categories [a] Decreased (≤ 1.2/1.0 mmol/L females /males), n (%)	105 (30.3%)	46 (32.2%)	59 (28.9%)	0.52
Low-Density Lipoprotein Cholesterol (LDL) (mmol/L)	2.43 ± 0.81	2.50 ± 0.86	2.38 ± 0.77	0.18
LDL categories [a] Elevated (≥ 3 mmol/L), n (%)	77 (22.2%)	35 (24.5%)	42 (20.6%)	0.39
Triglycerides (mmol/L)	0.98 (0.75- 1.39)	0.96 (0.73- 1.35)	1.00 (0.78- 1.39)	0.39
Triglycerides categories [a] Elevated (≥ 1.7 mmol/L), n (%)	56 (16.1%)	19 (13.3%)	37 (18.1%)	0.23
Glucose & HbA1c				
Fasting glucose (mmol/L)	4.70 (4.30- 5.20)	4.60 (4.30- 5.00) [b]	4.80 (4.40- 5.28) [b]	0.02
Fasting glucose categories [a] Elevated (≥ 5.6 mmol/L), n (%)	43 (12.4%)	16 (11.2%)	27 (13.2%)	0.57
Glycated Haemoglobin (HbA1c) (%)	5.30 (5.00- 5.50)	5.40 (5.00- 5.60) [c]	5.20 (5.00- 5.40) [c]	0.01
HbA1c categories [a] Elevated (≥ 5.9%), n (%)	27 (7.8%)	13 (9.1%)	14 (6.9%)	0.45

Data presented as mean ± SD or n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. Means with same superscript letter: differ significantly ($p < 0.05$). [a] Lipid profile and glucose and HbA1c % categories is based on guidelines by Seedat and Rayner, 2013 and Alberti *et al.*, 2009.

In HIV subgroup analyses, the HIV-infected on ART group demonstrated a significantly higher total cholesterol (p= 0.001), LDL cholesterol (p= 0.04) and HDL cholesterol (p= 0.013) in comparison to HIV-infected/ no ART group. Additionally, the prevalence of participants with low HDL levels was higher in the HIV-infected/ no ART group compared to the treated group (p= 0.002). (**Refer to table 4.10**).

Table 4.10: Fasting lipid, glucose and HBA1c measurements of the HIV/ no ART and HIV with ART study groups.

Variable	HIV-infected/ no ART	HIV-infected + ART	P value
Lipid profile			
Total cholesterol (mmol/L)	3.66 (2.75- 4.00) [a]	4.46 (3.89- 5.13) [a]	0.001
Total cholesterol categories [b] Elevated (≥ 5 mmol/L), n (%)	1 (6.3%)	47 (25.0%)	0.09
High-Density Lipoprotein Cholesterol (HDL) (mmol/L)	0.97 (0.85- 1.44) [c]	1.45 (1.12- 1.83) [c]	0.01
HDL categories [b] Decreased (≤ 1.2/1.0 mmol/L females /males), n (%)	10 (62.5%) [d]	49 (26.1%) [d]	0.002
Low-Density Lipoprotein Cholesterol (LDL) (mmol/L)	1.96 ± 0.80 [e]	2.41 ± 0.76 [e]	0.04
LDL categories [b] Elevated (≥3 mmol/L), n (%)	2 (12.5%)	40 (21.3%)	0.41
Triglycerides (mmol/L)	1.04 (0.64- 1.55)	1.00 (0,78- 1.39)	0.47
Triglycerides categories [b] Elevated (≥1.7 mmol/L), n (%)	2 (12.5%)	35 (18.6%)	0.54
Glucose & HbA1c			
Fasting glucose (mmol/L)	4.30 (4.10- 5.10)	4.8 (4.40- 5.30)	0.07
Fasting glucose categories [b] Elevated (≥ 5.6 mmol/L), n (%)	1 (6.3%)	26 (13.8%)	0.39
Glycated Haemoglobin (HbA1c) (%)	5.00 (4.90- 5.40)	5.20 (5.00- 5.55)	0.26
HbA1c categories [b] Elevated (≥ 5.9%), n (%)	3 (18.8%)	11 (5.9%)	0.05

Data presented as n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. Means or medians with same superscript letter: differ significantly ($p < 0.05$). [b] Lipid profile and glucose and HbA1c % categories is based on guidelines by Seedat and Rayner, 2013 and Alberti *et al.*, 2009.

4.2.6 Haemoglobin and GGT measurements

There was a significant difference in the prevalence of HIV infected participants with decreased Hb levels (anaemia) compared to HIV free participants (p= 0.05). Additionally, median GGT was significantly higher in the HIV-infected group in comparison to HIV-free group (p< 0.001).

Furthermore, the prevalence of participants with high GGT levels was higher in the HIV infected group compared to HIV free (p< 0.001) (**Refer to table 4.11**).

Table 4.11: Haemoglobin and GGT measurements of the total, HIV-free and HIV-infected study groups.

Variable	Total cohort	HIV-free	HIV-infected	P value
Haemoglobin (g/dL)	13.30 (12.45-14.40)	13.50 (12.60-14.60)	13.20 (12.30-14.30)	0.11
Haemoglobin categories [a] Decreased (< 12.0/ 13.0 g/dL females/males), n (%)	71 (20.5%)	22 (15.4%) [b]	49 (24.0%) [b]	0.05
Liver function				
GGT (U/L)	34.00 (23.00-59.00)	27.00 (18.00-38.00) [c]	42.50 (28.00-80.75) [c]	< 0.001
GGT categories [d] Elevated (≥ 40/ ≥ 60 U/L women/men), n (%)	131 (37.8%)	32 (22.4%) [e]	99 (48.5%) [e]	< 0.001

Data presented as n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. Means or medians with same superscript letter: differ significantly (p < 0.05). [a] Haemoglobin categories are based on WHO guidelines. [d] GGT categories are based on guidelines by NHLS, SA.

In HIV subgroup analyses, median Hb as well as GGT was significantly higher in the HIV-infected on ART group in comparison to the HIV-infected/ no ART group (p < 0.001; p= 0.01, respectively). Additionally, the prevalence of low Hb (anaemia) was higher in the untreated group compared to HIV-infected on ART (p= 0.002) (**Refer to table 4.12**).

Table 4.12: Haemoglobin and GGT measurements of the HIV/ no ART and HIV with ART study groups.

Variable	HIV-infected/ no ART	HIV-infected + ART	P value
Haemoglobin (g/dL)	11.20 (10.60- 12.70) [a]	13.35 (12.40- 14.40) [a]	p<0.001
Haemoglobin categories [b] Decreased (< 12.0/ 13.0 g/dL females/males), n (%)	9 (56.3%) [c]	40 (21.3%) [c]	0.002
Liver function			
GGT (U/L)	28.00 (22.00- 40.00) [d]	45.00 (29.00- 86.75) [d]	0.01
GGT categories [e] Elevated (≥ 40/ ≥ 60 U/L women/men), n (%)	5 (31.3%)	94 (50.0%)	0.15

Data presented as n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. Means or medians with same superscript letter: differ significantly ($p < 0.05$). [b] Haemoglobin cut-off values for anaemia are based on WHO guidelines. [e] GGT cut-off values are according to the NHLS, South Africa.

4.2.7 Kidney function measurements

With regards to kidney function measurements, the albumin-to-creatinine ratio (ACR) was significantly higher in the HIV-infected group in comparison to HIV-free group (p= 0.04). No significant differences were observed in serum creatinine, urine albumin and eGFR measurements between the two study groups (**refer to table 4.13**).

Table 4.13: Kidney function measurements of the total, HIV-free and HIV-infected study groups.

Variable	Total cohort	HIV-free	HIV-infected	P value
Serum creatinine (µmol/L)	61.00 (53.00- 71.00)	61.00 (54.00- 71.00)	61.00 (52.00- 71.00)	0.56
Urine albumin (mg/L)	9.00 (3.87- 26.80)	8.80 (3.00- 19.98)	9.05 (4.33- 34.13)	0.11
Albumin-to-creatinine ratio (mg/mmol)	0.76 (0.40- 2.00)	0.70 (0.40- 1.50) [a]	0.80 (0.41- 2.48) [a]	0.04
ACR categories [b] Increased (> 3 mg/mmol), n (%)	64 (18.4%)	22 (15.4%)	42 (20.6%)	0.22
eGRF (mL/minute/1.73 m3) categories [b] Decreased (< 60 mL/minute/1.73 m3), n (%)	4 (1.2%)	2 (1.4%)	2 (1.0%)	0.72

Data presented as n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. Means or medians with same superscript letter: differ significantly ($p < 0.05$). [b] ACR and eGFR categories used as previously reported (Du Plessis, 2013).

In HIV subgroup analyses, the median serum creatinine was significantly higher in the HIV-infected on ART group in comparison to HIV-infected/no ART group (p= 0.003). There were no other significant differences in kidney function variables between the HIV-infected ART and HIV-infected/ no ART group (**refer to table 4.14**)

Table 4.14: Kidney function measurements of the HIV/ no ART and HIV with ART study groups.

Variable	HIV-infected/ no ART	HIV-infected + ART	P value
Serum creatinine (µmol/L)	50.50 (45.00- 61.25) [a]	62.00 (53.25- 71.00) [a]	0.003
Urine albumin (mg/L)	22.05 (4.45- 67.15)	8.90 (4.20- 30.80)	0.33
Albumin-to-creatinine ratio (mg/mmol)	2.05 (0.63- 6.85)	0.80 (0.40- 2.20)	0.08
ACR categories [b] Increased (> 3 mg/mmol), n (%)	5 (31.3%)	37 (19.7%)	0.27
eGRF (mL/minute/1.73 m3) categories [a] Decreased (< 60 mL/minute/1.73 m3), n (%)	0 (0%)	2 (1.1%)	0.68

Data presented as n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. Means or medians with same superscript letter: differ significantly ($p < 0.05$). [b] ACR and eGFR categories used as previously reported (Du Plessis, 2013).

4.2.8 Systemic inflammation and biomarker measurements

In the HIV-infected group the median VCAM-1 was observed to be significantly higher (p= 0.01) in comparison to HIV-free group. PAI-1 was found to be significantly lower in the HIV-infected group in comparison to HIV-free (p= 0.01). No other significant differences were observed in the biomarkers of inflammation and endothelial dysfunction as shown in **table 4.15**.

Table 4.15: Systemic inflammation and biomarker measurements of the total, HIV-free and HIV-infected study groups.

Variable	Total cohort	HIV-free	HIV-infected	P value
hsCRP (mg/L)	4.70 (1.70- 10.60)	4.60 (1.80- 9.00)	4.90 (1.63- 11.98)	0.35
hsCRP categories [a] Elevated (> 3mg/L in HIV-free; > 3.3mg/L in HIV-infected), n (%)	207 (59.7%)	83 (58.0%)	124 (60.8%)	0.61
Tnf-α (pg/ml)	21.86 (18.83- 25.74)	22.27 (19.44- 26.07)	21.55 (18.24- 25.74)	0.49
VEGF (pg/ml)	82.74 (52.13 – 142.27)	95.62 (95.62- 142.06)	74.54 (50.73- 146.05)	0.37
VCAM-1 (ng/ml)	882.58 (618.04- 1180 30.0)	704.49 (553.82- 1008.73) [b]	954.12 (656.90- 1269.50) [b]	0.01

ICAM-1 (ng/ml)	324.35 (195.50-528.53)	381.50 (241.17-631.86)	309.93 (179.80-520.74)	0.17
E-selectin (ng/ml)	33.68 (25.76-44.68)	33.69 (28.01-48.38)	33.68 (24.58-24.58)	0.47
P-selectin (ng/ml)	33.42 (26.41-43.31)	35.50 (27.37-47.46)	32.99 (25.54-42.76)	0.37
PAI-1 (ng/ml)	87.09 (63.48-114.55)	97.68 (78.52-120.70) [c]	79.46 (59.58-108.67) [c]	0.01

Data presented as n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants. Means or medians with same superscript letter: differ significantly ($p < 0.05$). [a] HsCRP categories based on guidelines by Ridker *et al.*, 2000 and De *et al.*, 2013; Vishwanath, Quaiser and Khan, 2016 for HIV-free population and HIV-infected population, respectively.

In the HIV subgroup analyses, VCAM-1 was observed to be significantly lower in the HIV-infected on ART group in comparison to HIV-infected/ no ART group ($p= 0.004$). No other significant differences were observed in the biomarkers of inflammation and endothelial dysfunction as shown in **table 4.16**.

Table 4.16: Systemic inflammation and biomarker measurements of the HIV/ no ART and HIV with ART study groups.

Variable	HIV-infected/ no ART	HIV-infected + ART	P value
hsCRP (mg/L)	3.30 (0.58- 15.45)	5.00 (1.70- 11.85)	0.50
hsCRP categories [a] Elevated (> 3mg/L), n (%)	1 (6.3%)	116 (61.7%)	0.36
Tnf-α (pg/ml)	24.35 (19.23- 26.90)	21.53 (18.15- 25.77)	0.43
VEGF (pg/ml)	89.87 (62.83- 248.66)	73.57 (47.95- 147.48)	0.44
VCAM-1 (ng/ml)	1538.90 (1168.55-1793.05) [a]	915.13 (640.86-1200.98) [a]	0.004
ICAM-1 (ng/ml)	336.60 (227.87-336.60)	307.29 (179.21-514.79)	0.36
E-selectin (ng/ml)	40.30 (27.63- 51.04)	32.36 (24.06- 43.44)	0.23
P-selectin (ng/ml)	37.71 (31.64- 49.19)	32.48 (23.72- 42.28)	0.15
PAI-1 (ng/ml)	94.37 (75.96- 140.71)	79.26 (57.57- 108.02)	0.09

Data presented as n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants. Means or medians with same superscript letter: differ significantly ($p < 0.05$). a HsCRP categories based on guidelines by Ridker et al., 2000.

4.2.9 Carotid IMT and subclinical atherosclerosis measurements

There were no significant differences in the mean IMT or the prevalence of subclinical atherosclerosis and plaque between the HIV-infected and HIV-free groups (**refer to table 4.17**).

Table 4.17: Carotid IMT and subclinical atherosclerosis measurements of the total, HIV-free and HIV-infected study groups.

Variable	Total cohort	HIV-free	HIV-infected	P value
Mean IMT (µm)	627.74 ± 135.37	631.21 ± 136.61	625.33 ± 134.79	0.69
IMT categories				0.91
Subclinical atherosclerosis [a] (> 900 µm), n (%)	14 (4.0%)	5 (3.5%)	9 (4.4%)	
Plaque [b] (> 1000 µm), n (%)	7 (2.0%)	3 (2.1%)	4 (2.0%)	

Data presented as mean ± SD or n (% of group). n, number of participants; SD, standard deviation. [a] Subclinical atherosclerosis cut off was based on guidelines by European Society of Cardiology and European Society of Hypertension. [b] Plaque was defined as an average IMT of more than 1000 µm, as suggested by Roozen et al., 2020.

In the HIV subgroup analyses, the HIV-infected on ART group demonstrated a significantly higher mean IMT (p= 0.004) in comparison to HIV-infected/ no ART group. Additionally, subclinical atherosclerosis and plaque were only present in participants from the HIV-infected on ART group (**Refer to table 4.18**).

Table 4.18: Carotid IMT and subclinical atherosclerosis measurements of the HIV/ no ART and HIV with ART study groups.

Variable	HIV-infected/ no ART	HIV-infected + ART	P value
Mean IMT (µm)	530.33 ± 92.79 [a]	633.24 ± 135.17 [a]	0.004
IMT categories			0.55
Subclinical atherosclerosis [b] (> 900 µm), n (%)	0 (0%)	9 (4.8%)	
Plaque [c] (> 1000 µm), n (%)	0 (0%)	4 (2.1%)	

Data presented as mean ± SD or n (% of group). n, number of participants; SD, standard deviation. Means or medians with same superscript letter: differ significantly ($p < 0.05$). [b] Subclinical atherosclerosis cut off was based on guidelines by European Society of Cardiology and European Society of Hypertension. [c] Plaque was defined as an average IMT of more than 1000 µm, as suggested by Roozen et al., 2020.

4.2.10 Endothelial function measurements (flow-mediated dilatation)

There were no significant differences in the mean baseline brachial artery diameter and FMD% between HIV-infected and HIV-free groups (**Refer to table 4.19**).

Table 4.19: Endothelial function measurements of the total, HIV-free and HIV-infected study groups.

Variable	Total cohort	HIV-free	HIV-infected	P value
Baseline brachial artery diameter (mm)	3.39 ± 0.62	3.37 ± 0.60	3.40 ± 0.62	0.59
Flow-mediated dilatation (%)	6.78 ± 5.43	6.74 ± 5.18	6.81 ± 5.61	0.90

Data presented as mean ± SD. SD, standard deviation.

In the HIV subgroup analyses, HIV-infected on ART group demonstrated a significantly higher baseline brachial artery (p= 0.036) and FMD% (p= 0.01) in comparison to HIV-infected/no ART (**Refer to table 4.20**).

Table 4.20: Endothelial function measurements of the HIV/ no ART and HIV with ART study groups.

Variable	HIV-infected/ no ART	HIV-infected + ART	P value
Baseline brachial artery diameter (mm)	3.19 ± 0.35 [a]	3.42 ± 0.64 [a]	0.04
Flow-mediated dilatation (%)	3.47 ± 5.60 [b]	7.09 ± 5.54 [b]	0.01

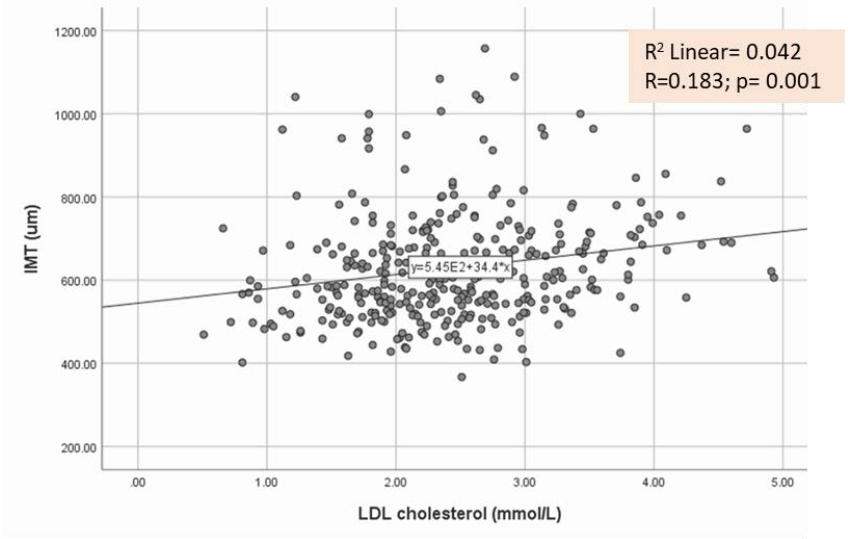
Data presented as mean ± SD. SD, standard deviation. Means or medians with same superscript letter: differ significantly ($p < 0.05$).

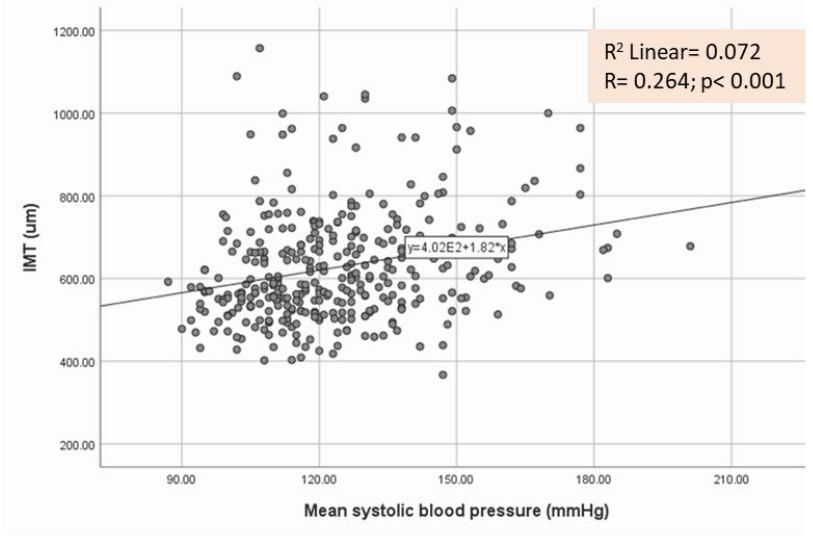
4.3 Associations of various demographic and cardiovascular risk variables with IMT in the total, HIV-free, and HIV-infected cohort.

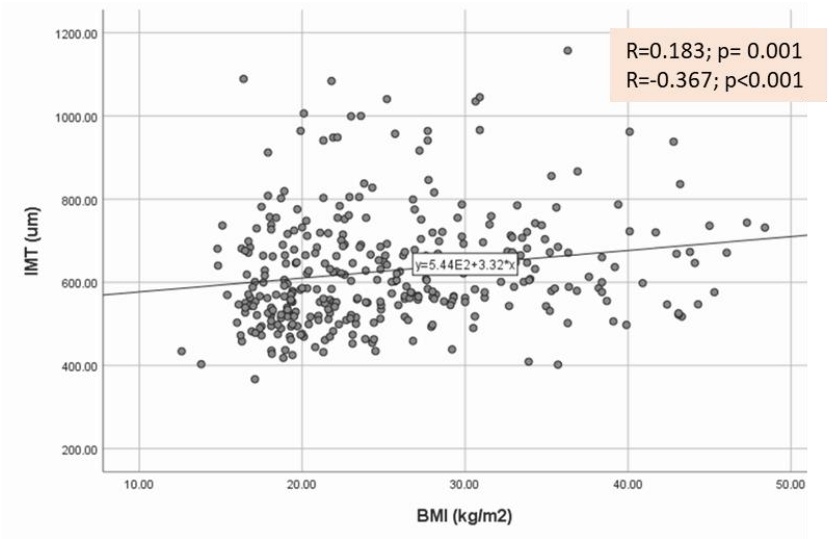
To evaluate associations with IMT, the Pearson's correlation test was conducted to determine the relationship between continuous variables and IMT (**Refer to figure 4.2, 4.3, 4.4** for scatter plot graphs demonstrating significant correlations of selected continuous variables with IMT; **refer to table 4.21** for a summary of correlations with IMT). For categorical variables and their relationship with IMT, one-way ANOVA was performed with Bonferroni post hoc test for four categories and t-test was performed for those variables with two categories (**Refer to table 4.21** for summary of significant categorical relationships with IMT).

Biomarkers of inflammation and endothelial dysfunction did not significantly correlate with IMT (**refer to table 4.22).**

Figure 4.2: Scatter plot graphs of selected significant correlations of variables with IMT in the total cohort.







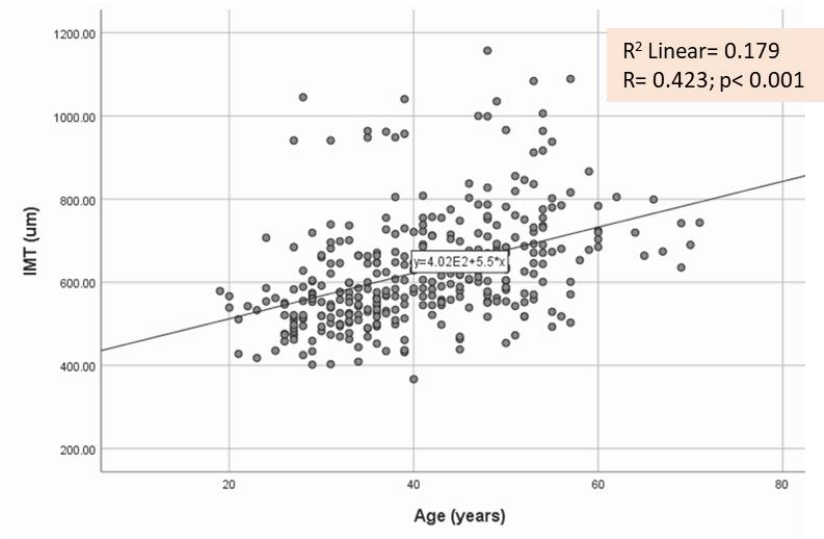
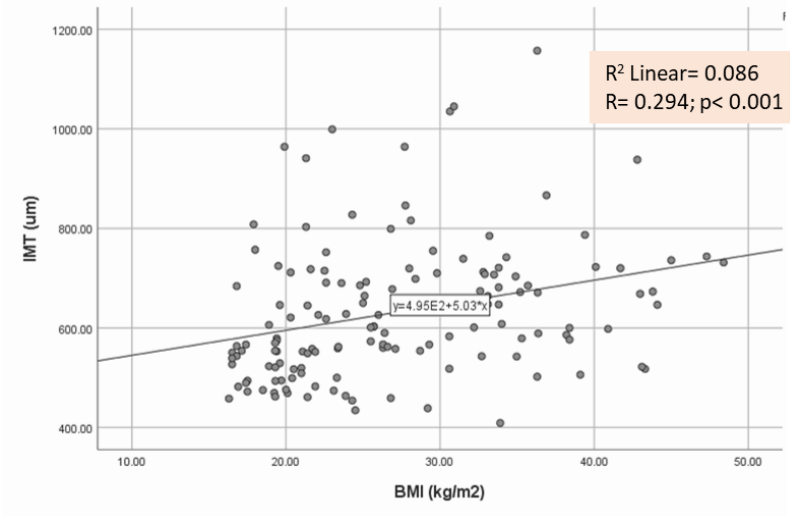


Figure 4.3: Scatter plot graphs of selected significant correlations of variables with IMT in the HIV-free group.



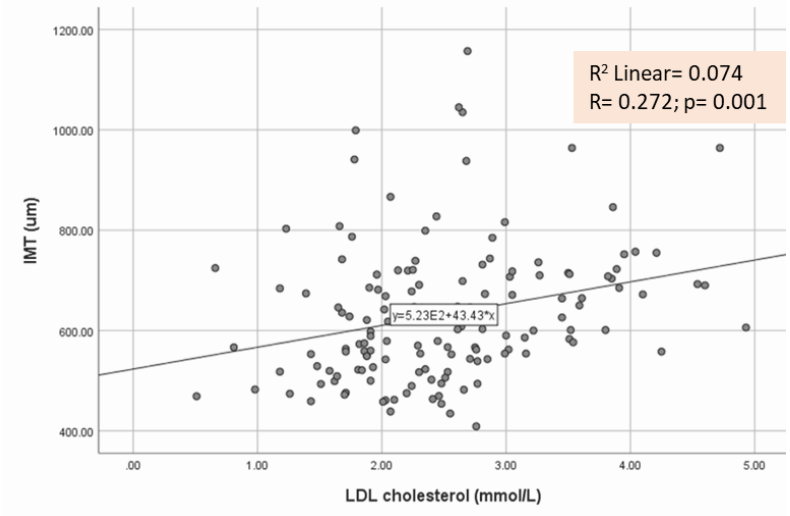
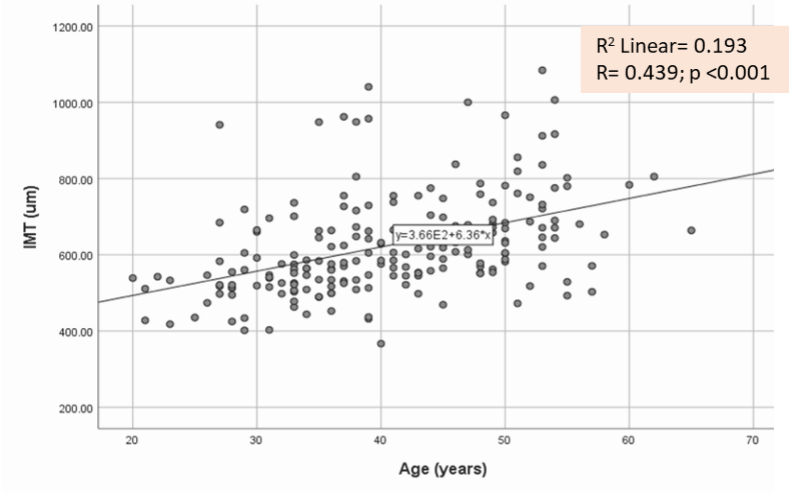


Figure 4.4: Scatter plot graphs of selected significant correlations of variables with IMT in the HIV-infected group.



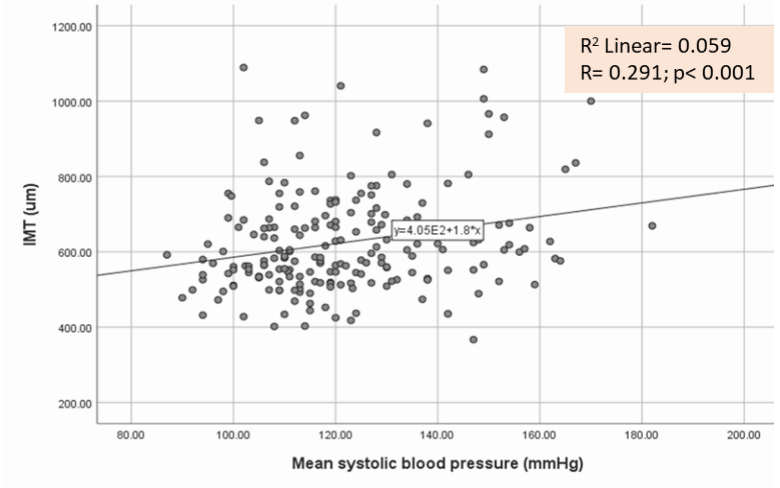


Table 4.21: Summary of significant correlations of variables with IMT.

Variables	Total cohort	HIV-free	HIV-infected
Continuous variables			
BMI (kg/m^2)	**R=0.183; p= 0.001**	**R= 0.294; p< 0.001**	R= 0.091, p= 0.20
Waist circumference (cm)	**R= 0.178; p= 0.001**	**R= 0.248; p= 0.003**	R= 0.116; p= 0.10
SBP (mmHg)	**R= 0.264; p< 0.001**	**R= 0.291; p < 0.001**	**R= 0.243; p< 0.001**
DBP (mmHg)	**R= 0.159; p= 0.003**	R= 0.143; p= 0.09	**R= 0.172; p= 0.014**
Total cholesterol (mmol/L)	**R= 0.165; p= 0.002**	**R= 0.210; p= 0.01**	R= 0.136; p= 0.05
Lipid LDL cholesterol (mmol/L)	**R=0.183; p= 0.001**	**R= 0.272; p= 0.001**	**R= 0.152; p= 0.030**
Baseline brachial artery diameter (mm)	**R= 0.189; p< 0.001**	R= 0.159; p= 0.06	**R= 0.212; p= 0.002**
Age (years)	**R= 0.423; p< 0.001**	**R= 0.411; p< 0.001**	**R= 0.439; p <0.001**
Categorical variables			
SBP categories	**p < 0.001**	**p= 0.01**	**p= 0.01**
DBP categories	**P= 0.03**	p= 0.23	p= 0.16
Age categories	**p < 0.001**	**P= 0.003**	**p <0.001**
BMI categories	**P = 0.01**	**p= 0.003**	**p= 0.01**
Waist circumference category	**p < 0.001**	**p= 0.001**	p= 0.09
Hypertension	**p= 0.01**	p= 0 .17	**p= 0.02**
ART status	-	-	**p= 0.004**
LDL categories	**p= 0.01**	**p= 0.01**	p= 0.19
Fasting glucose categories	**p= 0.05**	**p= 0.02**	**p= 0.05**
HbA1c categories	**p= 0.01**	**p= 0.03**	**p= 0.01**

R, Pearson correlation; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoproteins, BMI, body mass index; ART, antiretroviral treatment; HbA1c, Glycated Haemoglobin

Table 4.22: Correlations of biomarkers of inflammation and endothelial dysfunction with IMT.

Variables	Total cohort	HIV-free	HIV-infected
hsCRP (mg/L)	R= 0.058; p= 0.29	R= 0.100; 0.24	R= 0.056; p= 0.43
hsCRP categories [a] Elevated (> 3mg/L in HIV-free; > 3.3mg/L in HIV-infected), n (%)	p= 0.06	P= 0.13	p= 0.22
Tnf-α (pg/ml)	R= 0.029; p= 0.73	R= -0.111; p= 0.45	R= 0.079; p= 0.46
VEGF (pg/ml)	R= -0.073; p= 0.40	R= 0.113; p= 0.45	R= -0.123; p= 0.25
VCAM-1 (ng/ml)	R=0.074; p= 0.39	R= 0.032; p= 0.83	R= 0.099; p= 0.35
ICAM-1 (ng/ml)	R= -0.016; p= 0.85	R= 0.087; p= 0.56	R= -0.018; p= 0.87
E-selectin (ng/ml)	R= 0.128; p= 0.13	R= 0.031; p= 0.83	R= 0.175; p= 0.10
P-selectin (ng/ml)	R= 0.067; p= 0.43	R= 0.158; p= 0.28	R= 0.021; p= 0.84
PAI-1 (ng/ml)	R= 0.016; p= 0.85	R= 0.091; p= 0.54	R= -0.021; p= 0.84

R, Pearson correlation; [a] HsCRP categories based on guidelines by Ridker *et al.*, 2000 and De *et al.*, 2013, Vishwanath, Quaiser and Khan, 2016 for HIV-free population and HIV-infected population, respectively.

Variables that significantly correlated with IMT were shortlisted for generalized linear regression analysis to determine independent associations.

For the total population, HIV status was forced to remain in the model, and age categories, gender categories, systolic blood pressure categories, BMI categories and LDL cholesterol were adjusted for in the model. Age ≥ 50 years, high systolic blood pressure as well as obese BMI category and LDL cholesterol demonstrated a positive significant association with IMT (**refer to table 4.23a**).

For the HIV-free group, age categories, gender categories, systolic blood pressure categories, BMI categories and LDL cholesterol were adjusted for in the model. High systolic blood pressure as well as obese BMI category and LDL cholesterol demonstrated a significant positive association with IMT (**refer to table 4.23a**).

For the total HIV-infected group, HIV duration categories, ART status and CD4 count as well as viral load were forced to remain in the model. Age categories, gender, systolic blood pressure categories and LDL cholesterol were adjusted for in the model. Age ≥ 50 years demonstrated a significant positive association with IMT (**refer to table 4.23a**).

For HIV-infected group on ART, HIV duration categories, ART duration, CD4 count as well as viral load were forced to remain in the model. Age ≥ 50 years demonstrated a significant positive association with IMT (**refer to table 4.23b**).

Table 4.23a: Independent associations of various cardiovascular and HIV related factors with IMT.

Average IMT *Likelihood ratio Chi square*	**Total cohort** 66.185; p <0.001		**HIV-free group** 33.297; p <0.001		**HIV-infected group** 41.932; p <0.001	
	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
HIV status (yes)	10.85 (452.08; 566.14)	0.44	-	-	-	-
HIV duration: > 5 years	-	-	-	-	20.93 (-95.77; 137.63)	0.73
ART status (yes)	-		-	-	70.01 (-5.98; 145.99)	0.07
Age (years): ≥ 50 years	73.42 (40.91; 105.93)	**<0.001**	26.12 (27.07; -26.96)	0.34	99.32 (55.52; 143.12)	**<0.001**
Gender: female	-14.79 (-45.41; 15.83)	0.34	18.55 (-32.11; 69.21)	0.47	-14.27 (-51.79; 23.25)	0.46
Systolic blood pressure category:						
High (≥ 140 mmHg)	66.75 (30.39; 103.10)	**<0.001**	78.09 (19.97; 136.22)	**0.01**	40.58 (-8.75; 89.91)	0.46
Elevated (130- 139 mmHg)	6.53 (-34.80; 47.86)	0.76	-1.38 (-61.28; 58.52)	0.96	33.73 (-24.56; 92.02)	0.46
Normal (120- 129 mmHg)	16.22 (-19.45; 51.88)	0.37	3.71 (-53.22; 60.63)	0.90	11.46 (-35.28; 58.20)	0.46
BMI categories						
Obese (BMI > 30 kg/m^2), n (%)	56.28 (11.37; 101.19)	**0.01**	79.72 (4.35; 155.09)	**0.03**	-	-
Overweight (BMI 25 to < 30 kg/m^2)	34.51 (-11.57; 80.60)	0.14	50.71 (-31.75; 133.16)	0.23	-	-
Normal weight (BMI 18.5 to < 25 kg/m^2)	20.85 (-17.48; 59.18)	0.29	33.56 (-37.98; 105.11)	0.36	-	-
Low-Density Lipoprotein Cholesterol (LDL) (mmol/L)	25.09 (8.10; 42.09)	**0.004**	32.10 (6.90; 57.30)	**0.01**	12.02 (-11.15;35.19)	0.31
CD4 count (cells/mm^3)	-	-	-	-	0.04 (-0.04; 0.13)	0.30
Viral Load (copies mRNA/ml): high					-2.42 (-52.43; 47.60)	0.93

IMT, intima media thickness; β, partial regression coefficient; CI, confidence interval; BMI, body mass index; ART, antiretroviral treatment; HIV, human immunodeficiency virus. Associations were determined by generalized linear regression analyses. P < 0.05 is regarded as statistically significant.

Table 4.23b: Independent associations of various cardiovascular and HIV related factors with IMT in the HIV-infected on ART study population.

Average IMT *Likelihood ratio Chi square*	**HIV-infected +ART cohort** 35.507; p <0.001	
	β (95% CI)	p
HIV duration: > 5 years	-24.52 (-150.21; 101.17)	0.70
ART duration (weeks)	0.13 (-0.01; 0.26)	0.07
Age (years): ≥ 50 years	92.05 (47.64; 136.46)	**<0.001**
Gender: female	-8.40 (-47.05; 30.25)	0.67
Systolic blood pressure category:		
High (≥ 140 mmHg)	34.58 (-17.32; 86.47)	0.19
Elevated (130- 139 mmHg)	26.78 (-34.32; 87.88)	0.39
Normal (120- 129 mmHg)	13.91 (-33.05; 60.87)	0.56
Low-Density Lipoprotein Cholesterol (LDL) (mmol/L)	20.61 (-3.54; 44.77)	0.09
CD4 count (cells/mm^3)	0.05 (-0.03; 0.14)	0.23
Viral Load (copies mRNA/ml): high	-1.78 (-53.60; 50.05)	0.95

IMT, intima media thickness; β, partial regression coefficient; CI, confidence interval; BMI, body mass index; ART, antiretroviral treatment; HIV, human immunodeficiency virus. Associations were determined by generalized linear regression analyses. P < 0.05 is regarded as statistically significant.

4.4 Associations of various demographic and cardiovascular risk variables with FMD% in the total, HIV-free, and HIV-infected cohort.

To evaluate possible associations with FMD%, the Pearson's correlation test was conducted to determine the relationship between continuous variables and FMD% (**Refer to figure 4.5, 4.6, 4.7** for scatter plot graphs demonstrating significant correlations of selected continuous variables with FMD%; **refer to table 4.24** for a summary of correlations with FMD%). For categorical variables and their relationship with FMD%, one-way ANOVA was performed with Bonferroni post hoc test for four categories and t-test was performed for those variables with two categories (**Refer to table 4.24** for summary of significant categorical relationships with FMD%).

Biomarkers of inflammation and endothelial dysfunction did not significantly correlate with FMD% (**refer to table 4.25**). Additionally, there were no significant correlations noted between FMD% and IMT.

Figure 4.5: Scatter plot graphs of selected significant correlations of variables with FMD% in the total cohort.

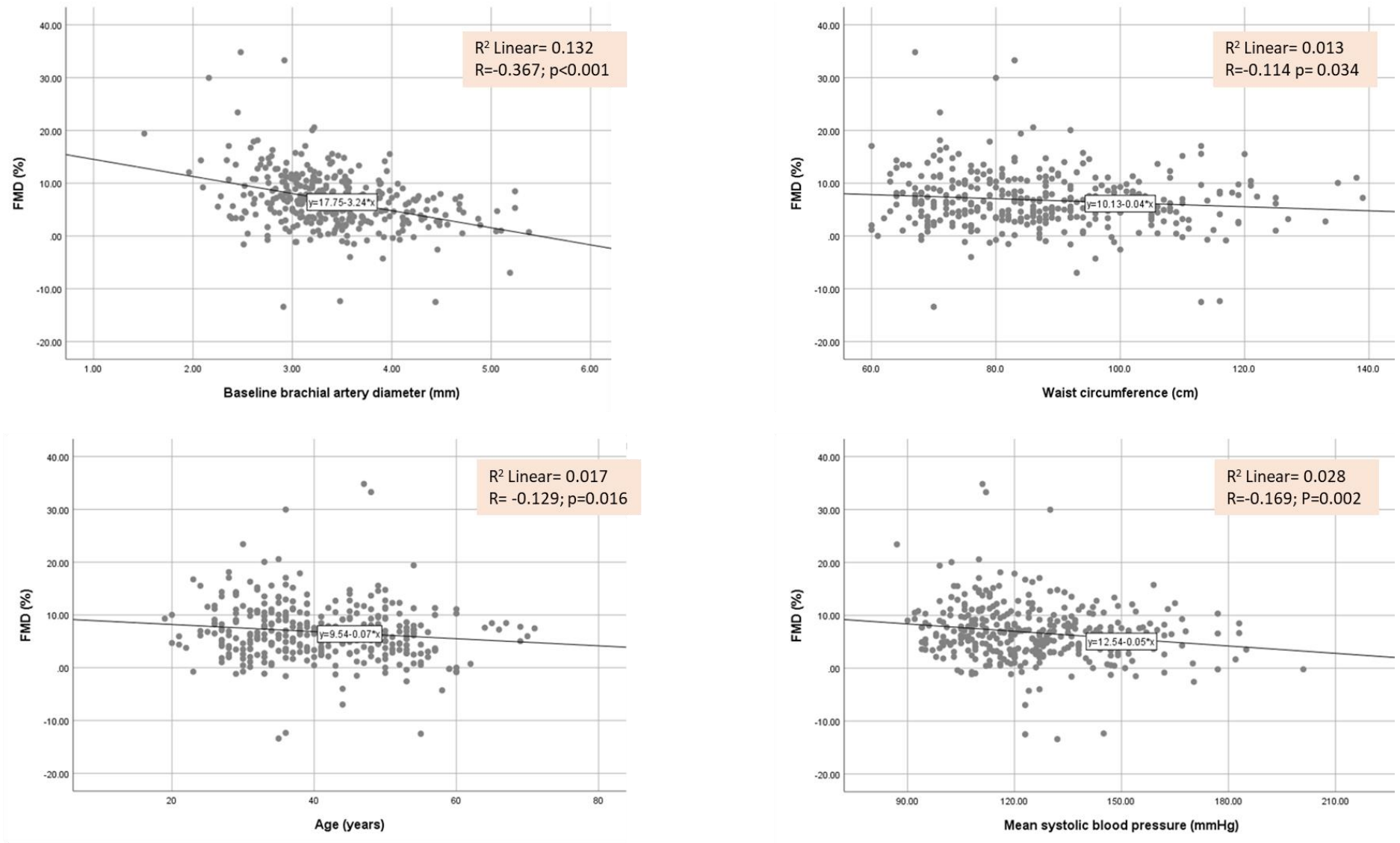


Figure 4.6: Scatter plot graphs of selected significant correlations of variables with FMD% in the HIV-free study group.

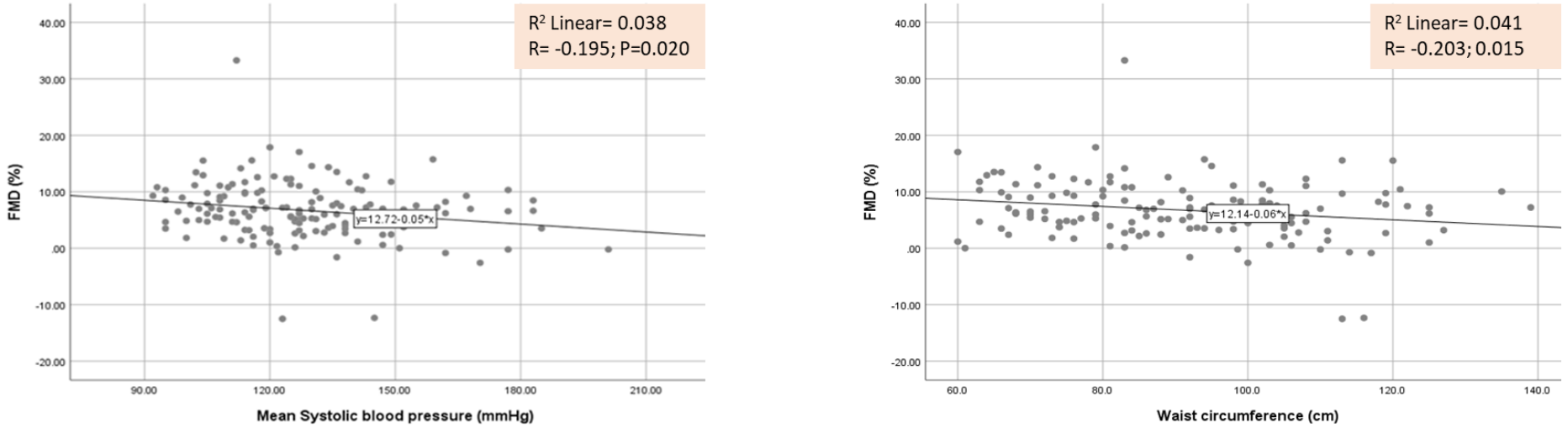


Figure 4.7: Scatter plot graphs of selected significant correlations variables with FMD% in the HIV-infected study group.

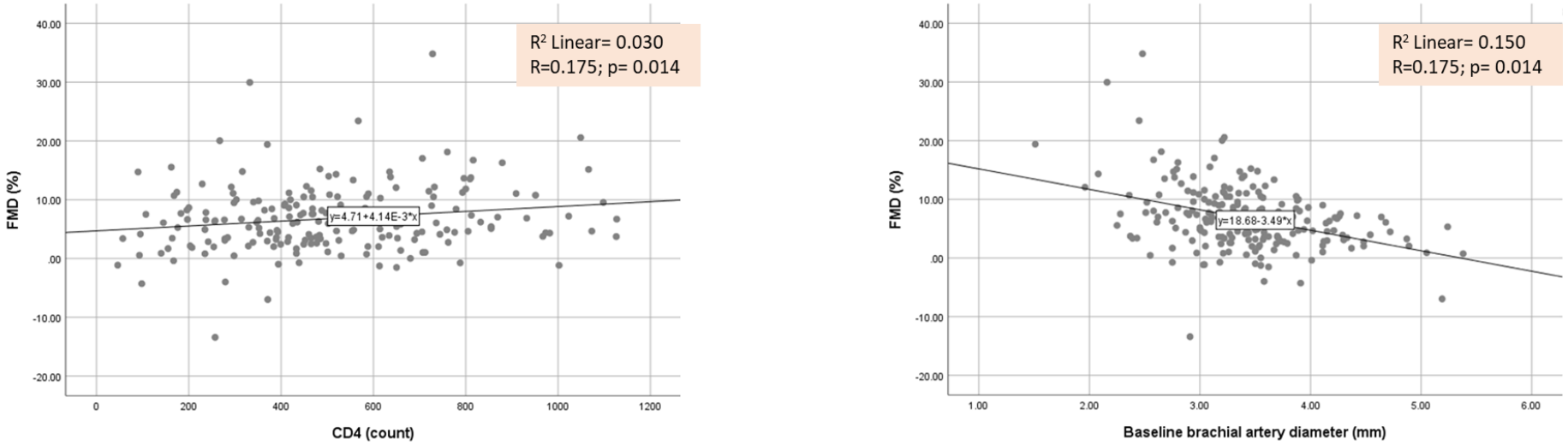


Table 4.24: Summary of significant correlations of variables with FMD%.

Variables	Total cohort	HIV-free	HIV-infected
Continuous variables			
BMI (kg/m^2)	R= -0.092; p= 0.09	**R= -0.210; p= 0.01**	R= -0.004; p= 0.96
Waist circumference (cm)	**R=-0.114; p= 0.03**	**R= -0.203; p= 0.02**	R= -0.045; p= 0.52
SBP (mmHg)	**R=-0.169; P=0.002**	**R= -0.195; P=0.02**	**R=0.152; p= 0.03**
Mean DBP (mmHg)	**R=-0.152; p= 0.01**	**R= -0.199; p= 0.02**	R= -0.118; p= 0.09
HBA1c %	**R=-0.110, p= 0.04**	R= -0.147; p= 0.08	R= -0.082; p= 0.25
CD4 (count)	**R=0.175; p= 0.01**	-	**R=0.175; p= 0.01**
Urine albumin (mg/L)	**R= -0.110; p= 0.04**	R= -0.018; p= 0.83	**R=0.175; p= 0.01**
Baseline brachial artery diameter (mm)	**R= -0.367; p<0.001**	**R=-0.334; p<0.001**	**R=-0.388; p< 0.001**
Maximum diameter (mm)	**R= -0.113; p= 0.04**	R=-0.068; p= 0.42	**R=-0.141; p= 0.04**
Recovery diameter (mm)	**R= -0.106; p= 0.05**	R= -0.092; p= 0.28	R=-0.113; p=0.11
Average carotid diameter (mm)	**R= -0.224; p <0.001**	**R= -0.333; p <0.001**	**R=-0.142; p= 0.04**
Age (years)	**R= -0.129; p=0.02**	R=- 0.161; p= 0.06	R=0.106; p= 0.13
Haemoglobin (g/dL)	R= -0.088; p= 0.10	**R=-0.212; p= 0.01**	R= -0.025; p= 0.73
Serum creatinine (µmol/L)	R= -0.101; p=0.06	**R=-0.283; p= 0.001**	R= -0.017; p= 0.81
Categorical variables			
Gender	**p= 0.02**	p= 0.06	p= 0.16
SBP categories	**p= 0.02**	P= 0.06	p= 0.06
DBP categories	**p= 0.03**	**p= 0.02**	p=0.95
Age categories	**p= 0.003**	**p= 0.01**	**p= 0.11**
BMI categories	p= 0.56	**p= 0.04**	p=0.92
GGT categories	p= 0.30	**P= 0.02**	**p= 0.01**
High Viral load	-	-	**p=0.002**
ART status	-	-	**p=0.01**

R, Pearson correlation; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoproteins, BMI, body mass index; ART, antiretroviral treatment.

Table 4.25: Correlations of biomarkers of inflammation and endothelial dysfunction with FMD%.

Variables	Total cohort	HIV-free	HIV-infected
hsCRP (mg/L)	R= -0.016; p= 0.77	R= 0.070; P= 0.41	R= -0.058; p= 0.42
hsCRP categories [a] Elevated (> 3mg/L in HIV-free; > 3.3mg/L in HIV-infected), n (%)	p= 0.69	p= 0.63	P=0.39
Tnf-α (pg/ml)	R= -0.031; p= 0.72	R= -0.151; p= 0.30	R= 0.011; p= 0.92
VEGF (pg/ml)	R= 0.053; p= 0.54	R= 0.108; p= 0.46	R= 0.044; p= 0.68
VCAM-1 (ng/ml)	R= -0.091; p= 0.29	R= 0.127; p= 0.38	R= -0.167; p=0 .11
ICAM-1 (ng/ml)	R= -0.051; p= 0.55	R= 0.010; p= 0.94	R= -0.096; p= 0.37
E-selectin (ng/ml)	R= -0.069; p= 0.42	R= -0180; p= 021	R= -0.018; p= 0.87
P-selectin (ng/ml)	R= 0.009; p= 0.92	R= 0.042; p= 0.77	R= -0.016; p= 0.88
PAI-1 (ng/ml)	R= -0.072; p= 0.40	R= 0.073; p= 0.62	R= -0.157; p= 0.14

R, Pearson correlation; [a] HsCRP categories based on guidelines by Ridker *et al.*, 2000, and De *et al.*, 2013, Vishwanath, Quaiser and Khan, 2016 for HIV-free population and HIV-infected population, respectively.

Variables that significantly correlated with FMD% were shortlisted for generalized linear regression analysis to determine independent associations. All regression models were repeated to observe associations when baseline brachial artery diameter is additionally adjusted for.

For the total population, HIV status was forced to remain in the model, and age categories, gender, systolic blood pressure categories, waist circumference, and urine albumin were adjusted for in the model. Age ≥ 50 years, high and normal systolic blood pressure as well as waist circumference and urine albumin demonstrated significant inverse associations with FMD%. Gender (female) demonstrated a significant positive association with FMD% (**refer to table 4.26a**). When brachial artery was adjusted for in the model, no significant associations were found (**refer to table 4.27a**).

In the HIV-free group, age categories, gender, systolic blood pressure categories, BMI categories and serum creatinine level were adjusted for in the model. Serum creatinine demonstrated a significant inverse association with FMD% (**refer to table 4.26a**). The significant association remained after adjusting for baseline brachial artery diameter (**refer to table 4.27a**).

In the total HIV-infected group, HIV duration categories, ART status and CD4 count as well as viral load were forced to remain in the model. Age categories, gender, systolic blood pressure categories and BMI categories were adjusted for in the model. Presence of ART showed significant positive association with FMD% and normal systolic blood pressure demonstrated a significant inverse

association with FMD (**refer to table 4.26a**). When brachial artery diameter was adjusted for in the model, presence of ART continued to demonstrate a significant positive association with FMD% while viral load demonstrated a significant inverse association with FMD% (**refer to table 4.27a**).

In the HIV-infected on ART group, HIV duration categories, ART duration, CD4 count as well as viral load were forced to remain in the model. Age categories, gender, systolic blood pressure categories and BMI categories were adjusted for in the model. Normal systolic blood pressure demonstrated a significant inverse association with FMD% (**refer to table 4.26b**). When brachial artery diameter was adjusted for in the model, viral load demonstrated a significant inverse association with FMD% (**refer to table 4.27b**).

Table 4.26a: Independent associations of various cardiovascular and HIV related factors with FMD%.

FMD% *Likelihood ratio Chi square*	**Total Cohort** 31.284; p <0.001		**HIV-free group** 28.335; p= 0.001		**HIV-infected group** 29.562, p= 0.009	
	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
HIV status (yes)	-0.14 (-1.21; 1.01)	0.81	-	-	-	-
HIV duration: > 5 years	-	-	-	-	-1.31 (-7.24; 4.62)	0.66
ART status	-	-	-	-	3.93 (0.43; 7.43)	**0.03**
Age (years): ≥ 50 years	-1.23 (-2.60; 0.14)	**0.08**	-1.02 (-3.01; 1.02)	0.33	-1.1 (-3.12; 0.92)	0.29
Gender: female	1.63 (0.37; 2.89)	**0.01**	1.41 (-0.95; 3.77)	0.24	1.03 (-0.75; 2.80)	0.26
Systolic blood pressure categories:						
High (≥ 140 mmHg)	-1.73 (-3.27; -0.20)	**0.03**	-2.02 (-4.25; 0.21)	0.08	-1.61 (-3.92; 0.69)	0.17
Elevated (130- 139 mmHg)	-.057 (-2.30; 1.16)	0.52	-0.60 (-2.91; 1.71)	0.61	0.32 (-2.42; 3.07)	0.82
Normal (120- 129 mmHg)	-1.75 (-3.26; -0.25)	**0.02**	-1.55 (-3.75; 0.65)	0.17	-3.27 (-5.43; -1.12)	**0.003**
Waist circumference (cm)	-0.04 (-0.08; -0.004)	**0.03**	-	-	-	-
Urine albumin (mg/L)	-0.01 (-0.013; 0.001)	**0.04**	-	-	-	-
Viral Load (copies mRNA/ml): high					-2.04 (-4.32; 0.24)	0.08
CD4 count (cells/mm^3)	-	-	-	-	0.001 (-0.002; 0.01)	0.47
BMI categories						
Obese (BMI > 30 kg/m^2), n (%)	-	-	-2.20 (-5.15; 0.752)	0.14	-1.03 (-3.80; 1.73)	0.46
Overweight (BMI 25 to < 30 kg/m^2)	-	-	-2.52 (-5.68; 0.65)	0.12	-0.04 (-2.59; 2.52)	0.98
Normal weight (BMI 18.5 to < 25 kg/m^2)	-	-	-0.31 (-3.07; 2.45)	0.83	-0.44 (-2.53; 1.65)	0.68
Serum creatinine (µmol/L)	-	-	-0.08 (-0.15; -0.01)	**0.03**	-	-

FMD, flow mediated dilatation; β, partial regression coefficient; CI, confidence interval; BMI, body mass index; ART, antiretroviral treatment; HIV, human immunodeficiency virus. Associations were determined by generalized linear regression analyses. $P < 0.05$ is regarded as statistically significant.

Table 4.26b Independent associations of various cardiovascular and HIV related factors with FMD% in the HIV-infected on ART study population.

FMD% *Likelihood ratio Chi square*	**HIV-infected + ART cohort** 23.012; p= 0.060	
	β (95% CI)	p
HIV duration: > 5 years	-2.48 (-8.98; 4.02)	0.46
ART duration (weeks)	0.002 [a] (-0.01; 0.01)	0.61
Age (years): ≥ 50 years	-1.21 (-3.27; 0.86)	0.25
Gender: female	0.93 (-0.92; 2.77)	0.33
Systolic blood pressure categories:		
High (≥ 140 mmHg)	-2.03 (-4.49; 0.43)	0.11
Elevated (130- 139 mmHg)	1.15 (-1.79; 4.08)	0.44
Normal (120- 129 mmHg)	-3.22 (-5.41; -1.04)	**0.004**
Viral Load (copies mRNA/ml): high	-2.12 (-4.51; 0.26)	0.08
CD4 count (cells/mm^3)	0.001[b] (-0.003[c]; 0.01)	0.55
BMI categories		
Obese (BMI > 30 kg/m^2), n (%)	-0.83 (-3.74; 2.09)	0.58
Overweight (BMI 25 to < 30 kg/m^2)	0.18 (-2.5; 2.86)	0.90
Normal weight (BMI 18.5 to < 25 kg/m^2)	0.02 (-2.25; 2.30)	0.98

FMD, flow mediated dilatation; β, partial regression coefficient; CI, confidence interval; BMI, body mass index; ART, antiretroviral treatment; HIV, human immunodeficiency virus. Associations were determined by generalized linear regression analyses. P < 0.05 is regarded as statistically significant.

Table 4.27a: Independent (after adjusting for brachial artery diameter) associations of various cardiovascular and HIV related factors with FMD%.

FMD% *Likelihood ratio Chi square*	**Total Cohort** 61.705; p <0.001		**HIV-free group** 32.956; p <0.001		**HIV-infected group** 56.310; p <0.001	
	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
HIV status (yes)	0.03 (-1.07; 1.13)	0.96	-	-		
HIV duration: > 5 years	-	-	-	-	-0.71 (-6.20; 4.79)	0.80
ART status	-	-	-	-	3.44 (0.19; 6.69)	**0.04**
Age (years): ≥ 50 years	-0.75 (-2.06; 0.57)	0.26	-0.50 (-2.53; 1.53)	0.63	-0.76 (-2.64; 1.12)	0.43
Gender: female	-0.437 (-1.84; 0.97)	0.54	0.449 (-2.03; 2.93)	0.72	-1.70 (-3.62; 0.22)	0.08
Systolic blood pressure categories						
High (≥ 140 mmHg)	-1.25 (-2.73; 0.23)	0.09	-2.07 (-4.26; 0.13)	0.07	-0.95 (-3.10; 1.20)	0.39
Elevated (130- 139 mmHg)	-0.58 (-2.23; 1.08)	0.49	-1.04 (-3.35; 1.27)	0.38	0.56 (-1.99; 3.10)	0.67
Normal (120- 129 mmHg)	-1.07 (-2.53; 0.39)	0.15	-1.54 (-3.70; 0.63)	0.16	-1.79 (-3.86; 0.28)	0.09
Waist circumference (cm)	-0.01 (-0.05; 0.03)	0.67	-	-	-	-
Urine albumin (mg/L)	-0.01 (-0.01; 0.00)	0.06	-	-	-	-
Baseline brachial artery diameter (mm)	-3.03 (-4.08; -1.98)	**<0.001**	-1.73 (-3.28; -0.17)	**0.03**	-4.08 (-5.57; -2.60)	**<0.001**
Viral Load (copies mRNA/ml): high	-	-	-	-	-2.21 (-4.32; -0.10)	**0.04**
CD4 count (cells/mm^3)	-	-	-	-	0.001 (-0.002; 0.01)	0.48
BMI categories						
Obese (BMI > 30 kg/m^2), n (%)	-	-	-1.19 (-4.23; 1.85)	0.44	0.01 (-2.58; 2.59)	0.10
Overweight (BMI 25 to < 30 kg/m^2)	-	-	-1.96 (-5.11; 1.20)	0.22	1.68 (-0.77; 4.13)	0.18
Normal weight (BMI 18.5 to < 25 kg/m^2)	-	-	-.015 (-2.75; 2.72)	0.99	0.44 (-1.53; 2.40)	0.66

Serum creatinine (µmol/L)	-	-	-0.07 (-0.138; 0.00)	**0.04**	-	-

FMD, flow mediated dilatation; β, partial regression coefficient; CI, confidence interval; BMI, body mass index; ART, antiretroviral treatment; HIV, human immunodeficiency virus. Associations were determined by generalized linear regression analyses. $P < 0.05$ is regarded as statistically significant.

Table 4.27b: Independent (after adjusting for brachial artery diameter) associations of various cardiovascular and HIV related factors with FMD% in the HIV-infected on ART study population.

FMD% *Likelihood ratio Chi square*	**HIV-infected + ART cohort** 53.084; p <0.001	
	β (95% CI)	p
HIV duration: > 5 years	-1.73 (-7.65; 4.20)	0.57
ART duration (weeks)	0.002 (-0.004; 0.01)	0.60
Age (years): ≥ 50 years	-0.85 (-2.74; 1.03)	0.38
Gender: female	-2.00 (-3.95; -0.05)	**0.05**
Systolic blood pressure categories		
High (≥ 140 mmHg)	-1.38 (-3.63; 0.87)	0.23
Elevated (130- 139 mmHg)	1.46 (-1.21; 4.13)	0.28
Normal (120- 129 mmHg)	-1.64 (-3.71; 0.42)	0.12
Baseline brachial artery diameter (mm)	-4.37 (-5.86; -2.88)	**<0.001**
Viral Load (copies mRNA/ml): high	-2.35 (-4.53; -0.17)	**0.03**
CD4 count (cells/mm^3)	0.001 (-0.003; 0.004)	0.67
BMI categories		
Obese (BMI > 30 kg/m^2), n (%)	0.27 (-2.42; 2.95)	0.85
Overweight (BMI 25 to < 30 kg/m^2)	2.03 (-0.50; 4.55)	0.12
Normal weight (BMI 18.5 to < 25 kg/m^2)	0.94 (-1.16; 3.03)	0.38

FMD, flow mediated dilatation; β, partial regression coefficient; CI, confidence interval; BMI, body mass index; ART, antiretroviral treatment; HIV, human immunodeficiency virus. Associations were determined by generalized linear regression analyses. P < 0.05 is regarded as statistically significant.

Section B: Results from longitudinal sub-study

4.5 Descriptive profile of the baseline population

4.5.1 Demographic profile, medical background, lifestyle and socioeconomic characteristics of the baseline population

For the longitudinal sub-study, 57 volunteering participants completed baseline and 12-month follow up visits. The mean age was 44.91 years, and 36.8% of the cohort was more than or equal to 50 years of age. The cohort was mostly represented by females (n= 50, 87.7%) and individuals who self-identified as being of mixed-race ancestry (n= 56, 98.2%). **Refer to table 4.28** for the baseline demographic profile of the cohort.

Table 4.28: Demographic profile of the study cohort at baseline.

Variable	Baseline characteristics
Age (years) (Mean ± SD)	44.91 ± 12.11
Age categories	
Age < 50, n (%)	36 (63.2%)
Age ≥ 50, n (%)	21 (36.8%)
Gender	
Female, n (%)	50 (87.7%)
Male, n (%)	7 (12.3%)
Ethnicity	
Mixed ancestry, n (%)	56 (98.2%)
African, n (%)	1 (1.8%)

Data presented as mean ± SD or n (% of group). n, number of participants; SD, standard deviation.

In the cohort, the most prevalent form of previous comorbidity was hypertension. History of all the other comorbidities was low (**Refer to table 4.29**). The current smoking status in the cohort was high (n= 40, 70.2%) while only 24.6 % of the participants reported to have never smoked before. Less than half (n= 24, 42.1%) of participants reported to have consumed alcohol in the last year prior to the baseline visit with almost all reporting a consumption frequency of less than 8 days per month (n= 23, 95.8%) (**Refer to table 4.29**).

Table 4.29: Medical background and lifestyle characteristics of the study cohort at baseline.

Variable	Baseline characteristics
History of comorbidities	
Previous TB n (%)	7 (12.3%)
Previous Stroke n (%)	2 (3.5%)
Type 2 Diabetes n (%)	4 (7%)
Previous hypertension	18 (31.6%)
Previous dyslipidaemia	11 (19.3%)
Previous heart disease	0 (0%)
Smoking	
Current smoker n (%)	40 (70.2%)
History of smoking n (%)	3 (5.3%)
No history of smoking n (%)	14 (24.6%)
Alcohol consumption	
Alcohol consumption in the past 12 months, n (%)	24 (42.1%)
< 8 days per month, n (%)	23 (95.8%)

Data presented as mean ± SD or n (% of group). n, number of participants; SD, standard deviation; TB, tuberculosis.

Approximately half of the study population was unemployed (n= 30, 52.6%) and those that were employed, most of them reported to be working part-time (n= 20, 35.1%). The majority of the participants (n= 34, 59.6%) reported to have a total household income between R1,000 - R4,999, with a substantial number of participants (n=19, 33.3%), reported to have a total household income of less than R1,000 (**Refer to table 4.30**).

Table 4.30: Socioeconomic characteristics of the study cohort at baseline.

Variable	Baseline characteristics
Employment status	
Unemployed, n (%)	30 (52.6%)
Employed full-time, n (%)	7 (12.3%)
Employed part-time, n (%)	20 (35.1%)
Monthly income	
< R1,000, n (%)	19 (33.3%)
R1,000 - R4,999, n (%)	34 (59.6%)
R5,000 - R9,999, n (%)	2 (3.5%)
R10,000 - R20,000, n (%)	1 (1.8%)

Data presented as mean ± SD or n (% of group). n, number of participants; SD, standard deviation.

4.5.2 Anthropometric characteristics of the baseline population

The mean BMI in the cohort was ~ 28.33 kg/m^2, which is in the overweight category according to the WHO guidelines. Additionally, the largest percentage of the cohort (n= 21, 36.8%) was in the obese category. Furthermore, the mean waist circumference was 89.93 cm and waist to hip ratio was 0.88, both of which falling under the high categories according to Alberti et al., 2009 (**Refer to table 4.31** for the composition of waist hip ratio and waist circumference categories).

Table 4.31: Anthropometric characteristics of the study cohort at baseline.

Variable	Baseline characteristics
BMI (kg/m^2)	28.33 ± 8.21
BMI categories [a]	
Underweight (BMI < 18.5 kg/m^2), n (%)	6 (10.5%)
Normal weight (BMI 18.5 to < 25 kg/m^2), n (%)	17 (29.8%)
Overweight (BMI 25 to < 30 kg/m^2), n (%)	13 (22.8%)
Obese (BMI > 30 kg/m^2), n (%)	21 (36.8%)
Waist circumference (cm)	89.93 ± 16.84
Waist circumference category [b]	
High (≥ 80/ ≥ 94 females/ males), n (%)	37 (64.9%)
Waist to hip ratio	0.88 ± 0.09
Waist to hip ratio categories [b]	
Low (≥ 0.80/ ≥ 0.95 females/ males), n (%)	13 (22.8%)
Moderate (0.81–0.85/0.96–1.0 females/ males), n (%)	14 (24.6%)
High (> 0.86/ > 1.0 females/ males), n (%)	30 (52.6%)

Data presented as mean ± SD or n (% of group). n, number of participants; SD, standard deviation; BMI, body mass index. [a] BMI category based on WHO. [b] Waist circumference and waist to hip ratio category based on guidelines by Alberti et al., 2009.

4.5.3 Blood pressure and heart rate measurements of the baseline population

The mean SBP and DBP (~ 125.98 mmHg; ~ 84.64 mmHg, respectively) in the cohort fell within the normal range according to Seedat and Rayner, 2013. About half of the cohort (n= 29, 50.9%) presented with low SBP, while less than half of the cohort (n= 20, 35.1%) presented with low DBP (**Refer to table 4.5** for the SBP and DBP categorical composition in the total cohort). Furthermore, less than half of the cohort (n=20, 35.1%) presented with hypertension according to guidelines by Seedat and Rayner, 2013 (**Refer to table 4.32**).

Table 4.32: Blood pressure and heart rate measurements of the study cohort at baseline.

Variable	Baseline characteristics
Systolic Blood Pressure (mmHg)	125.98 ± 19.78
Systolic blood pressure categories [a]	
Low (< 120 mmHg), n (%)	29 (50.9%)
Normal (120- 129 mmHg), n (%)	9 (15.8%)
Elevated (130- 139 mmHg), n (%)	4 (7.0%)
High (≥ 140 mmHg), n (%)	15 (26.3%)
Diastolic Blood pressure (mmHg)	84.64 ± 11.73
Diastolic blood pressure categories [a]	
Low (< 80 mmHg), n (%)	20 (35.1%)
Normal (80- 84 mmHg), n (%)	12 (21.1%)
Elevated (85- 89 mmHg), n (%)	9 (15.8%)
High (≥ 90 mmHg), n (%)	16 (28.1%)
Hypertension [a] (SBP > 140 mmHg or DBP >90 mmHg), n (%)	20 (35.1%)
Heart Rate (bpm)	70.96 ± 11.73

Data presented as mean ± SD or n (% of group). n, number of participants; SD, standard deviation; SBP, systolic blood pressure; DBP, diastolic blood pressure. [a] Systolic, diastolic blood pressure category, as well as hypertension cut off based on guidelines by Seedat and Rayner, 2013

4.5.4 Fasting lipid, glucose and HBA1c measurements of the baseline population

The mean total cholesterol, HDL level, LDL level as well as triglycerides, were all within the normal range according to guidelines by Seedat and Rayner, 2013 and Alberti et al., 2009. **Refer to table 4.33** for the mean values and categorical composition of the lipid profile.

Additionally, the median fasting glucose level as well as the HbA1c % (~ 4.9 mmol/L; ~ 5.4 %, respectively) were within the normal range according to guidelines by Seedat and Rayner, 2013 and Alberti et al., 2009. Furthermore, less than quarter of the study cohort presented with elevation in these variables (**refer to table 4.33**).

Table 4.33: Fasting lipid, glucose and HBA1c measurements of the study cohort at baseline.

Variable	Baseline characteristics
Lipid profile	
Total cholesterol	4.32 ± 0.70
Total cholesterol categories [a] Elevated (≥ 5 mmol/L), n (%)	8 (14.0%)
High-Density Lipoprotein Cholesterol (HDL) (mmol/L)	1.37 ± 0.30
HDL categories [a] Decreased (≤ 1.2/1.0 mmol/L females /males), n (%)	14 (24.6%)
Low-Density Lipoprotein Cholesterol (LDL) (mmol/L)	2.45 ± 0.67
LDL categories [a] Elevated (≥ 3 mmol/L), n (%)	13 (22.8%)
Triglycerides (mmol/L)	0.92 (0.67 - 1.43)
Triglycerides categories [a] Elevated (≥1.7 mmol/L), n (%)	6 (10.5%)
Fasting glucose and HbA1c	
Fasting glucose (mmol/L)	4.90 (4.35 - 5.60)
Fasting glucose categories [a] Elevated (≥ 5.6 mmol/L), n (%)	13 (22.8%)
Glycated Haemoglobin (HbA1c) (%)	5.40 (4.95 - 5.70)
HbA1c categories [a] Elevated (≥ 5.9%), n (%)	13 (22.8%)

Data presented as mean ± SD or n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. [a] Lipid profile and glucose and HbA1c % categories is based on guidelines by Seedat and Rayner, 2013 and Alberti *et al.*, 2009.

4.5.5 Haemoglobin and GGT measurements of the baseline population

The mean Hb level and the median GGT level in the cohort was within the normal range, according to guidelines by WHO and NHLS, South Africa, respectively. **Refer to table 4.34** for the categorical composition of these parameters in the cohort.

Table 4.34: Haemoglobin and GGT measurements of the study cohort at baseline.

Variable	Baseline characteristics
Haemoglobin (g/dL)	13.17 ± 1.22
Haemoglobin categories [a] Decreased (< 12.0/ 13.0 g/dL females/males), n (%)	12 (21.1%)
Liver function	
γ-Glutamyl transferase (U/L)	27.00 (17.50 - 40.00)

GGT categories [b] Elevated (≥ 40/ ≥ 60 U/L women/men), n (%)	14 (24.6 %)

Data presented as mean ± SD or n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. [a] Haemoglobin categories are based on WHO guidelines. [b] GGT categories are based on guidelines by NHLS, SA.

4.5.6 Kidney function measurements of the baseline population

The median ACR ratio in the cohort was 0.60, which is below the normal range according to Du Plessis, 2013. Furthermore, very few participants (n= 4, 7.0%; n= 2, 3.5%) presented with increased ACR ratio or decreased eGFR, respectively (**refer to table 4.35**).

Table 4.35: Kidney function measurements of the study cohort at baseline.

Variable	Baseline characteristics
Kidney function	
Serum creatinine (µmol/L)	63.95 ± 14.78
Urine albumin (mg/L)	6.80 (3.05 - 15.80)
Albumin-to-creatinine ratio (mg/mmol)	0.60 (0.31 - 1.30)
ACR categories [a] Increased (> 3 mg/mmol), n (%)	4 (7.0%)
eGRF (mL/minute/1.73 m3) categories [a] Decreased (< 60 mL/minute/1.73 m3), n (%)	2 (3.5%)

Data presented as mean ± SD or n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. [a] ACR and eGFR categories used as previously reported (Du Plessis, 2013).

4.5.7 Systemic inflammation and biomarker measurements of the baseline population

The mean hsCRP of the cohort was 5.20 mg/L, which falls within the elevated range according to guidelines by Ridker *et al.*, 2000. Additionally, more than half of the study cohort (n= 36, 63.2%) represented with elevated hsCRP. TNF-α, VEGF as well as adhesion molecules such as VCAM-1, ICAM-1, E-selectin, P-selectin and PAI-1 were all presented as median and are described in **table 4.36.**

Table 4.36: Systemic inflammation and biomarker measurements of the study cohort at baseline.

Variable	Baseline characteristics
hsCRP (mg/L)	5.20 (1.20 - 10.70)
hsCRP categories [a] Elevated (> 3mg/L), n (%)	36 (63.2%)

Tnf-α (pg/ml)	22.95 (20.07- 27.13)
VEGF (pg/ml)	98.75 (79.91- 146.12)
VCAM-1 (ng/ml)	727.45 (578.20 - 1041.60)
ICAM-1 (ng/ml)	415.94 (255.84 - 651.28)
E-selectin (ng/ml)	34.35 (28.27 - 46.48)
P-selectin (ng/ml)	36.87 (30.01- 45.97)
PAI-1 (ng/ml)	91.52 (77.32- 114.13)

Data presented as n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants. [a] HsCRP categories based on guidelines by Ridker *et al.*, 2000.

4.5.8 Endothelial function measurements of the baseline population

The mean baseline brachial artery diameter and FMD% is described in **table 4.37.**

Table 4.37: Endothelial function measurements of the study cohort at baseline.

Variable	Baseline characteristics
Baseline brachial artery diameter (mm)	3.41 ± 0.61
Flow-mediated dilatation (%)	5.72 ± 4.62

Data presented as mean ± SD. SD, standard deviation.

4.5.9 Subclinical atherosclerosis measurements of the baseline population

The mean IMT of the cohort was 651.10 µm, which is within the normal range according to guidelines supplied by the European Society of Cardiology and European Society of Hypertension (Mancia *et al.*, 2013). Subclinical atherosclerosis was present in n=2, 3.5% of the cohort and no participants presented with plaque (**refer to table 4.38**).

Table 4.38: Subclinical atherosclerosis measurements of the study cohort at baseline.

Variable	Baseline characteristics
Mean IMT (µm)	651.10 ± 124.70
Subclinical atherosclerosis (> 900 µm), n (%)	2 (3.5%)
Plaque (> 1000 µm), n (%)	0 (0%)

Data presented as mean ± SD or n (% of group). n, number of participants; SD, standard deviation

4.6 Differences in variables between baseline and 12-month follow up

As the study comprised of both males and females, additional gender subgroup analyses were conducted to note differences between the two time points (**refer to appendix C**).

4.6.1 Differences in anthropometric measurements between baseline and 12-month follow up

The cohort's mean BMI did not significantly change over the 12-month time. **Refer to appendix C, table C3,** for cross tabulation of BMI categories using McNemar-Bowker Test. The majority (n= 48) of the individuals did not change categories over time. n=7 moved to a higher BMI category, while n= 2, moved to a lower BMI category. There were no significant changes in the BMI categories between the two time points.

Although there were no significant changes in the waist circumference categories between the two time points, the mean waist circumference was significantly higher at 12-month follow up in comparison to baseline (p = 0.03). No significant differences in the mean waist to hip ratio were found between the two time points (**table 4.39**). With regards to waist to hip ratio categories, the majority (n= 41) of the participants did not change categories over time. N= 5 tended to move to a higher category, while n=11 moved to a lower category (**refer to appendix C, table C4** for cross tabulation for waist to hip ratio). There were no significant changes in the waist to hip ratio categories between the two time points.

Table 4.39: Differences in anthropometric measurements between baseline and 12-month follow up.

Variable	Baseline (time point 1)	12-month follow up (time point 2)	N	Difference (Time point 1- Time point 2)[a]	P value
BMI (kg/m2)	28.33 ± 8.21	28.51 ± 7.97	**54**	**-0.18**	**0.47**
BMI categories [b]	-	-	**57**	-	**0.34**
Waist circumference (cm)	89.93 ± 16.84	91.70 ± 17.43	**56**	**-1.77**	**0.03**
Waist circumference [c] **categories**	-	-	**57**	-	**0.29**
High, n (%)	37 (64.9%)	41 (71.9%)	-	**- 4 (11%)**	-
Waist to hip ratio	0.88 ± 0.09	0.87 ± 0.09	**56**	**0.01**	**0.35**
Waist to hip ratio [c] **categories**	-	-	**57**	-	**0.23**

Data presented as mean ± SD or n (% of group). n, number of participants; SD, standard deviation. [a] **Negative difference indicates an increase in the variable at 12-month follow up**. [b] BMI category based on WHO. [c] Waist circumference and waist to hip ratio category based on guidelines by Alberti *et al.*, 2009. For normally distributed data, a paired samples t-test was conducted and for comparison

between categories at the two time points, cross tabulation using the McNemar-Bowker test was conducted.

4.6.2 Differences in heart rate and blood pressure measurements between baseline and 12-month follow up

Mean systolic blood pressure was significantly higher at 12-month follow up in comparison to baseline ($p < 0.001$). Similarly, the mean diastolic blood pressure was also significantly higher at 12-month follow up in comparison to baseline ($p < 0.001$). Furthermore, at baseline, the mean values fell within the normal clinical range for both systolic and diastolic blood pressure according to guidelines by Seedat and Rayner, 2013, while at 12-month follow up, the mean values were in the elevated blood pressure range based on the same guidelines.

There were no significant changes between systolic blood pressure categories and diastolic blood pressure categories between the two time points. **Refer to appendix C, table C5 & C6,** for cross tabulation of systolic blood pressure and diastolic blood pressure categories using McNemar-Bowker Test. Both for the systolic and diastolic blood pressure, n= 27, did not change categories over time, while n= 9 changed to a lower category and n=21 moved to a higher category between time point 1 and 2.

There was a significant difference (p= 0.03) in the hypertension category between baseline and 12-month follow up. 50% more individuals presented with hypertension at 12-month follow up in comparison to baseline time point **(table 4.40**). No significant difference was observed in the heart rate measurements between the two time points.

Table 4.40: Differences in blood pressure and heart rate measurements between baseline and 12-month follow up.

Variable	Baseline (time point 1)	12-month follow up (time point 2)	N	Difference (Time point 1- Time point 2) [a]	P value
Systolic Blood Pressure (mmHg)	125.98 ± 19.78	132.26 ± 21.31	**56**	**-6.28**	**<0.001**
Systolic blood pressure categories [b]	-	-	**57**	-	**0.06**
Diastolic Blood pressure (mmHg)	84.64 ± 11.73	89.21 ± 13.59	**56**	**-4.57**	**<0.001**
Diastolic blood pressure categories [b]	-	-	**57**	-	**0.07**
Hypertension, n (%) [b]	20 (35.1%)	30 (52.6%)	**56**	**-10 (50.0%)**	**0.03**

Heart Rate (bpm)	70.96 ± 11.73	67.87 ± 11.82	**57**	**3.10**	**0.29**

Data presented as mean ± SD or n (% of group). n, number of participants; SD, standard deviation.

[a] **Negative difference indicates an increase in the variable at 12-month follow up**. [b] Systolic, diastolic blood pressure category, as well as hypertension cut off based on guidelines by Seedat and Rayner, 2013 category based on WHO. For normally distributed data, a paired samples t-test was conducted and for comparison between categories at the two time points, cross tabulation using the McNemar- Bowker test was conducted.

4.6.3 Differences in fasting lipid, glucose and HBA1c measurements between baseline and 12-month follow up

There were no significant differences in the lipid profile parameters between baseline and 12-month follow up time point. However, the median fasting glucose was significantly higher at 12-month follow up in comparison to baseline (p= 0.03). There were no significant differences in fasting glucose categories and HbA1c measurements (both continuous and categorical) between the two time points (**Table 4.41**).

Table 4.41: Differences in fasting lipid, glucose and HbA1c measurements between baseline and 12-month follow up.

Variable	Baseline (time point 1)	12-month follow up (time point 2)	N	Difference (Time point 1- Time point 2) [a]	P value
Lipid profile					
Total cholesterol (mmol/L)	4.32 ± 0.70	4.29 ± 0.88	**56**	**0.03**	**0.68**
Total cholesterol categories[b]	-	-	**56**	-	**1.00**
Elevated, n (%)	8 (14.0%)	9 (15.8%)	-	**-1 (12.5%)**	-
High-Density Lipoprotein Cholesterol (HDL) (mmol/L)	1.37 ± 0.30	1.35 ± 0.36	**56**	**0.02**	**0.42**
HDL categories[b] Decreased, n (%)	14 (24.6%)	19 (33.3%)	**57**	**-5 (35.7%)**	**0.27**
Low-Density Lipoprotein Cholesterol (LDL) (mmol/L)	2.45 ± 0.67	2.43 ± 0.81	**56**	**0.09**	**0.75**
LDL categories[b]	-	-	**56**	-	**0.45**
Elevated, n (%)	13 (22.8%)	10 (17.5%)	-	**3 (23.1%)**	-

Triglycerides (mmol/L)	0.92 (0.67 - 1.43)	1.07 (0.74-1.43)	**57**	**0.15**	**0.67**
Triglycerides categories[b]	-	-	**57**	-	**1.00**
Elevated, n (%)	6 (10.5%)	5 (8.8.%)	-	**1 (16.7%)**	-
Glucose and HbA1c					
Fasting glucose (mmol/L)	4.90 (4.35 - 5.60)	4.60 (4.30 - 5.10)	**57**	**-0.10**	**0.03**
Fasting glucose categories[b]	-	-	**57**	-	**0.44**
Elevated, n (%)	13 (22.8%)	6 (10.5%)	-	**7 (53.8%)**	-
Glycated Haemoglobin (HbA1c) (%)	5.40 (4.95 - 5.70)	5.50 (5.10-5.75)	**57**	**0.10**	**0.07**
HbA1c categories[b]	-	-	**57**	-	**0.12**
Elevated, n (%)	13 (22.8%)	8 (14.0%)	-	**5 (38.5%)**	-

Data presented as mean ± SD or n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. [a] **Negative difference indicates an increase in the variable at 12-month follow up**. [b] Lipid profile and glucose homeostasis categories is based on guidelines by Seedat and Rayner, 2013 and Alberti *et al.*, 2009. For normally distributed data, a paired samples t-test was conducted, for non-normally distributed data, Wilcoxon signed ranked test was conducted, and for comparison between categories at the two time points, cross tabulation using the McNemar-Bowker test was conducted.

4.6.4 Differences in Hb and GGT measurements between baseline and 12-month follow up

There were no significant differences in Hb and GGT measurements between the two time points of the study (**refer to table 4.42**).

Table: 4.42: Differences in Hb and GGT measurements between baseline and 12-month follow up.

Variable	Baseline (time point 1)	12-month follow up (time point 2)	N	Difference (Time point 1-Time point 2) [a]	P value
Haemoglobin (g/dL)	13.17 ± 1.22	13.19 ± 1.51	**56**	**-0.03**	**0.81**
Haemoglobin categories [b]	-	-	**57**	-	**0.73**
Decreased, n (%)	12 (21.1%)	14 (24.6%)	-	**-2 (16.7%)**	-
		Liver function			
GGT (U/L)	27.00 (17.50 - 40.00)	27.00 (18.50 - 38.50)	**57**	**0.00**	**0.62**
GGT categories [c]	-	-	**57**	-	**0.72**

Elevated, n (%)	14 (24.6 %)	12 (21.1%)	-	**2 (14.3%)**	-

Data presented as mean ± SD or n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. [a] **Negative difference indicates an increase in the variable at 12-month follow up**. [b] haemoglobin categories based on WHO guidelines and GGT categories are based on guidelines by NHLS, SA. For normally distributed data, a paired samples t-test was conducted, for non-normally distributed data, Wilcoxon signed ranked test was conducted, and for comparison between categories at the two time points, cross tabulation using the McNemar- Bowker test was conducted.

4.6.5 Differences in kidney function measurements between baseline and 12-month follow up

There were no significant differences in the kidney function measurements between baseline and 12-month follow up (**refer to table 4.43**).

Table 4.43: Differences in kidney function measurements between baseline and 12-month follow up.

Variable	Baseline (time point 1)	12-month follow up (time point 2)	N	Difference (Time point 1- Time point 2) [a]	P value
Serum creatinine (µmol/L)	63.95 ± 14.78	63.02 ± 15.85	**55**	**0.930**	**0.38**
Urine albumin (mg/L)	6.80 (3.05 - 15.80)	8.10 (3.10- 17.80)	**57**	**0.40**	**0.35**
Albumin-to-creatinine ratio (mg/mmol)	0.60 (0.31 - 1.30)	0.60 (0.40- 1.35)	**57**	**0.02**	**0.45**
ACR categories[b]	-	-	**57**	-	**0.23**
Increased, n (%)	4 (7%)	9 (15.8%)	-	**-5 (125%)**	-
eGRF categories [b]	-	-	**57**	-	**1.00**
Decreased, n (%)	2 (3.5%)	1 (1.8%)	-	**1 (50%)**	-

Data presented as mean ± SD or n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. [a] **Negative difference indicates an increase in the variable at 12-month follow up**. [b] ACR and eGFR categories used as previously reported (Du Plessis, 2013). For normally distributed data, a paired samples t-test was conducted, for non-normally distributed data, Wilcoxon signed ranked test was conducted, and for comparison between categories at the two time points, cross tabulation using the McNemar-Bowker test was conducted.

4.6.6 Differences in systemic inflammation and biomarker measurements between baseline and 12-month follow up

There were no significant differences in systemic inflammation and biomarker measurements between the two time points of the study (**table 4.44**).

Table 4.44: Differences in systemic inflammation and biomarker measurements between baseline and 12-month follow up.

Variable	Baseline (time point 1)	12-month follow up (time point 2)	N	Difference (Time point 1- Time point 2) [a]	P value
hsCRP (mg/L)	5.20 (1.20 - 10.70)	5.80 (1.30- 9.80)	**56**	**-0.50**	**0.20**
hsCRP categories[b]	-	-	**55**	-	**0.45**
Elevated, n (%)	36 (63.2%)	33 (57.9%)	-	**3 (8.3%)**	-
Tnf-α (pg/ml)	22.95 (20.07- 27.13)	22.72 (19.44- 28.08)	**39**	**-0.66**	**0.54**
VEGF (pg/ml)	98.75 (79.91- 146.12)	104.24 (70.38- 144.93)	**38**	**-6.27**	**0.11**
VCAM-1 (ng/ml)	727.45 (578.20 - 1041.60)	697.55 (555.01- 972.86)	**39**	**0.42**	**0.44**
ICAM-1 (ng/ml)	415.94 (255.84 - 651.28)	380.85 (253.96- 691.60)	**38**	**5.18**	**0.80**
E-selectin (ng/ml)	34.35 (28.27 - 46.48)	35.50(28.83- 48.33)	**39**	**1.85**	**0.70**
P-selectin (ng/ml)	36.87 (30.01- 45.97)	36.60 (27.68- 41.03)	**39**	**0.37**	**0.62**
PAI-1 (ng/ml)	91.52 (77.32- 114.13)	97.68 (78.47 - 121.46)	**39**	**1.90**	**0.66**

Data presented as mean ± SD or n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. [a] **Negative difference indicates an increase in the variable at 12-month follow up**. [b] HsCRP categories based on guidelines by Ridker *et al.*, 2000. For normally distributed data, a paired samples t-test was conducted, for non-normally distributed data, Wilcoxon signed ranked test was conducted, and for comparison between categories at the two time points, cross tabulation using the McNemar-Bowker test was conducted.

4.6.7 Differences in endothelial function measurements between baseline and 12-month follow up

There were no significant differences in FMD% between baseline and 12-month follow up (**table 4.45).**

Table 4.45: Differences in endothelial function measurements between baseline and 12-month follow up.

Variable	Baseline (time point 1)	12-month follow up (time point 2)	N	Difference (Time point 1-Time point 2) [a]	P value
Baseline brachial artery diameter (mm)	3.41 ± 0.61	3.41 ± 0.65	**54**	**0.00**	**0.89**
Flow-mediated dilatation (%)	5.72 ± 4.62	6.49 ± 6.19	**55**	**-0.78**	**0.36**

Data presented as mean ± SD or n (% of group). n, number of participants; SD, standard deviation. [a] **Negative difference indicates an increase in the variable at 12-month follow up**.

4.6.8 Differences in Subclinical atherosclerosis measurements between baseline and 12-month follow up

There were no significant differences in mean IMT or subclinical atherosclerosis between baseline and 12-month follow up (**table 4.46).** Two participants presented with plaque at 12-month follow up.

Table 4.46: Differences in subclinical atherosclerosis measurements between baseline and 12-month follow up.

Variable	Baseline (time point 1)	12-month follow up (time point 2)	N	Difference (Time point 1-Time point 2) [a]	P value
Mean IMT (µm) (mean ± SD)	651.10 ± 124.70	639.55 ± 169.09	**57**	**11.54**	**0.59**
Subclinical atherosclerosis, n (%) [b]	2 (3.5%)	2 (3.5%)	**57**	**0**	-
Plaque, n (%) [c]	0 (0%)	2 (3,5%)	**57**	**-2 (3.5%)**	-

Data presented as mean ± SD or n (% of group). n, number of participants; SD, standard deviation. [a] **Negative difference indicates an increase in the variable at 12-month follow up**. [b] Subclinical atherosclerosis cut off was based on guidelines by European Society of Cardiology and European Society of Hypertension. [c] Plaque was defined as an average IMT of more than 1000 µm, as suggested by Roozen *et al.*, 2020.

Chapter 5: Discussion

5.1 Introduction

This chapter evaluates the findings generated from the PhD study and critically analyses its implications and relevance to the PhD aims and objectives, as well as in the scientific research field applicable. Results from both the cross-sectional main study and the longitudinal sub-study will be argued in a combined and logical manner in this chapter.

5.2 Demographic, medical background and lifestyle related findings

The cross-sectional main study as well as the longitudinal sub-study were largely represented by participants who self-identified as being of mixed ancestry, which is plausible considering the geographical location of the studies. According to the Department of Statistics of South Africa, South Africans of mixed ancestry predominantly reside in the Western Cape Province (Statistics South Africa, 2012) and thus the cohort is reasonably representative of the demographic make-up of the region.

Additionally, both studies were represented mostly by females. According to literature, there are cultural as well as economical barriers related to gender in healthcare (Camlin *et al.*, 2016; Mbokazi *et al.*, 2020). For example, culturally, males often view 'care-seeking' as an activity for females and children, and clinics are said to be 'female spaces' by males (Camlin *et al.*, 2016). Economically, men are often more likely to be employed in the South African context and may find it difficult to go to the clinic and miss a day's work (Mbokazi *et al.*, 2020). Furthermore, literature suggests that generally males have been 'missing' from HIV testing and care (both prevention and treatment) (Cornell, Cox and Wilkinson, 2015; Mbokazi *et al.*, 2020). This could potentially be the reason why it was difficult to recruit more males for the current study. The study participants were recruited from local clinics by registered nurses. These clinics are often used more by females than males to access healthcare. Thus, generally, females were easier to recruit and available to volunteer for the assessments. Although no significant differences were noted in gender between the cross-sectional study groups of HIV-infected and HIV-free (**refer to chapter 4, table 4.1**), additional statistical subgroup gender analyses were conducted (**refer to appendix B**) to understand gender differences in the results found and this will be included in the discussion of the relevant findings in this chapter. Additionally, in all the independent association analyses, gender was always adjusted for. Overall, it is noteworthy that in all the cross-sectional study groups, there were no significant differences in the demographic data such as age, gender and ethnicity **(refer to chapter 4, table 4.1)**. This further ensured that the study groups were reasonably matched for the purposes of this PhD research.

With regards to medical background, the most prevalent form of previous comorbidity in the cross-sectional total cohort was TB (**refer to chapter 4, table 4.2**). Furthermore, the HIV-infected group had a significantly higher prevalence of previous history of TB in comparison to HIV-free group. The risk of developing TB in HIV is well known. Research confirms that HIV-infected individuals infected with TB are 20- 40 times at a higher risk at developing active TB than those that are HIV-free (Sandhu, 2011; Méda *et al.*, 2013). Additionally, TB is known to be the most common HIV-associated opportunistic disease globally, where it can poorly impact the HIV-infected individual by accelerating HIV disease progression and reducing ART efficacy (Lawn, 2005; Dagnra *et al.*, 2010). Hence, this finding in the cross-sectional main study is very much in line with existing research. It is further noteworthy that the burden of TB is particularly high in South Africa, where the country is one of the six countries contributing to 60% of the global TB burden (Naidoo *et al.*, 2017).

In the HIV-free group of the cross-sectional main study, as well as the longitudinal total study cohort (comprising of only HIV-free participants), a history of hypertension was the most prevalent form of reported comorbidities (**refer to chapter 4, table 4.2**). Research suggests that the incidence of hypertension is on the rise in SSA and this could be as a result of urbanization and westernization in this region (Seedat, Ali and Ferdinand, 2018). Furthermore, in South Africa, hypertension prevalence rates of up to 60% have been reported in studies (Gaziano *et al.*, 2017). Interestingly, in the cross-sectional main study, the prevalence of hypertension was the second most common form of comorbidity in the HIV-infected group (**refer to chapter 4, table 4.2**). Previous studies have shown that hypertension is increasingly observed in HIV-infected individuals (Medina-Torne *et al.*, 2012), however, in the current study, the HIV-infected group demonstrated a significantly lower prevalence in comparison to HIV-free group (~14% vs. ~23%) (**refer to chapter 4, table 4.2**). Global data suggest that 35% of all HIV-infected individuals on ART have hypertension in comparison to an estimated 30% of HIV-free individuals (Fahme, Bloomfield and Peck, 2018). Furthermore, HIV-infected individuals on ART are found to be at a higher risk of hypertension in comparison to those that are HIV-free (Fiseha *et al.*, 2019). However, the PI class of ART has been largely implicated in the development of hypertension (Fahme, Bloomfield and Peck, 2018), and it is noteworthy, that the majority of the HIV-infected participants in the current study were reported to be receiving NRTI / NNRTI-containing ART. Additionally, it is interesting to note that studies also suggest that the presence of hypertension is often under-diagnosed in people living with HIV (Fiseha *et al.*, 2019), which should be considered with regards to the findings of the present study.

In the cross-sectional main study and the longitudinal sub-study, the prevalence of smoking was high (~59% and ~70%, respectively). The prevalence rates observed in the present cohort are high compared to the prevalence of approximately 20% reported in general South African population (World Health Organization, 2018). Although smoking prevalence has declined country-wide over the years, disparities in smoking based on gender, ethnic background and socioeconomic status persist (Reddy *et*

al., 2013; Lau *et al.*, 2018). Particularly, the prevalence of both smoking and alcohol consumption is found to be relatively higher in the lower socioeconomic status groups (Lau *et al.*, 2018). Both the cross-sectional and longitudinal studies presented with participants from a lower socioeconomic status, for example, more than half of the study population reported to be unemployed and had a household income below R4,999 per month (**refer to chapter 4, table 4.3**). Both the employment status as well as household income are measures of socioeconomic status of an individual.

Interestingly, there were no significant differences in smoking between HIV-infected and HIV-free groups of the cross-sectional main study (**refer to chapter 4, table 4.2**). Literature suggests that HIV-infected individuals are more likely to smoke in comparison to the HIV-free individuals (Jackson-Morris, Fujiwara and Pevzner, 2015; Okello *et al.*, 2020). Furthermore, a study from South Africa, reports an alarmingly high prevalence of smoking in people living with HIV (Elf *et al.*, 2018). However, contradicting the said study, another South African study reports a relatively low prevalence of smoking in HIV-infected population (Waweru *et al.*, 2013). However, it is important to note that the current study did not clinically validate tobacco consumption; for instance, no urine cotinine measurements were conducted. All lifestyle related questions were self-reported, and the possibility of under-reporting of lifestyle parameters need to be considered. Overall, there were no significant differences between smoking, alcohol consumption or socioeconomic characteristics between the HIV-infected and HIV-free cross-sectional study groups (**refer to chapter 4, table 4.2 & 4.3**).

5.3 HIV- related and ART descriptive findings from the cross-sectional main study

Although the latest guidelines from the WHO and the South African National Department of Health recommend the initiation of ART immediately after HIV diagnosis (WHO, 2016; South African National Department of Health, 2019), not all the HIV-infected participants in the present study reported to be receiving ART at the time of data collection (2017-2018). This is concerning but can be justified by the fact that the HIV ART-naïve participants were still transitioning from the previous HIV treatment guidelines (that used CD4 count cut-off values to determine commencement of ART) to the revised treatment guidelines at the time of data collection. South Africa is known to have the largest ART roll-out program globally, however as of 2020, only 70% of HIV-infected individuals are receiving ART. With regards to the 90-90-90 target set by UNAIDS, which aims to provide ART for 90% of those diagnosed with HIV, unfortunately, the country is yet to achieve this goal (UNAIDS, 2020). The current PhD study included the group of HIV-infected participants not receiving ART. Although a relatively small group of participants was not receiving ART, statistically, in the majority of the comparisons, there were not only significant statistical differences but also clinical differences in several important parameters between the HIV-infected on ART vs HIV-infected without ART groups. Furthermore, the

statistical effect sizes for many of the parameters were large enough (above the cut off values as suggested by Lakens, 2013), which further validated the results found. **Refer to appendix D, table D1** for the Cohen's D effect size categories used to validate the results when comparing the HIV-infected receiving ART group with HIV-infected without ART group.

As expected, in the cross-sectional main study, the HIV-infected group receiving ART had a significantly lower viral load and a higher CD4 count in comparison to the HIV-infected group without ART (**refer to chapter 4. Table 4.4**). It is well known that ART acts on various stages of the HIV life cycle and inhibits key steps in viral replication which ultimately reduces viral load (as measured clinically) (Arts and Hazuda, 2012). Furthermore, it is known that HIV gp120 attacks and binds to the CD4 receptor in the HIV replication cycle (Arts and Hazuda, 2012). In a way, measurement of CD4 count can represent the extent of immune deficiency and declines in CD4 count are a measure of disease progression (Martinson *et al.*, 2014). Literature confirms that ART can reduce viral load (even achieve viral suppression) and increase CD4 count in majority of the patients (Autran *et al.*, 1997; Wilson and Sereti, 2013). Furthermore, research from South Africa suggests that CD4 count in the HIV-infected individual without ART will decline over time (Martinson *et al.*, 2014).

Additionally, as expected, there was a significantly higher prevalence of participants with high viral load measurements (> 1000 mRNA/ml) in the HIV-infected group without ART (~81%) in comparison to the group receiving ART (~17%) (**refer to chapter 4, table 4.4**). It is well known that without ART, viral load will increase over time as a result of the HIV replication cycle. Furthermore, the WHO classifies viral load above 1000 mRNA/ml as virologic failure, which generally implies ART failure (WHO, 2019). In the present cohort, ~17% of the HIV-infected receiving ART group demonstrated high viral load/ virologic failure. This could mean that there is presence of either drug resistance, or toxicity or poor adherence to ART among some participants in our study population. Research suggests that virologic failure on first-line regimens (i.e. NRTI/ NNRTI-containing first line ART) largely occurs due to pre-existing (transmitted) drug resistance or due to poor adherence (Usach, Melis and Peris, 2013). Literature has consistently suggested socio-economic variables such as unemployment, poverty, food insecurity, and transport costs as culprits of poor adherence in HIV (Tuller *et al.*, 2010; Weiser *et al.*, 2010). Furthermore, in the South African context, financial constraints, substance abuse, alternative therapy use, as well as stigma of HIV have known to obstruct adherence (Azia and Mukumbang, 2016).

5.4 Cardiovascular risk profile findings

Anthropometric characteristics

In the HIV-free group of the cross-sectional main study, as well as the longitudinal total study cohort (comprising of only HIV-free participants), the mean and median BMI, respectively, fell within the

overweight range according to WHO criteria (**refer to chapter 4, table 4.5 for cross-sectional data; table 4.31 for longitudinal data**). This is in line with the current weight and obesity trends in South Africa. Due to epidemiological transition, there is an increase in prevalence of obesity nationwide. In fact, South Africa is known to have the highest prevalence of obesity in SSA (Ramlal and Govender, 2016). Interestingly, when gender analysis was conducted (**refer to appendix B, table B3**), the males in the HIV-free cohort of the cross-sectional study presented with a normal mean BMI, while in the females, the mean BMI fell within the overweight BMI range. Furthermore, females demonstrated a significantly higher mean BMI in comparison to men in the HIV-free group of the cross-sectional study. This finding too, is in line with literature. Existing research indicates that 68% of females and 31% of males are overweight or obese based on BMI categories in South Africa (South Africa demographic and health survey, 2016). The higher prevalence of overweight or obesity in females in comparison to males in the South African context is further consistent with results from other low and middle-income countries (Kruger, 2018).

In the longitudinal sub-study, the mean waist circumference of the HIV-free participants was significantly higher at 12-month follow up in comparison to baseline (**refer to chapter 4, table 4.39**). This is in line with the current obesity and weight trends across the world. Particularly in South Africa, literature suggests that obesity prevalence is likely to increase in the country due to the continued epidemiological transition (Cois and Day, 2015). Interestingly, in the current study's gender subgroup analysis (**refer to appendix C, table C2**), female participants demonstrated a borderline significant (p= 0.053) increase in waist circumference at 12-month follow up. Males showed no significant differences between the two time points (**refer to appendix C, table C1**). This is further in line with existing literature where, globally, females are increasingly gaining weight more rapidly than males (Cois and Day, 2015). Several factors can contribute to this, including socioeconomic, behavioural drivers as well as the effects of menopause, which can impact metabolism and body fat which can promote weight gain (Westerman, Engberding and Wenger, 2015). It is noteworthy that majority of the longitudinal sub-study cohort comprised of females. Thus, although the overall cohort included both male and females, it is likely that our overall anthropometric findings are largely driven by the female group (as demonstrated by gender subgroup analysis).

In the cross-sectional study, the HIV-infected group had significantly lower mean BMI and waist circumference values in comparison to the HIV-free group (**refer to chapter 4, table 4.5**). This is in line with some previous research findings from SSA and South Africa, where HIV has been shown to be associated with a lower BMI and a lower prevalence of obesity and overweight (Malaza, Mossong *et al.*, 2012, Bärnighausen, Welz *et al.*, 2008, Manne-Goehler *et al.*, 2017). However, a large body of literature points to the emergence of increased obesity and visceral adiposity in HIV-infected populations. Previously, HIV infection was known to be riddled with under-nutrition, weight loss, loss of lean body mass and micronutrient deficiencies (Lemmer, Badri *et al.*, 2011). Clinically, however, it

is important to note that the current study's HIV-infected group presented with a mean BMI (~22.3 kg/m^2) that fell within the normal weight range. However, despite this, 22% of the HIV-infected participants presented with a BMI in the underweight category, suggesting that one in five of the participants may have shown signs of HIV-related wasting effects.

On the other hand, approximately 35% of the HIV-infected participants were in the overweight or obese category. Furthermore, 49% of the HIV-infected participants presented with visceral obesity as measured by waist circumference. This is an interesting finding, since increased body fat composition measures are often associated with certain ART drug classes. In particular, PI-based ART drug formulations have been shown to have adverse effects such as weight gain, visceral adiposity and eventually lipodystrophy (Price *et al.*, 2018; Okello *et al.*, 2020). However, in the current cohort the majority of the treated participants were receiving NRTI/ NNRTI containing ART, which is not readily known to be associated with abnormal adiposity or obesity related effects (Godfrey *et al.*, 2019). It has to be noted that some research findings do point to weight gain with NRTI/ NNRTI containing ART, especially the second generation NNRTI such Rilpivirine (Sax *et al.*, 2020). However, none of the current study participants did not report to be receiving any second generation NNRTI. Thus, it can be speculated that the overweight and obesity findings in our HIV-infected participants may have been mainly due to lifestyle factors (resembling the general population), and were unlikely the result of ART. Additionally, when conducting statistical analysis in the female and male sub-populations separately (**refer to appendix B, table B2**), only females demonstrated evidence of underweight (significantly lower BMI and waist circumference) in the HIV-infected group compared to the HIV-free group. This finding is in line with another study from South Africa, which noted that lower BMI and prevalence of overweight and obesity is more pronounced in HIV-infected females in comparison to HIV-free females (Malaza *et al.*, 2012). Additionally, in the current study, there were no differences in BMI and waist circumference between the two groups in the male population (**refer to appendix B, table B1**), which further suggests that the body composition related anthropometric findings in the HIV-infected cohort may have been largely driven by the female participants.

In the HIV sub-group analyses, the HIV-infected group receiving ART demonstrated a significantly higher mean BMI and waist circumference in comparison to HIV-infected without ART group (**refer to chapter 4, table 4.6**). Once again, it is important to note that, clinically, both parameters fell within the normal ranges according to WHO criteria for BMI and according to waist circumference cut-off values suggested by Alberti et al., 2009. Furthermore, interestingly, 37% of the HIV-infected without ART group presented with a BMI in the underweight category in comparison to 20% in the HIV-infected receiving ART group. This could be due to the HIV associated wasting syndrome, which is a well-known effect of the virus and was common in the pre-HAART era. In fact, initially, wasting was the AIDS defining diagnosis (Weiss *et al.*, 1993). Wasting in HIV is characterized by a loss of fat mass and lean body mass. Since the advent of HAART, the incidence of wasting has reduced remarkably

(Smit *et al.*, 2002; Erlandson *et al.*, 2015). It is well-established that ART can reverse HIV-related weight loss and wasting as well as improve overall health (Coodley, Loveless and Merrill, 1994; Malaza *et al.*, 2012). Thus, it can be speculated that the 20% of the HIV-infected receiving ART group, which presented with a BMI in the underweight category are likely the proportion which also presented with virologic failure as discussed in earlier sections. This further points to the presence of poor adherence or drug resistance in some of the ART receiving group. In the literature, several studies suggest that initiation of ART is associated with weight gain. Some of that weight gain following ART initiation may be attributed to a "return to health" phenomenon; however, excessive weight gain can also occur depending on the type of ART and other patient factors (McComsey *et al.*, 2016; Lake, 2017).

Blood pressure and heart rate measurements

In the longitudinal sub-study, blood pressure (both SBP and DBP) was significantly higher at 12-month follow up in comparison to baseline (**refer to chapter 4, table 4.40**). Additionally, at baseline, mean blood pressure values (both SBP and DBP) fell within the normal clinical range, while at 12-month follow up, the values were in the elevated blood pressure range. Furthermore, 50% more individuals than in baseline presented with hypertension at 12-month follow up (**refer to chapter 4, table 4.40**). These findings are mostly in agreement with the current blood pressure and hypertension trends present in low and middle-income countries such as South Africa. Countries in this region are experiencing a constant increase in mean blood pressure over time (Cois and Ehrlich, 2018). This can be attributed to rapid economic development, urbanization and adoption of westernized lifestyles which often accompanies low levels of exercise, increased consumption of salt-rich, processed, and energy-dense food (Ibrahim and Damasceno, 2012; Cois and Ehrlich, 2018). Contradicting this, some literature also suggests that South Africa, may in fact be in full demographic and epidemiological transition (Kahn, 2011), and there may actually be a decrease in blood pressure trends (Cois and Ehrlich, 2018). However, more recent longitudinal studies suggest otherwise, such as the study by Davids et, al., 2019, which concluded that there is a significant increase in blood pressure over 7 years in the mixed-race ancestry population of South Africa (Davids *et al.*, 2019). It is notable, that in the current cohort, there was also an increase in visceral obesity as shown by waist circumference measurements (**refer to chapter 4. Table 4.39**). There is general agreement in the literature that an interaction exists between blood pressure and obesity, with a particularly strong relationship between increased waist circumference and blood pressure (Siani *et al.*, 2002). Interestingly, as was shown with the waist circumference findings in this cohort, the blood pressure findings were also more relevant to the females. In gender subgroup analysis (refer to **appendix C, table C2**), females demonstrated a significantly higher blood pressure (both SBP and DBP) at 12-month follow up, which was not observed in the males. Previously, studies have demonstrated that males are more prone to increased blood pressure, however menopausal and post-menopausal changes in females may increase blood pressure even higher than males (Reckelhoff,

2001; Everett *et al.*, 2015). Female reproductive hormones are implicated in this phenomenon (Everett *et al.*, 2015). In the current cohort, however, it can be speculated that in addition to menopause (mean age of the female participants was approximately 45 years), increase in waist circumference may have influenced blood pressure or vice versa.

In the cross-sectional study, mean blood pressure values (both SBP and DBP) were found to be significantly lower in the HIV-infected group in comparison to HIV-free group (**refer to chapter 4, table 4.7**). Additionally, there was significant difference in DBP categories between HIV-infected and HIV-free group. More HIV-infected individuals were found to have low DBP. This contradicts the literature as studies suggest that HIV-infected individuals have a higher prevalence of diastolic dysfunction which can influence the DBP. Although it is noteworthy that ART plays a role in this mechanism (Hsue, *et al.*, 2010). Furthermore, the overall prevalence of hypertension was significantly lower in the HIV-infected group (~29%) versus the HIV-free group (~46%). Existing literature suggests that HIV-infected individuals on ART, specifically, have higher rates of hypertension in comparison to HIV-free (Diouf *et al.*, 2012; Sachithananthan, Loha and Gose, 2013; Fiseha *et al.*, 2019). However, in the current study's HIV sub-group analyses, no significant differences were found between the HIV-infected receiving ART group in comparison to HIV-infected without ART group (**refer to chapter 4, table 4.8**). With regards to the type of treatment, PIs have particularly been implicated in the pathogenesis of hypertension due to their mechanistic effect on RAAS activation, endothelial dysfunction, arterial stiffness and dyslipidaemia (Fahme, Bloomfield and Peck, 2018; Rodolphe Thiébaut, Wafaa M El-Sadr, Nina Friis-Møller Rickenbach, Martin *et al.*, 2005). Rather, fewer studies suggest that use of NNRTI-containing HAART is associated with elevation of both SBP and DBP in HIV-infected individuals (Chow *et al.*, 2003). Thus, the findings of the present study could be attributed to the type of treatment as the majority of our participants received NRTI/ NNRTI ART. Interestingly, studies also suggest that an increase in blood pressure in HIV-infected populations is often related to other cardiovascular risk factors such as elevated BMI (Baekken *et al.*, 2008; Medina-Torne *et al.*, 2012). Since our HIV-infected group did not present with any other elevated cardiovascular risk factors compared to their HIV-free counterparts, the blood pressure related findings appear to be plausible. On the other hand, the HIV-free group has consistently presented with higher cardiovascular risk related findings (discussed in earlier sections and will also be discussed further). For instance, the prevalence of hypertension was significantly higher in the HIV-free group in comparison to HIV-infected group.

Finally, in the cross-sectional study, the HIV-infected group demonstrated a significantly higher heart rate in comparison to HIV-free group (**refer to chapter 4, table 4.7**). Interestingly, HIV is known to affect the cardiac autonomic function (Benseñor *et al.*, 2011). Studies have evaluated heart rate variability in HIV patients with most suggesting a decreased variability (Godijk *et al.*, 2020). The current PhD finding, although interesting, is difficult to explain and falls outside the scope of the study.

Fasting lipid, glucose and HBA1c measurements

In the longitudinal study, there were no differences in the lipid profile measurements between the two time points (**refer to chapter 4, table 4.41**). Additionally, the mean values of all the lipid parameters fell within the normal range. Interestingly, this same cohort demonstrated no changes in BMI at 12-month follow up, which in the literature has been found to associate with lipids (Shamai *et al.,* 2011). Thus, the current finding is plausible and can be expected. However, there was a significant increase in fasting glucose levels at 12-month follow up in comparison to baseline (**refer to chapter 4, table 4.41**). Literature has long suggested that on average fasting blood glucose levels increase over time and with age (O'Sullivan, 1974). Although, clinically, it is important to note that the current study's fasting glucose level were in the normal range at both time points, the effect of change in blood glucose levels in the literature has been associated with increased cardiovascular risk in healthy individuals (Jin *et al.*, 2017). For example, a study suggests that increasing fasting glucose in a non-diabetic population is associated with risks of CVD such as MI and stroke (Lee *et al.*, 2018). Additionally, in the literature there is a particularly stronger link between visceral fat (measured by waist circumference) and insulin resistance (known to cause increased blood glucose levels) (Wahrenberg *et al.*, 2005). To this end, the current cohort also demonstrated a significant increase in waist circumference at 12-month follow-up. Thus, it can be speculated that increase in waist circumference may have influenced the increasing fasting glucose or vice versa.

In the cross-sectional study, there were no significant differences in any of the lipid parameters between the HIV-infected and HIV-free groups (**refer to chapter 4, table 4.9**). However, when the HIV group was further analysed in terms of ART treatment status, the HIV-infected group receiving ART demonstrated significantly higher total cholesterol, LDL cholesterol as well as HDL cholesterol levels, in comparison to HIV-infected without ART group (**refer to chapter 4, table 4.10**). In the literature, it is well known that ART can increase total cholesterol, LDL-cholesterol, and triglycerides (Hemkens and Bucher, 2014). It is further known that the type of ART and drug classes may impact the extent of lipid changes. Drugs from the NRTI class are particularly known to increase total and LDL cholesterol (Sax et al., 2009; Hemkens and Bucher, 2014). In the literature, ART is also known to lower HDL-cholesterol (Hemkens and Bucher, 2014). In contrast, the current study showed a higher mean HDL level in the HIV-infected group receiving ART compared to the untreated group. Furthermore, the prevalence of participants with clinically low HDL levels was higher in the untreated group. Studies have observed a decrease in total, HDL and LDL cholesterol in untreated HIV individuals (Riddler *et al.*, 2003; Hemkens and Bucher, 2014; Feeney, 2011; Baza *et al.*, 2007; Njoroge *et al.*, 2017). The HI virus itself is implicated in inducing these effects through persistent inflammation and immune activation as well as through its increased thrombotic activity (Hemkens and Bucher, 2014; Beltrán *et al.*, 2015). Particularly, the function of cholesterol ester transfer protein (CETP) which is known to transfer cholesterol esters from HDL-cholesterol to apolipoprotein-B containing proteins, are found to

be elevated in HIV-infection (Feeney, 2011). The activity of these CETP inversely correlates with HDL levels (Rose *et al.*, 2008). This is a potential mechanism that could explain the lower mean HDL levels and higher prevalence of participants with clinically low HDL levels in the HIV-infected without ART group of the current study (Feeney, 2011).

In the cross-sectional study, fasting glucose was found be elevated in the HIV-infected group in comparison to HIV-free group, whilst HbA1c% was found to be significantly lower in HIV-infected group in comparison to HIV-free (**refer to chapter 4, table 4.9**). Although these differences are statistically significant, their clinical or physiological relevance is not clear. It is also notable that both mean fasting glucose and HbA1c levels fell within the normal clinical range in both the study groups. However, in general, impaired glucose regulation is common in HIV (Spollett, 2006). Particularly certain classes of antiretroviral drugs have been associated with impaired glucose regulation and insulin resistance. Additional HIV subgroup analysis did not show any significant effects in the glucose parameters between untreated and treated HIV-infected groups (**refer to chapter 4, table 4.10**).

Haemoglobin and GGT measurements

In the longitudinal sub-study, the mean Hb and GGT levels in the total cohort fell within the normal clinical ranges (**refer to chapter 4, table 4.34**). Furthermore, there were no differences between baseline and 12-month follow-up. In the literature, elevated GGT levels have been demonstrated in individuals with high alcohol consumption, since the current cohort did not report on increased alcohol consumption at 12-month follow up, our finding is plausible in this cohort.

In the cross-sectional study, there was a higher prevalence of participants presenting with clinically low Hb levels (anaemia) in the HIV-infected group in comparison to HIV-free group (**refer to chapter 4, table 4.11**). These findings are very much in line with existing literature. It is well established that the prevalence of anaemia is high in people living with HIV, particularly those with an increased disease progression (Curkendall *et al.*, 2007). Studies suggest that approximately 95% of HIV-infected individuals have anaemia prior to ART initiation, while up to 46% of HIV-infected individuals receiving ART develop anaemia at some point in the disease (Ssali *et al.*, 2006; Tamir, Alemu and Tsegaye, 2018). Anaemia appears to be particularly common in HIV-infected individuals from low and middle-income regions such as SSA, as these individuals are more likely to be malnourished, have increased immunosuppression, and comorbidities such as TB, than those in high income regions (Takuva *et al.*, 2013). The pathophysiology of anaemia in HIV includes impaired erythropoiesis (due to increased inflammatory cytokines, and deceased production of hematopoietic growth factors), malabsorption and impaired recycling of iron, nutritional deficiencies, malignant bone marrow infiltration, bone marrow infection and haemolysis (Jacobson *et al.*, 1990; Volberding *et al.*, 2004). Interestingly, in the literature, to some extent ART is known to reverse HIV-associated anaemia by

reducing HIV replication (Curkendall *et al.*, 2007; Moor *et al.*, 2002). Thus, as expected, in the current study's HIV subgroup analysis, the prevalence of low Hb (anaemia) was higher in the untreated group compared to HIV-infected receiving ART group (**refer to chapter 4, table 4.12**).

In the cross-sectional study, the HIV-infected group presented with a higher prevalence of high GGT levels in comparison to HIV-free group (**refer to chapter 4, table 4.11**). Furthermore, in additional HIV subgroup analyses, mean GGT levels were significantly higher in the HIV-infected receiving ART group in comparison to the HIV-infected without ART group (**refer to chapter 4, table 4.12**). This observation is in line with other studies which have also reported high GGT levels in HIV-infected on ART populations in comparison to HIV-infected/ no ART populations (De Socio *et al.*, 2007; Peluso *et al.*, 2020). Clinically, elevated GGT levels are mainly used as a marker of abnormal liver or biliary tract function and alcohol consumption, and in the literature both HIV and ART are well known for their adverse effects on the liver (Soriano, Barreiro and Sherman, 2013; Mayanja *et al.*, 2017). The literature suggests several mechanisms of HIV-related liver injury, which includes cytotoxicity, oxidative stress, systemic inflammation, immune mediated injury and mitochondrial injury (Kaspar and Sterling, 2017). Furthermore, increased GGT has been associated with CVD risk in the literature (Jiang, Jiang and Tao, 2013), where studies suggest that elevated GGT may be a marker of increased oxidative stress (Ndrepepa and Kastrati, 2016). In addition, circulating GGT levels have been association with markers of inflammation such as ICAM-1 and VCAM-1 (Bradley *et al.*, 2014), interestingly the HIV-infected group in this cohort also demonstrated increased VCAM-1 in comparison to HIV-free (discussed later under section 5.5). Thus, it can be speculated that the heightened inflammatory state of HIV may have influenced the GGT levels. Additionally, in gender analyses, in the male population (**refer to appendix B, table B1**) as well as the female population (refer to appendix B, table B2), HIV-infected groups demonstrated a higher GGT level in comparison to HIV-free groups. However, when comparing males versus females (**refer to appendix B, table B3 & B4**), males demonstrated a significantly higher GGT in comparison to females in both the HIV-infected and HIV-free populations. Considering that GGT is also a marker for alcohol consumption, literature is in agreement that males exceed females in alcohol consumption, especially high-volume consumption (Wilsnack *et al.*, 2009), and thus often present with higher GGT levels than females as also reported by Ha *et al.*, 2014. Thus, it can be speculated that in addition to HIV, there may be alcohol consumption related increase in GGT, especially in the males of the current study.

Kidney function measurements

In the longitudinal sub-study, there were no significant differences in the measured markers of kidney function between baseline and 12-month follow-up (**refer to chapter 4, table 4.43**). This is plausible

as this cohort demonstrated a relatively healthy kidney function at baseline as well, with less than 10% presenting with increased ACR ratio or decreased eGFR (**refer to chapter 4, table 4.35**).

In the cross-sectional study, ACR was significantly higher in the HIV-infected group in comparison to HIV-free group (**refer to chapter 4, 4.13**), which supports previous findings in the literature. HIV can induce direct as well as indirect adverse effects on the kidneys and result in HIV-associated kidney disease (Yilma *et al.*, 2019). Microalbuminuria is a well-known recognized early marker of renal dysfunction, which is expressed by urine albumin to creatinine ratio (Glassock, 2010). Increased ACR in HIV has been demonstrated in previous studies (Szczech *et al.*, 2007; Pirro *et al.*, 2016), and has been suggested as a marker of CVD risk in HIV (Tongma *et al.*, 2013). On the other hand, creatinine is widely known in the literature to be increased in individuals receiving ART (Lucas *et al.*, 2014; Cristelli *et al.*, 2017). Mechanistically, some ART drugs may increase serum creatinine through their effect of the excretion of creatinine by the proximal renal tubule, causing an apparent change in glomerular function, without altering the eGFR. Other drugs are known to inhibit the excretion of serum creatinine by proximal renal tubular cells by the blockade of specific transporters (Australasian Society for HIV Medicine, 2019). To this end, in the current study's HIV subgroup analyses, serum creatinine levels were significantly higher in HIV-infected receiving ART group in comparison to HIV-infected without ART group (**refer to chapter 4. 4.14**). Interestingly, previous studies suggest that increased serum creatinine levels may be predictive of future CVD (Matts *et al.*, 1993). Both increased inflammation and oxidative stress have been implicated in the relationship between renal impairment and CVD (Ryom *et al.*, 2016). It is highly interesting that there seems to be presence of 'non-traditional' markers of CVD in the HIV-infected group of this cohort, as supported by both increased ACR and creatinine levels as well as presence of elevated GGT (as discussed earlier).

5.5. Biomarker findings

In the longitudinal sub-study, no significant differences were found in biomarker levels between the baseline and 12-month time points (**refer to chapter 4, table 4.44**). In the literature, the biomarkers of inflammation and endothelial dysfunction measured in the present study have all been associated with atherosclerosis and cardiovascular diseases (Stoner *et al.*, 2013; Gimbrone and García-Cardeña, 2016). Since the current longitudinal study did not demonstrate differences in the clinical measurements of endothelial function (FMD) or subclinical atherosclerosis (IMT), our temporal biomarker related findings were not unexpected.

In the cross-sectional main study, VCAM-1 was observed to be significantly higher in the HIV-infected group in comparison to HIV-free group (**refer to chapter 4, table 4.15**). This is in line with the findings from previous studies. Several studies suggest elevation of biomarkers such as VCAM-1 in HIV-infected populations in comparison to HIV-free populations, including studies from South Africa

(Melendez *et al.*, 2008; Ross *et al.*, 2009; Fourie and Fourie, 2011; Miller and Coppola, 2011; Graham, Mwilu and Conrad Liles, 2013; Fourie *et al.*, 2015). Mechanistically, HIV can activate several inflammatory pathways causing the release of cytokines and endothelial adhesion molecule expression which facilitate adhesion and transmigration of leukocytes (Fisher, Miller and Lipshultz, 2006). HIV is known to produce direct endothelial cell damage, increasing endothelial permeability, favoring apoptosis and increasing the expression of adhesion molecules such as VCAM-1 and ICAM-1 (Ren, Yao and Chen, 2002; Beltrán *et al.*, 2015). Interestingly, in gender analysis, only females demonstrated increased VCAM-1 in the HIV-infected group versus HIV-free group (**refer to appendix B, table B2**). No differences in VCAM-1 were found in the male population, between the HIV-infected and HIV-free groups (**refer to appendix B, table B1**). A study from Kenya, also demonstrated a significantly higher VCAM-1 in HIV-infected women in comparison to HIV-free women (Graham *et al.*, 2013). However, the literature is not clear about gender differences regarding biomarkers in the HIV-context. Interestingly, *in vivo* studies have emerged suggesting the role of sex hormones (oestrogen and progesterone) in females which may lead to a higher expression of inflammatory or cytotoxic pathways (Scully, 2018). However, more research is required to shed clearer light regarding the role of gender in HIV-related inflammation.

In the additional HIV subgroup analyses, VCAM-1 was observed to be significantly lower in the HIV-infected on ART group in comparison to HIV-infected/ no ART group (**refer to chapter 4, table 4.16**). Existing research demonstrates that levels of VCAM-1 are elevated in HIV, while presence of ART reduces the expression of this marker of endothelial activation (Calmy *et al.*, 2009; McComsey *et al.*, 2012; Hemkens and Bucher, 2014). Although the present study could not demonstrate differences in any of the other biomarkers between the ART vs no/ART group, it has been reported that ART is associated with lower levels of biomarkers of inflammation, particularly, reduced levels of VCAM-1, ICAM-1 and CRP were reported in a study of 115 HIV-infected individuals after 2 months and 14 months of ART (Duprez *et al.*, 2012). Furthermore, another study showed that VCAM-1 and ICAM-1 levels were significantly decreased after 24 weeks and 96 weeks of ART (McComsey *et al.*, 2012). **Refer to table 5.1**, for an overview of the effects of HIV and ART on selected biomarkers.

Table 5.1: Overview of reported effects of HIV infection and antiretroviral therapy on selected biomarkers based on contents from (Hadigan et al., 2001; Kamin and Grinspoon, 2005; Nyagol *et al.*, 2008; Ross *et al.*, 2009; Keating et al., 2011; Hunt *et al.*, 2012; McComsey *et al.*, 2012; Vaidya *et al.*, 2014; Janssen *et al.*, 2017; Pasquereau, Kumar and Herbein, 2017)

Biomarkers	Effect of HIV-infection	Effect of ART
ICAM-1, VCAM-1	↑ in blood plasma and serum levels	↓ after initiation of ART ↑ after stopping ART
PAI-1	↑ in blood plasma and serum levels ↑ In HIV-associated lipodystrophy	↑ in ART-associated lipodystrophy and obesity
hsCRP	↑ in blood levels	↓ ART
e-selectin, p selectin	↑ in serum levels	↑↓ in serum levels
TNF-α	↑ serum levels	↑↓ in serum levels
VEGF	↑ in serum levels	↑↓ in serum levels

↑ , increase; ↓ decrease; ↓↑ mixed results

In the cross-sectional main study, PAI-1 was found to be the significantly lower in the HIV-infected group in comparison to HIV-free group (**refer to chapter 4, table 4.15**), which is in contrast to the findings of previous studies. PAI-1 is mainly produced in the liver, adipose tissue and vascular endothelium (Yasar Yildiz *et al.*, 2014), and strongly and irreversibly inhibits plasminogen activators which are responsible for the conversion of plasminogen to plasmin which accelerates fibrosis (Mira *et al.*, 2020). Many studies have reported that PAI-1 levels are generally elevated in HIV, despite ART and viral suppression (C Hadigan *et al.*, 2001; Kamin and Grinspoon, 2005; Janssen *et al.*, 2017). It is difficult to explain why PAI-1 levels were lower in the HIV-infected group of the current cohort. Existing literature suggests that PAI-1 is found to be elevated in HIV-infected individuals who have HIV-associated lipodystrophy syndrome and obesity in comparison to HIV-free individuals (Wirunsawanya *et al.*, 2017). The current study's HIV-infected group did not present with significantly higher rates of obesity (high BMI) or other detectable body composition abnormalities (increased waist circumference), which could have provided some speculative explanation for the PAI-1 findings.

There were no other differences observed in the biomarkers between HIV-infected and HIV-free groups of the cross-sectional study (**refer to chapter 4, table 4.15 & 4.16**). Literature largely suggests that biomarkers of inflammation such as hsCRP, and adhesion molecules such as ICAM-1 are elevated in HIV-infected populations in comparison to HIV-free populations**. Refer to table 5.1** for current research on the effect of HIV and ART on these biomarkers. However, the levels of these biomarkers can be affected by the type of ART (Laurence, Elhadad and Ahamed, 2018). Research on this has been contradictory so far with some studies suggesting that ART may have a protective effect in HIV-infected populations (Kurz *et al.*, 2012; Beltrán *et al.*, 2015). In the current cohort, it appears that ART may be imposing a more protective effect in the HIV-infected group, however the current study's results are not conclusive, considering only two out of the eight biomarkers measured demonstrated a difference supporting the premise. It can be speculated that the current study's biomarker analysis was based on a

relatively small sample size as not all participants were included in the analysis due to budgetary constraints, but rather chosen by randomization. This may have influenced the statistical power and results. Additionally, in the literature, these markers of inflammation and endothelial dysfunction have been known to increase with age, including in the HIV context (Aberg *et, al.*, 2012), thus it can be speculated that the relatively young age of the present study's cohort (~41 years) may have also impacted our results. Furthermore, in the literature, the progression of HIV and duration of ART are known to influence inflammation. For instance, increased biomarkers are correlated with increased progression of HIV (Muswe *et al.*, 2017), while the duration of ART has been shown to reduce biomarkers of inflammation, such as TNF-α (Muswe *et al.*, 2017). However, it is important to note that although ART is known to reduce inflammation, existing literature also suggests that the inflammation persists even at undetectable viremia, suggesting that despite ART, HIV can elevate these biomarkers (Hearps *et al.*, 2012; Paiardini and Müller-Trutwin, 2013). Thus, it can be speculated, that it is rather the HIV duration or progression that is influencing the biomarker results in our cohort. Especially since, in the current study only 47% (less than half) had HIV for more than 5 years. Overall, it appears that, in the current cohort, endothelial activation and dysfunction may not yet be sufficiently established to be biochemically and clinically detectable, despite the presence of traditional risk factors such as obesity and hypertension and non-traditional risk factors such as increased GGT and creatinine.

5.6 Subclinical atherosclerosis findings

In the longitudinal sub-study, there were no significant differences in mean IMT measurements or subclinical atherosclerosis between baseline and 12-month follow-up (**refer to chapter 4, table 4.46**). However, interestingly**,** two participants presented with plaque at 12-month follow-up. The lack of temporal changes may suggest that the 12-month follow up period was too short to observe differences in the cohort. However, this cohort demonstrated increase in obesity related parameters at 12-month follow-up, which are known to be associated with subclinical atherosclerosis and plaque. Additionally, there were no differences in the lipid profile of this cohort at 12-month time point. Lipid levels have been associated with the progression of IMT in the general population and since in the current cohort, no changes in lipid levels were demonstrated, the lack of difference in IMT progression is plausible. Furthermore, IMT is known to be associated with age in literature (Madhuri, Chandra and Jabbar, 2010), thus it can be speculated that the relatively young age (~45 years) of this cohort may have also influenced the results. Overall, it is noteworthy that not many previous studies have evaluated IMT in this demographically distinct population from the Western Cape; hence, our findings should be regarded as novel in this regard.

In the cross-sectional study, the mean IMT measurements fell within the normal range in the HIV-infected as well as the HIV-free group (**refer to chapter 4, table 4.17**). Furthermore, there were no

significant differences in the mean IMT or the prevalence of subclinical atherosclerosis and plaque between the HIV-infected and HIV-free groups. Our findings agree with studies from the USA, Ghana, Botswana as well as a South African study which also demonstrated no differences in this measurement between the HIV-infected and HIV-free groups (Currier *et al.*, 2005; Johnsen *et al.*, 2006; C M T Fourie *et al.*, 2015; Mosepele *et al.*, 2018; Sarfo *et al.*, 2019). On the other hand, our findings contradict several other studies which have demonstrated a higher IMT in HIV-infected individuals in comparison to HIV-free individuals (Chironi, *et al.*, 2003; Lorenz *et al.*, 2008; Ross *et al.*, 2009; Grunfeld *et al.*, 2009; Van Vonderen *et al.*, 2009). As suggested by Fourie et al., 2015 and Hsue et al., 2012, contradictions in the literature can be explained by the choice of the anatomical location of the IMT measurement, for instance, the current study measured IMT in the common carotid artery, while differences have been noted by some studies in the carotid bifurcation region (Hsue *et al.*, 2012). Interestingly, in the carotid bifurcation region, shear stress is low which may enhance the effect of chronic inflammation which potentially can induce increase expression of vascular adhesion molecules such as VCAM-1 (Chatzizisis *et al.*, 2007; Fourie *et al.*, 2015), which the current study also demonstrated. In the additional gender analyses, it was interesting to note that in the male participants, the mean IMT was higher in the HIV-infected group compared to the HIV-free group (~649 µm vs ~595 µm; p=0.05), which was not observed in female participants (**refer to appendix B, table B1 for male population and table B2 for female population**). This finding could suggest that HIV-infected male participants are more susceptible to pro-atherosclerotic changes than their female counterparts.

It is important to note that the prevalence of participants presenting with subclinical atherosclerosis and plaque in the cross-sectional cohort was low. This could be explained by the cut-off value used in the current study. In comparison to two other studies from Africa (Ssinabulya *et al.*, 2014; Sarfo *et al.*, 2019) and one from South Africa (Schoffelen *et al.*, 2015), the current study's cut-off value for subclinical atherosclerosis (> 900 µm), may be perceived to be too high. The cut-off value used by these mentioned studies for subclinical atherosclerosis was > 780 µm based on the observation that on average a healthy adult reaches an IMT of 780 µm by the age of 76 (de Groot *et al.*, 2004). The current study also performed supplementary statistical analyses using the cut-off value of 780 µm (**refer to appendix E**). With this new cut-off category, it is interesting that although the prevalence of subclinical atherosclerosis increased slightly in the total cohort from 4% to 10% (**refer to appendix E, table E1**), there were still no differences in subclinical atherosclerosis between HIV-infected and HIV-free groups (**refer to appendix E, table E1**). Thus, it can be speculated that, in the present cohort, the choice of IMT cut-off value did not significantly influence the findings, and that there may in fact be a low presence of subclinical atherosclerosis in this cohort. It can be suggested that our findings are influenced by the relatively young age of the cohort (~45 years) or the technique used to measure IMT. However, it is noteworthy, that there are several cut-off values present for IMT- related subclinical atherosclerosis

in clinical research, and future studies are recommended to standardize this definition for specific populations.

Interestingly, in the HIV subgroup analyses, the HIV-infected on ART group demonstrated a significantly higher mean IMT in comparison to HIV-infected/ no ART group (**refer to chapter 4, table 4.18**). Clinically, however, it is noteworthy that both groups presented with mean IMT values that fell within the normal range. Although both HIV and ART are known to influence IMT, several studies have demonstrated increased IMT in HIV-infected on ART versus HIV-infected/ no ART (Priscilla Y. Hsue *et al.*, 2009; Hanna *et al.*, 2016; Msoka *et al.*, 2019). Thus, in this respect, the findings from our cohort are in agreement with previous studies. However, literature is not conclusive on whether these modifications in the IMT are due to the direct effects of ART or caused indirectly, by their metabolic side-effects (Schoffelen *et al.*, 2015). Interestingly, one study showed an increase in IMT after initiation of ART, especially with tenofovir based therapy (which forms part of the first line therapy in South Africa) (Delaney, Scherzer et al. 2010). However, the results are contradictory with regards to the type of ART. For example, another study found faster progression of IMT among HIV-infected individuals on PI-containing ART compared to those on NNRTI-based regimens (Baker *et al.*, 2009). The same study showed that HIV infected individuals on PIs have a significantly higher IMT (Baker *et al.*, 2009). Overall, in the current study, ART certainly appears to influence IMT. It is further notable that presence of subclinical atherosclerosis and plaque was only present in the HIV-infected ART group, additionally suggesting adverse, potentially pro-atherogenic effects of ART in our cohort.

5.7 Endothelial function findings

In the longitudinal study, there were no significant differences in endothelial function as measured by FMD%, between baseline and 12-month follow-up (**refer to chapter 4, table 4.45**). This finding is plausible in the current cohort, with HIV-free participants who were generally healthy and did not demonstrate changes in many cardiovascular risk parameters. However, the current cohort did demonstrate an increase in blood pressure at 12-month follow up. In the literature, it has been shown that blood pressure inversely associates with FMD% and endothelial function (Benjamin *et al.*, 2004; Yufu *et al.*, 2009). Decreased FMD% is often observed with elevated blood pressure and hypertension in individuals (Juonala *et al.*, 2006). It has been suggested that high blood pressure may interfere with the integrity of endothelial cells causing endothelial activation and dysfunction (Cohn *et al.*, 2004; Juonala *et al.*, 2006). Similarly, obesity and waist circumference have been known to inversely correlate with FMD% (Williams *et al.*, 2005; Arkin *et al.*, 2008). One can speculate that although there were differences in blood pressure and waist circumference in this population, clinically there was a very small change over time (clinically, only blood pressure changed from normal to elevated category), hence the absence of temporal change towards an endothelial dysfunction phenotype is not totally

unexpected. In the literature, endothelial dysfunction has been associated with aging in the general population (Seals, Jablonski and Donato, 2011), thus it can be speculated that the relatively young age (~45 years) of the current cohort may have influenced our finding. Furthermore, it can be suggested that the 12-month follow up period may have been too short to observe any detectable temporal changes in endothelial function; thus, a longer follow-up period is recommended for future studies.

In the cross-sectional study, there were no significant differences in FMD% between HIV-infected and HIV-free groups (**refer to chapter 4, table 4.19**). The absence of changes in the clinical measurement of endothelial function by FMD was generally underpinned by the endothelial biomarker findings which showed that the majority of biomarkers (except VCAM-1 and PAI-1) were not different between the groups (see Section 5.5). The current study's findings are in agreement with some studies in literature which also demonstrated no differences in endothelial function as measured by FMD% between HIV-infected and HIV-free groups (Nolan *et al.*, 2003; Blanco, 2006; Mondy *et al.*, 2008). However, generally, several studies suggest a lower FMD% or endothelial dysfunction in HIV-infected individuals (with or without ART) in comparison to HIV-free individuals (Solages *et al.*, 2006; Van Wijk *et al.*, 2006; Stein, Currier and Hsue, 2014; Sharma, Gupta and Srivastava, 2018). Both the treatment and the virus itself have been implicated as potential mechanisms. ART is known for its adverse effects of increasing oxidative stress and endothelial recruitment of mononuclear cells, in addition to its lipid related effects (Skowyra *et al.*, 2012). With regards to the virus itself, especially, viral proteins gp120, Tat and Nef have been implicated (Anand, Rachel and Parthasarathy, 2018). The pathogenesis of HIV and ART related endothelial dysfunction has been described in detail in chapter 2, section C of this dissertation.

Particularly, with regards to ART and FMD, studies have demonstrated mixed results. Variability in this regard could be attributed to differences in ART combinations, treatment duration, as well the cardio-metabolic profiles of the study population. The current cross-sectional study demonstrated a significantly higher FMD% (implying improved endothelial function) in the HIV-infected on ART group in comparison to HIV-infected/ no ART group (**refer to chapter 4, table 4.20**). In this regard, our findings contradict many studies. For instance, a study by Andrade et al., 2008 demonstrated that FMD was significantly lower in their ART receiving HIV group in comparison to their untreated HIV group (Andrade *et al.*, 2008). Additionally, another study from Ethiopia observed that FMD was lower in younger adults receiving ART (efavirenz and lopinavir in combination with ritonavir) in comparison to untreated younger adults (Gleason *et al.*, 2016). It appears as though certain classes of ART, especially those that are PI-based, are implicated in lower FMD in HIV-infected individuals. For example, a study of adult HIV-infected individuals by Stein et al. 2001 reported on impaired (lower FMD% relative to study's control group FMD%) endothelial function in HIV-infected individuals using PI-containing regimens while the HIV-infected ART naïve individuals presented with normal FMD% ("normal FMD%" was considered more than or equal to the mean FMD% in the study's control group).

This study further concluded that PIs are associated with pro-atherogenic lipoprotein changes and with endothelial dysfunction (Stein, Currier *et al.*, 2014, Stein, Klein *et al.*, 2001). It is notable that the current study's participants were not receiving ART from the PI class but from the NRTI/ NNRTI class. It therefore appears that the vascular endothelial function of participants on ART in the current cohort was protected from the harmful effects of HIV. Our finding supports some existing studies in the literature. For instance, a study by Torriani et, al., 2008 demonstrated improved endothelial function in their cohort after commencement of ART (Torriani *et al.*, 2008). Their study demonstrated improvement of FMD after four weeks of treatment and then again after 24 weeks of treatment (Torriani *et al.*, 2008).

5.8 Summary of descriptive findings from the PhD.

The current study has yielded interesting descriptive findings from both the cross-sectional main study as well as the longitudinal sub-study. **Refer to figure 5.1** for a schematic overview of the descriptive findings.

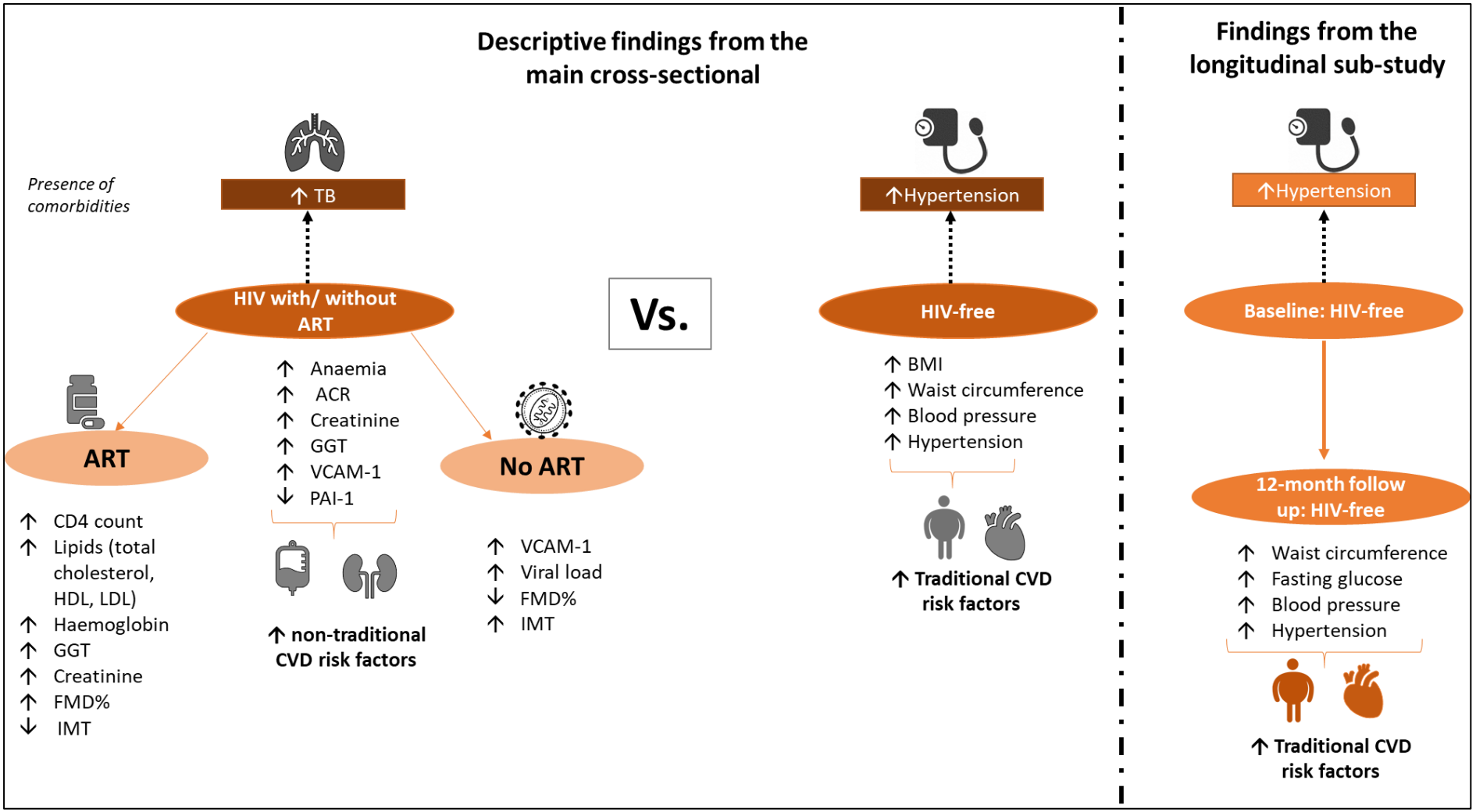


Figure 5.1: Summary of descriptive findings from the cross-sectional main study as well as the longitudinal sub-study showing potential pathophysiological changes. Figure created by the author of this dissertation. BMI, body mass index; ACR, albumin creatinine ratio; FMD, flow mediated dilatation, IMT, intima

media thickness, TB, tuberculosis, GGT, γ-Glutamyl transferase; VCAM-1, Vascular Cellular Adhesion Molecule-1; PAI-1, Plasminogen activator inhibitor-1.

5.9 Independent associations with IMT

Biomarker related associations with IMT

Overall, no correlations were observed between any of the biomarkers of inflammation and endothelial dysfunction with IMT in any of the study groups. Our findings are to some extent in line with existing literature which have also demonstrated no relationships of biomarkers with the common carotid IMT (as measured in the current PhD). In the HIV context, there are a few notable studies, especially relevant to the current PhD, which will be discussed.

A study by Ross et. al., 2009 demonstrated that CRP positively correlated with internal carotid IMT in both HIV-infected and HIV-free groups. Furthermore, their study showed that endothelial markers were not correlated with common carotid IMT in HIV-infected study participants, while only VCAM-1 negatively correlated with internal carotid IMT in the HIV-free group. Regression analysis showed that VCAM-1 and TNF-α independently associated with internal carotid IMT in the HIV-infected population (Allison C. Ross *et al.*, 2009). Similarly, a more recent study by Subramanya et. al., demonstrated CRP and TNF-α to be correlated with bifurcation IMT in total population including HIV-infected and HIV-free individuals. However, in regression analysis, after adjusting for demographic and cardiovascular factors, the association was lost. Similarly, in their HIV-infected group, correlations were found between ICAM-1, CRP and bifurcation IMT, but the associations were lost after adjusting for traditional risk factors (Subramanya *et al.*, 2019).

Overall, interestingly, both studies demonstrated that these biomarkers largely associated with internal carotid artery IMT (Allison C. Ross *et al.*, 2009) or bifurcation IMT (Subramanya *et al.*, 2019), and it is important to note that these studies also measured common carotid artery IMT. This is interesting and there seems to be no plausible explanation in literature on why biomarkers do not correlate with common carotid artery IMT, as one would expect the development of atherosclerosis to occur in a diffuse and systematic process (also suggested by Ross et. al., 2009). Furthermore, there are other studies that demonstrated biomarker relationships with IMT other than common carotid artery IMT such as Thakore et al., 2007. Thus, the lack of relationship between the biomarkers and IMT in the current study may be ascribed to the choice of the anatomical region of the carotid artery for the measurement. Additionally, as mentioned in earlier sections, the current study's biomarker analysis was based on a relatively small sample size as not all samples were included in the analysis but rather chosen by randomization. This may have influenced the statistical power and results.

HIV and ART-related associations with IMT

HIV infection status did not significantly associate with IMT in the current study (**refer to chapter 4, table 4.23a**). This contradicts many studies in the literature, for instance, a large case control study with 1500 participants showed that HIV is an independent predictor of early carotid atherosclerosis as measured by IMT (Lorenz *et al.*, 2008; Margaritis, 2019). Additionally, another study reported that HIV was independently associated with IMT even after adjusting for demographics and cardiovascular risk factors. In fact, the study reported the strength of the association was similar in magnitude to that of a traditional cardiovascular risk factor such as diabetes (Grunfeld *et al.*, 2009). However, it is important to note that the said study also measured internal carotid IMT (including the bulb region) and they found a stronger association of HIV with this measurement in comparison to the common carotid IMT (as measured in the present study). Furthermore, interestingly, studies that found little to no association of HIV with IMT, measured only the common carotid artery (Depairon *et al.*, 2001; Currier *et al.*, 2005, 2007; Johnsen *et al.*, 2006). Thus, the current finding could be explained by the differences in the anatomical location at which IMT was measured.

Additionally, several studies in the literature have demonstrated that ART is an independent predictor of IMT (Jericó *et al.*, 2006; Lorenz *et al.*, 2008). In the current study, ART status demonstrated a significant correlation with IMT (**refer to chapter 4, table 4.21**), however when included in the regression model for the HIV-infected group where several other factors were adjusted for, independent associations were not found (**refer to chapter 4, table 4.23a**). Literature regarding this has demonstrated mixed results, where some studies demonstrate that cardiovascular risk factors rather than HIV-related factors associate with IMT (Depairon *et al.*, 2001; Schoffelen *et al.*, 2015). The current study findings are in agreement with another study from South Africa, albeit in a cohort from a different geographical region, which also found no association between HIV, ART and IMT (Schoffelen *et al.*, 2015). However, it is important to note that in the said study, participants were largely receiving PI-based ART. Another study demonstrated similar results (no independent associations of ART and HIV with IMT) (Chironi, Escaut, Gariepy, Cogny, J. Monsuez, *et al.*, 2003), however, their participants received NRTI/ NNRTI based treatment, similar to the participants of our study. It could therefore be speculated that in our cohort, the absence of PI-based ART may explain the lack of association between ART and IMT. Furthermore, in the HIV-infected on ART group, ART duration did not significantly associate with IMT. Our finding is in line with existing studies (including a study from South Africa) which also demonstrated no association (Jericó *et al.*, 2006; Schoffelen *et al.*, 2015).

Cardiovascular related associations with IMT

In our total cohort, as well as the HIV-free group, systolic hypertension (high systolic blood pressure), obesity (as measured by BMI) and LDL cholesterol all demonstrated significant, independent positive

associations with IMT (**refer to chapter 4, table 4.23a**). These findings are in line with existing literature. In both the HIV and non-HIV context, traditional cardiovascular risk factors such as the aforementioned parameters, are known to associate with IMT. Several studies demonstrate obesity (as measured by BMI) to be an independent predictor of IMT (Tan and Chuang, 2012; Dalmas *et al.*, 2013; Jin *et al.*, 2018). Furthermore, the impact of LDL cholesterol on IMT is well known. Several previous studies have demonstrated LDL to be an independent predictor of IMT (Salonen and Salonen, 1991; Sun *et al.*, 2005; Yang *et al.*, 2014, 2020). Physiologically, the role of LDL is well established in the development of atherosclerosis (as described in chapter 2). With regards to hypertension, the literature also confirms its association with IMT (Salonen and Salonen, 1991; Sun *et al.*, 2005; Takase *et al.*, 2017). Furthermore, studies suggest that systolic blood pressure, and not the diastolic blood pressure associates with IMT (Salonen and Salonen, 1991; Zanchetti *et al.*, 2001), and systolic hypertension (high systolic blood pressure) is associated with increased IMT (Lim *et al.*, 2009). According to the widely accepted 'response to injury' model of atherogenesis, various factors such as hemodynamic forces and chemical agents, induce endothelial dysfunction, which is followed by aggregation of platelets, lipids and smooth muscle cells in the intimal layer. All the mentioned factors increase IMT, which ultimately leads to the formation of plaques (Sun *et al.*, 2005). Accordingly, it is known that atherosclerotic deposition of LDL cholesterol may require prior injury to the endothelium by factors such as hypertension and obesity (which is what the current study also suggests). Overall, hypertension, obesity and LDL cholesterol are established independent predictors of IMT, and the current study confirms that, even after adjusting for age and gender in both groups, as well as HIV status in the total study group. It is interesting that these cardiovascular parameters did not associate with IMT in the HIV-infected group, when a study has demonstrated BMI, hypertension and types of cholesterols (such as total and triglycerides) to be associated with IMT in the HIV-context (Currier *et al.*, 2005). It can be speculated that the type of treatment as well as the viral load may influence these associations. For instance, the in the study by Currier et, al., 2005, the HIV-infected participants were on PI treatment, while the current study participants were largely receiving NRTI/ NNRTI ART.

In the total, HIV-infected, and HIV-infected on ART study groups, age ≥ 50 years demonstrated a significant positive association with IMT (**refer to chapter 4, table 4.23a and 4.23b**). In fact, age was the only variable that independently associated with IMT in the HIV-infected group. The positive association between increasing age and IMT is well established in the literature. It has been shown that carotid IMT increases with age among both HIV-infected and HIV-free individuals ('Hsue PY *et al.*, 2004; Fitch *et al.*, 2013). Aging is a well-known risk factor of CVD (Nonterah *et al.*, 2019), particularly age of 50 years and above is known to be a risk factor of CVD (Lloyd-Jones *et al.*, 2006). Additionally, it is established that aging is independently associated with a decline in physiological function (Chia, Egan and Ferrucci, 2018). Within the HIV-context, the virus can lead to a premature aging phenotype, where patients experience immune aging through HIV-induced alterations in monocyte phenotype and

function (Fitch *et al.*, 2013). Several studies have found age to be an independent predictor of IMT in HIV-infected populations (Ross *et al.*, 2009; Fitch *et al.*, 2013; Ssinabulya *et al.*, 2014; Hanna *et al.*, 2016; Nonterah *et al.*, 2019). The current study confirms this finding in the HIV context.

Finally, it is highly interesting that age did not associate with IMT in the HIV-free group. Our finding suggests that in the general population, modifiable risk factors such as LDL-cholesterol, hypertension and obesity are independent predictors of IMT. However, in our cohort, the HIV-infected participants appeared to be distinctly susceptible to the vascular wall thickening effects of increasing age.

5.10 Independent associations with FMD%

IMT associations with FMD%

The current study found no significant association between IMT and FMD% in any of the study populations. This is in line with other studies which also included HIV-infected and HIV-free groups (Odueyungbo *et al.*, 2009; Sharma, Gupta and Srivastava, 2018). However, there are studies which also demonstrated a significant inverse correlation between FMD% and IMT such as by Ghiadoni et al., 2015. Interestingly, in the current study, there was a significant inverse correlation between carotid artery diameter and FMD% in all the study groups (**refer to chapter 4, table 4.24**). This is interesting and plausible as the brachial artery diameter is also inversely associated with FMD% (**refer to chapter 4, table 4.24, 4.27a & 4.27b**). This finding suggests that the relative changes in the diameters of medium-sized arteries in the body, other than the brachial artery, may be inversely related to the FMD%.

Brachial artery association with FMD%

For independent associations with FMD% in the current study, all regression analyses were repeated with baseline brachial artery diameter adjustment. The confounding impact of brachial artery diameter with FMD% has often been debated. Baseline arterial diameter has been known to independently influence vasodilation, with small arteries dilating relatively more than the large arteries (Barac, Campia and Panza, 2007). Hence, studies recommend to adjust for the diameter in all statistical models (Longenecker and Hoit, 2012). However, statistically, it is also suggested that by adjusting for brachial artery diameter, it may be a covariate adjustment in the model as brachial artery diameter is already used to calculate FMD% (Thijssen *et al.*, 2011). Hence in the current study, we explored both methods (inclusion and exclusion of brachial artery diameter in the model) to assess associations with FMD%. However, it is notable that independent associations even after adjusting for brachial artery, may demonstrate higher clinical relevance but further research is recommended to standardize methodologies.

Biomarker related associations with FMD%

As with the IMT, the biomarkers of inflammation and endothelial dysfunction did not correlate with FMD% in any of the study groups (**refer to chapter 4, table 4.25**). Existing literature has demonstrated mixed results on the relationship between biomarkers of inflammation and endothelial function, and FMD. For instance, the relationship between CRP and FMD% in individuals appears to be controversial; two studies suggested that higher CRP was associated with impaired endothelial function in HIV (Ho *et al.*, 2012; Grome *et al.*, 2017), while other studies demonstrated no associations in HIV-infected on ART populations (Nolan *et al.*, 2003; Deanfield Muriel Caslake *et al.*, 2005; Van Wijk *et al.*, 2006; Mondy *et al.*, 2008; Torriani *et al.*, 2008). Overall, our findings agree with a few studies that also could not demonstrate correlations between FMD % and biomarkers such TNF-α, VEGF, VCAM-1, ICAM-1, PAI-1, e-selectin, and p-selectin (Masiá *et al.*, 2010; Dysangco *et al.*, 2017; Grome *et al.*, 2017). Interestingly, these inflammatory molecules are known in the literature for their role in endothelial activation and endothelial dysfunction (discussed in detail in chapter 2), as well as in HIV-infection, however, not many studies have demonstrated their relationship with endothelial dysfunction as measured by FMD%. It has been suggested that these markers, especially VCAM-1 and ICAM-1 are expressed on numerous cells apart from the endothelium, such as inflammatory leukocytes and smooth muscle cells, and therefore they may be imposing a stronger effect on systemic inflammation in HIV rather than in vascular inflammation (Blankenberg, Barbaux and Tiret, 2003; Masiá *et al.*, 2010). Similarly, other markers such as PAI-1 are also expressed in adipocytes, platelets, and hepatocytes in addition to endothelial cells (Yasar Yildiz *et al.*, 2014). Thus, a similar mechanism can be speculated to be at play in this regard (Masiá *et al.*, 2010). However, on the other hand, it is also possible that the variability in findings from different studies may be due the technique used to measure endothelial function as well as the current study's limited sample size for biomarker analysis (as described earlier).

HIV and ART related associations with FMD%

In the total population, HIV infection status did not associate with FMD% (**refer to chapter 4, table 4.26a % 4.27a**). Existing literature suggests that both HIV and ART are inversely associated with FMD%. For instance, a study demonstrated that untreated HIV is associated with endothelial dysfunction (Oliviero *et al.*, 2009). Other cross-sectional studies, as well as a meta-analysis, reported that both HIV and ART are associated with decreased FMD% (Solages *et al.*, 2006; Priscilla Y Hsue *et al.*, 2009; Sun *et al.*, 2015). Interestingly, in the current study's HIV-infected group, ART demonstrated a significant positive association with FMD%, suggesting a more vasculo-protective role in HIV. Furthermore, viral load demonstrated a significant inverse association with FMD% (**refer to chapter 4, table 4.47a and 4.27b**). It is notable that associations remained even after adjusting for brachial artery diameter. Very few studies demonstrate this cardioprotective effect of ART on FMD% (Deanfield Muriel Caslake *et al.*, 2005; Torriani *et al.*, 2008), however, this could be attributed to the type of ART as well. For instance, ART from the PI class has shown an inverse association with FMD% in several studies (Stein *et al.*, 2001; Charakida *et al.*, 2010). Viral load on the other hand has been

known to be an independent predictor of FMD% in the HIV-context (Blum *et al.*, 2005; Solages *et al.*, 2006; Oliviero *et al.*, 2009; Ho *et al.*, 2012; Torriani *et al.*, 2008). This suggests that the virus itself may influence endothelial function while the ART may in fact provide some protection to the HIV-infected population not only from harmful effects of HIV but also from vascular dysfunction.

Overall, our study confirms that in the HIV-context, ART is an independent predictor of endothelial function, where the virus (using viral load as a surrogate marker) is an independent predictor of endothelial dysfunction.

Cardiovascular related associations with FMD%

In the total population, female gender demonstrated a significant positive association with FMD% (**refer to chapter 4, table 4.26a**). It can be speculated that the finding is due to hormonal changes in female body. Literature suggests that hormonal changes related to the menstrual cycle may affect FMD% (Corretti *et al.*, 2002; Charakida *et al.*, 2010). Especially one of the main female hormones, estrogen, is known to enhance endothelial vasodilation (Arora *et al.*, 1998). In the literature, it has been suggested that estrogen may be able to stimulate endothelial nitric oxide synthesis as well as release nitric oxide or prevent nitric oxide degradation due its antioxidant properties (Rainbow *et al.*, 1980; Arora *et al.*, 1998). However, in addition to female hormones, our finding is more likely due to the diameter of the female brachial artery in comparison to males. In the literature it is established that females generally have a smaller baseline brachial artery diameter than males (Pham, Kim *et al.*, 2016, Kapuku, Treiber *et al.*, 2004). Since brachial artery diameter has a significant inverse association with FMD%, this finding is plausible when brachial artery diameter is not adjusted for in the model. As expected, once brachial artery diameter was adjusted for, this finding was lost in the total population (**refer to chapter 4, table 4.27a**).

Additionally, in the total population, high and normal systolic blood pressure, as well as waist circumference and urine albumin demonstrated significant inverse associations with FMD% (**refer to chapter 4, table 4.26a**). All these parameters have been associated with FMD% in the literature (Benjamin *et al.*, 2004; Cohn *et al.*, 2004; Williams *et al.*, 2005; Juonala *et al.*, 2006; Arkin *et al.*, 2008; Yufu *et al.*, 2009). Furthermore, literature suggests that microalbuminuria as expressed by albumin and creatine ratio (ACR) is associated with endothelial dysfunction. Studies have demonstrated inverse associations between, albumin, creatinine levels as well as ACR with FMD% (Gardin *et al.*, 2008; Younis, Nafady and Mahmoud, 2011; Pirro *et al.*, 2016). The mechanisms linking microalbuminuria to increased renal and cardiovascular risk are not fully understood, but it has been proposed that microalbuminuria is a reflection of generalized endothelial dysfunction (Ochodnicky *et al.*, 2006). Thus, all mechanisms of endothelial dysfunction such as increased ROS, oxidative stress, inflammation and endothelial activation are implicated (Ochodnicky *et al.*, 2006). It is interesting that in the current study, all these associations in the total population were lost once brachial artery diameter was adjusted for

(**refer to chapter 4, table 4.27a**). However, in the HIV-free group, creatinine remained as an independent, inverse predictor of FMD% even after adjusting for brachial artery diameter, suggesting that creatinine is an independent predictor of impaired endothelial function (as measured by FMD%) in the HIV-free population.

5.11 Summary of independent associations

In summary no significant associations were found between biomarkers, IMT and FMD % in the PhD study. Refer to figure 5.2 for a summary of independent associations with IMT and FMD% in the different study populations.

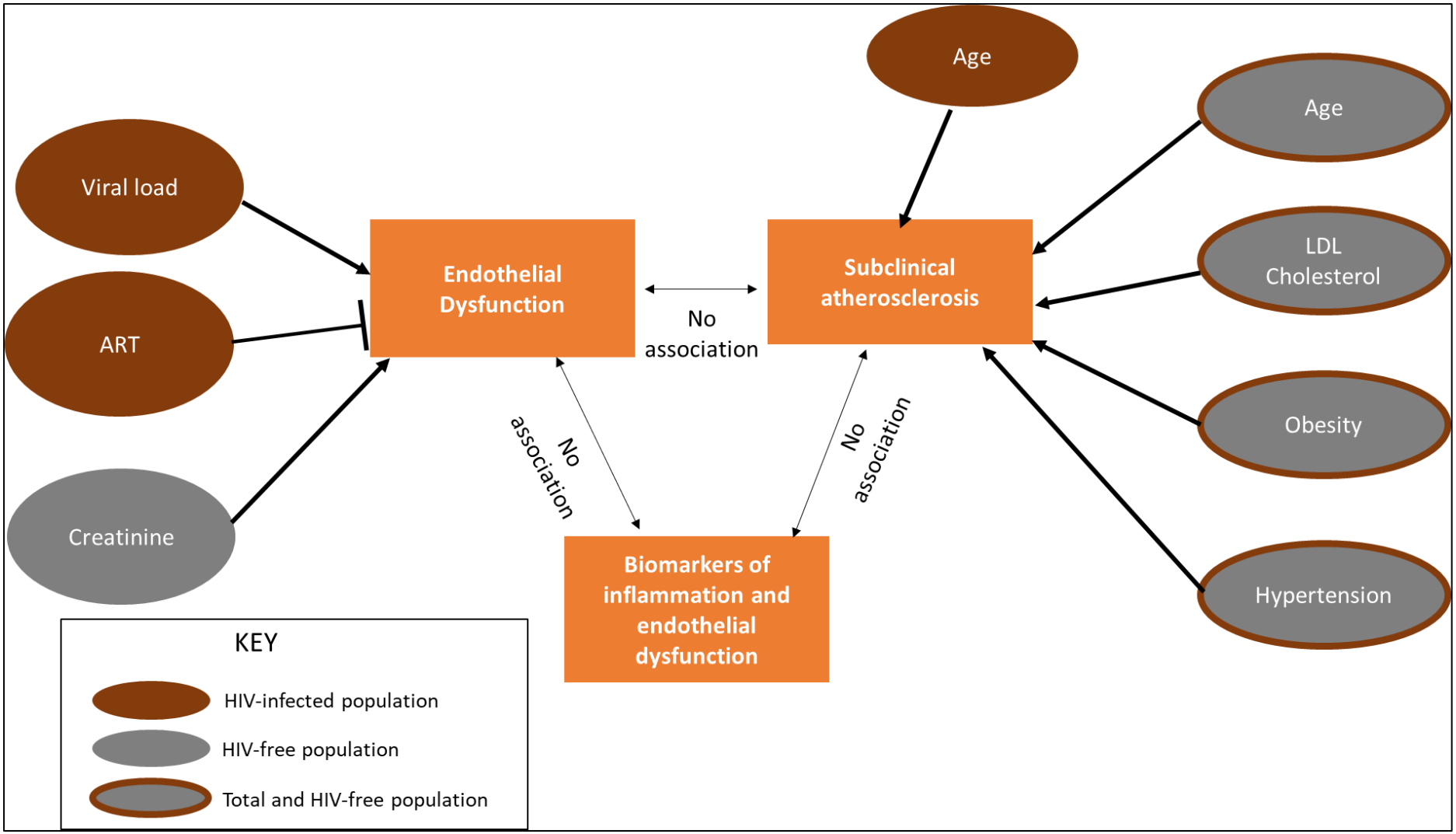


Figure 5.2: Independent associations shown in the study.

Chapter 6: Conclusion

6.1 Introduction

This chapter describes the conclusion with respect to the overall aims and objectives of the PhD study as well as a reflection of the advantages, shortcomings, and future directions of the study.

6.2 Reflection of the PhD aims

At present, CVD is one of the major causes of death in people living with HIV. The underlying pathophysiological origins of many types of CVD are endothelial dysfunction and atherosclerosis, which can both be measured by non-invasive methods such as FMD and carotid IMT measurements. Both atherosclerosis (especially subclinical atherosclerosis) and endothelial dysfunction can potentially be prevented, if detected early. Interestingly, in the HIV-context, both the HI virus and ART have been implicated in the development of endothelial dysfunction and atherosclerosis. Additionally, circulating biomarkers of inflammation, endothelial activation and dysfunction are known to be associated with endothelial dysfunction and atherosclerosis, which can be screened and measured to predict cardiovascular outcomes in the HIV-infected population. Furthermore, the interaction between traditional cardiovascular risk factors, HIV, ART and endothelial dysfunction and atherosclerosis is vastly complex, and not well researched in South African populations, particularly not in the Western Cape Province. Hence, the current study aimed to investigate the putative relationship between subclinical atherosclerosis, endothelial dysfunction, biomarkers of CVD, and HIV-infection in a South African population from the Western Cape Province.

6.3 Final conclusion

The current PhD study successfully addressed its overarching aim by conducting a clinical cross-sectional study in the HIV-context as well as by conducting an exploratory longitudinal study to assess the 12-month temporal progression of selected CVD outcomes, including endothelial function and subclinical atherosclerosis, in a sub-group of HIV-free participants.

The longitudinal study showed that measurements of visceral obesity, fasting blood glucose, and blood pressure, including the prevalence of hypertension, increased over the period of 12-months. None of the other risk factors, nor subclinical and endothelial function measurements showed any changes between the two time points of the study, suggesting that the 12-month follow up period may have been too short to observe differences in the cohort.

Findings from the cross-sectional study showed a significantly lower prevalence of traditional cardiovascular risk factors, such as BMI, waist circumference, blood pressure, hypertension in the HIV-

infected population in comparison to HIV-free population. In contrast, other markers of cardiovascular risk and future CVD, such as anaemia, ACR, creatinine and GGT were significantly elevated in the HIV-infected group in comparison to HIV-free group. What is more, our study demonstrates key differences between HIV-infected without ART group in comparison to HIV-infected receiving ART group. For instance, viral load and VCAM-1 levels were significantly higher in the HIV-infected without ART group in comparison to HIV-infected receiving ART group. In contrast, CD4 count, total cholesterol, HDL, LDL, haemoglobin, GGT, creatinine levels were higher in the ART group in comparison to the without ART group. Collectively, these findings suggest a mixed cardiovascular risk profile in the HIV-infected participants of this cohort. Overall, although there were no differences in sub-clinical atherosclerosis and endothelial function between the two main study groups (HIV-infected vs HIV-free), endothelial function (as measured by FMD) was better and subclinical atherosclerosis (measured by IMT) reduced in the ART group in comparison to no ART group. The results demonstrate that, in the cohort of the current study, ART was associated with a more favourable cardiovascular risk profile, reduced subclinical atherosclerosis, and improved endothelial function.

A key aim of the PhD study was to investigate the relationships between subclinical atherosclerosis and serum biomarkers of endothelial dysfunction and vascular inflammation in HIV-infected (with or without ART) and HIV-free participants. Interestingly, our study demonstrated no relationship between subclinical atherosclerosis and the serum biomarkers measured. Additionally, biomarkers showed no associations with endothelial function in this cohort. With regards to independent associations, the current study confirms that age, systolic hypertension, obesity, and LDL-cholesterol levels are independently associated with subclinical atherosclerosis in the total cohort, even after adjusting for HIV status. The study shows that age was the only factor that independently associated with subclinical atherosclerosis in the HIV-infected group, even after adjusting for ART. With regards to the FMD analyses, the study findings showed that, in the HIV-free context, creatinine is an independent predictor of impaired endothelial function, even after adjusting for brachial artery diameter. In the HIV-context, viral load was an independent, inverse predictor of endothelial function even after adjusting for brachial artery diameter, HIV and ART duration, suggesting that endothelial function was associated with viral suppression. **Refer to figure 6.**1 for a schematic representation of the overall findings from the PhD.

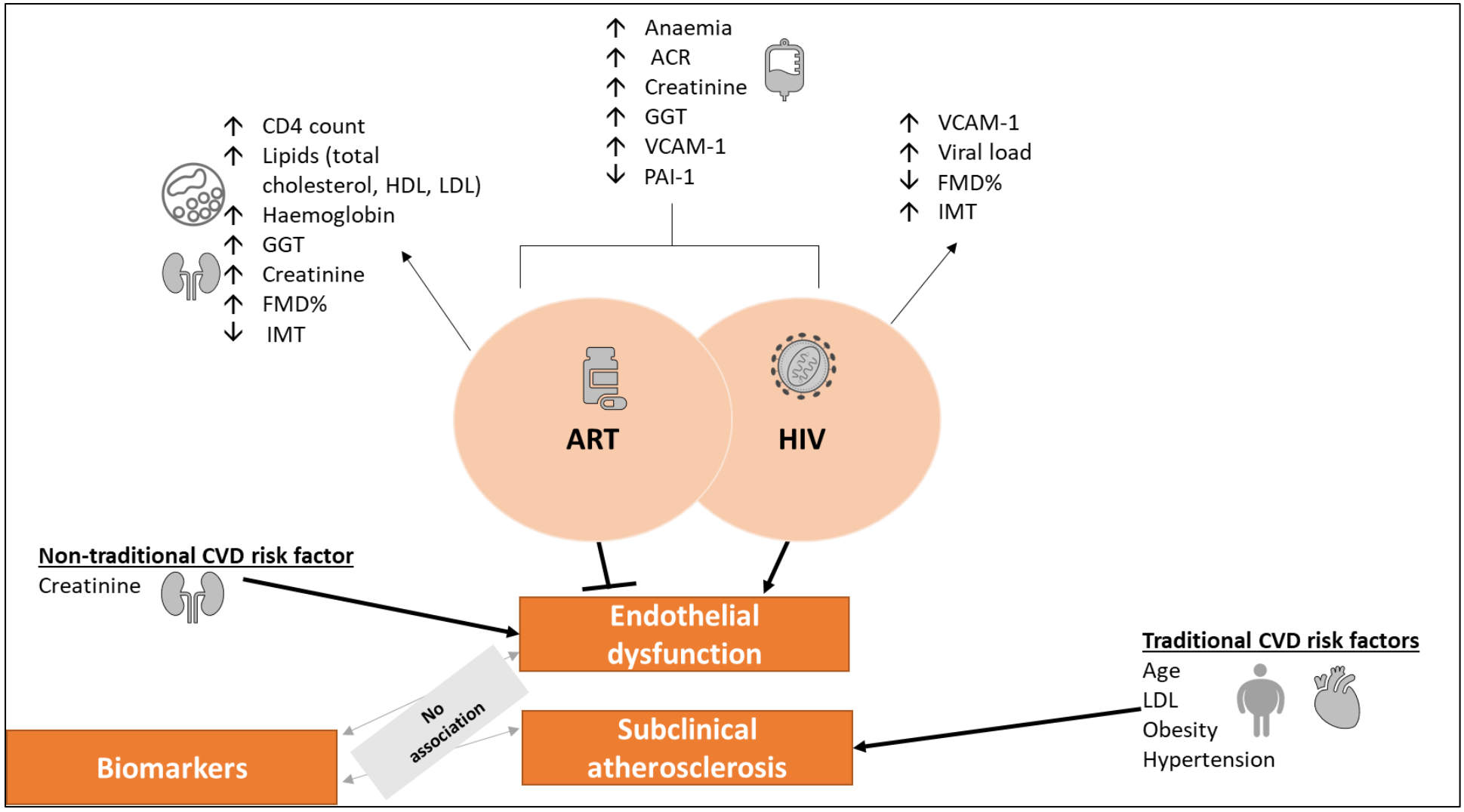


Figure 6.1 Schematic representation of the overall findings from the PhD. Figure designed by the author of this dissertation.

In conclusion, our study demonstrates a more cardioprotective and immune-protective effect of ART in HIV-infection, while a relatively higher presence of cardiovascular risk parameters in the HIV-free population. It is important to note that our study comprised of more females than males and although, no significant differences were noted in gender between the HIV-infected and HIV-free groups of the cross-sectional study, several findings were only found in the female groups when gender analysis was conducted, suggesting that many of the findings may have been driven by the female participants rather than their male counterparts. Similarly, the longitudinal study also noted findings in females rather than males when gender analysis was conducted, further suggesting the relevance of the current study's descriptive findings to the female population.

6.4 Advantages, limitations, and future directions

The study has several advantages. The total sample size in the cross-sectional study of n= 347, is higher than many studies in the literature such as Ross *et al.*, 2009, exploring similar research questions. The current study further used two separate epidemiological methodologies in addressing its aims, namely cross-sectional and longitudinal approach. In addition, the study utilized, for the first time, two non-invasive ultrasound-based vascular assessment methods (IMT and FMD) in identifying subclinical atherosclerosis and endothelial function in a Western Cape study population of people living with HIV. Additionally, the current study included both males and female adults from the Western Cape population of South Africa and to the best of our knowledge we are the first to report on endothelial function as measured by FMD% and subclinical atherosclerosis as measured by IMT, in a study population representative of the Western Cape's largest demographic group.

Furthermore, although it was concerning to report that not all HIV-infected individuals were receiving ART, we were able to include these individuals and conduct HIV subgroup analyses to compare the HIV-infected group receiving treatment with the HIV-infected group without treatment. This provided the present study with a unique opportunity to evaluate the role of ART in the HIV context.

However, the current study also has some limitations. The lack of temporal changes in subclinical atherosclerosis, endothelial function, biomarkers and cardiovascular risk parameters in the longitudinal study suggests that the 12-month follow up period may not have been sufficient in the current setting, and thus a longer follow up period is recommended to evaluate the progression of these parameters in the general population. Furthermore, the current study did not include HIV-infected individuals in the longitudinal study, thus cause and effect conclusions cannot be made in the HIV context. The cross-sectional study is also limited due its observational nature and can only address associations, not causality. Thus, longitudinal studies, of longer follow-up duration and including HIV-infected individuals are recommended. Additionally, our sample size in the longitudinal study was relatively

low with only n= 57, which may have influenced the statistical power and results. Thus, overall a larger sample size with a longer follow-up period is recommended.

Additionally, in both the cross-sectional and the longitudinal study, it was difficult to recruit equal number of male and female participants. Although there was no significant difference in gender in the cross-sectional HIV-infected and HIV-free groups, and although all regression models were adjusted for gender, many of the findings found were seemingly driven by the female participants (as demonstrated in the gender analysis). Similarly, many of the longitudinal study findings were apparently driven more by the female gender. Thus ideally, it would be valuable to either include equal participants from each gender group or rather study the research question in male and female populations separately in order to make specific conclusions in each sub-population. The current study, however, benefited from our attempts to analyse gender differences in each finding even though it was not specified as one of the original aims of the PhD study.

Furthermore, the method of measuring FMD% has been debated in the literature for its operator dependencies and lack of reproducibility. However, we did mitigate this risk to best of our ability by selecting only a few highly trained and experienced researchers (which included the author of this dissertation) to measure FMD%. Additionally, each operator was assigned specific tasks in the assessment which remained constant throughout the study. For quality control purposes, FMD results were also randomly selected and assessed by blinded experts. However, research on standardization in the FMD method is recommended. Similarly, with regards to the IMT measurement, standardization is required for the categorization of subclinical atherosclerosis and plaque. There are different cut off values present in the literature, thus standardization of these cut-offs in specific populations is warranted. Equally, endothelial dysfunction, measured by FMD% has not been defined clearly in clinical research. Literature largely evaluates relative FMD% measurements, thus clear standardization and definition are required to conclusively identify endothelial dysfunction. Finally, the current study measured common carotid IMT while many studies have also measured internal carotid IMT and bifurcation IMT. Thus, it would be valuable to measure IMT at different anatomical locations to conclusively identify subclinical atherosclerosis, and its associations with biomarkers and cardiovascular risk factors.

With regards to the biomarker analysis, the current study's biomarker analysis was based on a relatively small sample size as not all samples were included in the analysis due to budgetary restrictions, but rather chosen by randomization. This may have influenced the statistical power and results. Additionally, in the literature, several other biomarkers have been identified such as asymmetric dimethylarginine (ADMA), IL-6 and vWF (as described in chapter 2) for endothelial dysfunction and atherosclerosis. Thus, it is recommended to evaluate the relevance of these other biomarkers in the HIV-context with sufficient power. Furthermore, the current study did not clinically measure tobacco

consumption by evaluating cotinine. All lifestyle related questions were self-reported, and the possibility of under-reporting of lifestyle parameters is possible. Thus, it is recommended, where possible lifestyle data needs to be validated clinically, for instance with the measurement of cotinine to validate smoking. Ultimately the current study did not predict cardiovascular risk in the participants using known prediction and scoring methods such as Framingham risk score. Future studies are warranted, considering the above limitations.

6.5 Role of the PhD candidate in the current study

The PhD candidate performed all the IMT, FMD and blood pressure measurements in the participants of the current study. The candidate underwent intense training in both the IMT and FMD techniques. Additionally, as an active member of the EndoAfrica research team, the candidate also assisted in general laboratory work for both the studies, which included receiving blood and urine samples from research nurses, preparing the samples for storage as well as delivering the samples to the NHLS for further analyses. Some of the qualitative data was also collected by the PhD candidate, which included conducting the health and demographic questionnaire interviews. With regards to biomarker analysis, the PhD candidate prepared all serum samples according to the requirements of the Luminex analyses and the Division of Molecular Biology and Human Genetics, University of Stellenbosch. Meetings were conducted with the Luminex technician to acquire knowledge of the multiplex system and the overall methodology used to analyze biomarkers. Additionally, the PhD candidate captured and verified all data collected for the current study. Furthermore, the PhD candidate performed all statistical analysis for the PhD after consultation with a professional biostatistician from the Biostatistics Unit (Faculty of Medicine and Health Sciences, Stellenbosch University). Several engagements were conducted with the biostatistician to ensure the validity of the statistics and accurate interpretation of all statistical models completed for the PhD. Finally, the PhD candidate presented data from the current study at several conferences and intends to complete 2 original manuscripts for publications.

6.6 Research outputs related to the PhD candidate

Published Peer Reviewed articles originating from the EndoAfrica cohort

Everson F, Martens DS, Nawrot TS, Goswami N, Mthethwa M, Webster I, Mashele N, **Charania S**, Kamau F, De Boever P, Strijdom H. Personal exposure to NO2 and benzene in the Cape Town region of South Africa is associated with shorter leukocyte telomere length in women. Environmental Research. 2020 Mar 1;182:108993.

Everson F, De Boever P, Nawrot TS, Goswami N, Mthethwa M, Webster I, Martens DS, Mashele N, **Charania S**, Kamau F, Strijdom H. Personal NO2 and Volatile Organic Compounds Exposure Levels

are Associated with Markers of Cardiovascular Risk in Women in the Cape Town Region of South Africa. International Journal of Environmental Research and Public Health. 2019 Jan;16(13):2284.

Published Peer reviewed conference abstracts

Strijdom H, De Boever P, Nawrot T, Goswami N, Webster I, Mthethwa M, Mashele N, Kamau F, Martens D, **Charania S**, Everson F. Personal air pollution exposure is associated with markers of cellular aging and cardiovascular risk:Findings from the EndoAfrica study. SA Heart 2019;16(3):241.

Strijdom, H., Essop, M.S., Goswami, N., De Boever, P., Webster, I., Everson, F., Kamau, F.M., **Charania, S**., Nawrot, T.S. HIV-infected participants on combination ART (tenofovir, emtricitabine, efavirenz) have improved endothelial function and smaller retinal venular calibers compared to treatment naive participants. European Heart Journal 2019; 40 (Supplement_1): ehz746.0308. European Society of Cardiology Congress, Paris, France, 31 August – 4 September 2019.

Charania S, Webster I, Mashele N, Mthethwa M, Kamau F, Espach Y, Everson F, Cyster H, Goswami N, Boever P, Nawrot T, Strijdom H. Determinants of carotid intima-media thickness (CIMT) in a Western Cape study population with and without HIV infection. SA Heart 2018; 15(4):276.

Charania S, Webster I, Mashele N, Mthethwa M, Kamau F, Espach Y, Everson F, Cyster H, Goswami N, Boever P, Nawrot T, Essop F Strijdom.Endothelial function and sub-clinical atherosclerosis in a Western Cape study population with and without HIV-infection. First conference of Biomedical and Natural Sciences and Therapeutics. 7-10 October 2018.

Everson F, Goswami N, De Boever P, Nawrot T, Essop MF, Mthethwa, Mashele N, **Charania S**, Espach Y, Webster I, Strijdom H. The effect of a fixed-dose combination ART regimen on retinal microvascular calibres in a South African HIV-infected study population. 19th Annual SA Heart Congress, Sun City, South Africa, 4-7 October 2018. SA Heart. 2018; 15(4): 277-278.

Strijdom H, Kamau F, Goswami N, De Boever P, Nawrot TS, Essop MF, Mashele N, Mthethwa M, Espach Y, **Charania S**, Everson F, Webster I. Abdominal obesity and antiretroviral therapy are associated with improved endothelium-dependent vascular function in HIV-infected individuals: Results from the EndoAfrica study. 19th Annual SA Heart Congress, Sun City, South Africa, 4-7 October 2018. SA Heart. 2018; 15(4): 308.

Strijdom H, Goswami N, De Boever P, Nawrot T, Essop F, Mthethwa M, Mashele N, Everson F, **Charania S**, Espach Y, Webster I. Determinants of endothelial function in a cohort of HIV-infected and HIV-free participants: The role of cardiovascular risk factors, biomarkers of inflammation and HIV-dependent parameters. 86th European Atherosclerosis Society Congress, Lisbon, Portugal, 5-8 May 2018. Atherosclerosis. 2018; 275: e127.

Strijdom H, Goswami N, De Boever P, Nawrot TS, Kessler HH, Stelzl E, Webster I, Everson F, Mashele N, Mthethwa M, **Charania S**, Espach Y, Kamau F, Essop MF. Vascular health in HIV: the role of cardiovascular risk factors, biomarkers of inflammation, HIV disease status and antiretroviral therapy (results from the EndoAfrica study, Cape Town, South Africa). 12th International Symposium on Molecular Diagnostics, Graz, Austria, May/June 2018. Clin Chem Lab Med 2018; 56(6): eA106.

Strijdom H, **Charania S**, Goswami N, De Boever P, Nawrot T, Mashele N, Webster I, Westcott C, Everson F, Mthethwa M, Essop F. Cardiovascular health and flow-mediated dilatation (FMD) in a South African cohort of HIV-infected participants: Findings from the EndoAfrica Study. 85th European Atherosclerosis Society Congress, Prague, Czech Republic, 23-26 April 2017. Atherosclerosis. 2017; 263: e141.

Mashele, N, **Charania, S**, Essop, F, Webster, I, Westcott, C, Goswami, N, De Boever, P, Nawrot, T. and Strijdom, H, 2016. The effects of HIV-infection and anti-retroviral treatment on endothelial function in a South African cohort. 84th European Atherosclerosis Society Congress, Innsbruck, Austria, 29 May – 1 June 2016Atherosclerosis 2016, 252, pp.e162-e163.

Other Conference outputs

Charania S, Webster I, Mashele N, Mthethwa M, Kamau F, Espach Y, Everson F, Cyster H, Goswami N, Boever P, Nawrot T, Essop F Strijdom H. Sub-clinical atherosclerosis and endothelial function in a study population of HIV positive and negative individuals of South Africa. Stellenbosch University Biomedical Research Day November 2018.

Charania S, Webster I, Mashele N, Mthethwa M, Kamau F, Espach Y, Everson F, Cyster H, Goswami N, Boever P, Nawrot T, Strijdom H. Carotid intima media thickness (CIMT) in a cohort of HIV positive and negative individuals of South Africa. Stellenbosch University 62nd Annual Academic Day 29 August, 2018

Appendix A: Questionnaires used in the study

General Data

Study ID	_____
Date subject signed consent	_____ ((dd-mm-yyyy))
Date and time start of interview	_____
Date of birth	_____
Age when signed consent	_____
Gender	○ Male ○ Female
Ethnicity	○ Black ○ White ○ Coloured ○ Indian ○ Other (please specify) ○ Refused
Ethnicity other	_____
Please indicate the option that describes your current marital status.	○ Never married ○ Married ○ Living together ○ Widowed ○ Seperated or Divorced
Field site where patient was recruited	○ Elsies River ○ Ravensmead ○ Bishop Lavis ○ Durbanville ○ Uitsig ○ Adriaanse ○ Potchefstroom ○ Mthatha ○ Fisantekraal ○ Worcester
Field worker taking interview	○ Charmaine Abrahams ○ Shirley Mc Anda ○ Susan van Zyl ○ Cathy Swartz ○ Carol Stryers ○ Lila Apollis ○ Bernadine Fransman

Medical Background

Study ID	_____

MEDICAL BACKGROUND

Past

Have you had a heart attack in the past?	◯ Yes ◯ No
Have you been diagnosed with cancer in the past?	◯ Yes ◯ No
Have you previously had TB?	◯ Yes ◯ No
If yes, when did you previously have TB?	_____ (If patient cannot recall date, enter year (yyyy))
Have you had a stroke in the past?	◯ Yes ◯ No

Present

Do you have high blood pressure (hypertension)?	◯ Yes ◯ No ◯ Unknown
If yes, what year were you first diagnosed?	_____
Do you have a heart disease?	◯ Yes ◯ No ◯ Unknown
If yes, what year were you diagnosed?	_____
Do you have high cholesterol?	◯ Yes ◯ No ◯ Unknown
If yes, what year were you diagnosed?	_____
Do you have any other long lasting health problems? (For example: kidney stones, arthritis, asthma, bilharzia, malaria)	◯ Yes ◯ No
If yes, please specify what the health problem is and what year you were diagnosed?	_____
Do you currently have pulmonary Tuberculosis (TB)?	◯ Yes ◯ No
If yes, are you on treatment?	◯ Yes ◯ No
When did you start treatment?	_____ (If the patient cannot recall date, enter year (yyyy))

Diabetes

Do you have Diabetes	○ Yes ○ No
What type of Diabetes do you have?	○ Type I Diabetes (also known as Juvenile Onset or Insulin Dependent Diabetes) ○ Type II Diabetes (also known as Non-insulin Dependent Diabetes) ○ Don't know
How long ago were you told you have diabetes?	○ < 12 months ○ 1-5 years ○ 6-15 years ○ 15+ years
Which of the following do you use to manage your diabetes?	☐ Diet ☐ Pills ☐ Injection ☐ Nothing ☐ Other ((tick all that apply))
If other, please specify.	_____

Family History

Stroke And Heart Disease

	Don't know	No	Yes, under the age of 60	Yes, over the age of 60	Yes, but I don't know the age
Has your mother had any heart disease?	○	○	○	○	○
Has your father had any heart disease?	○	○	○	○	○
Has your mother ever had a stroke?	○	○	○	○	○
Has your father ever had a stroke?	○	○	○	○	○

Diabetes

	Don't know	No	Yes, Type I	Yes, Type II	Yes, but I don't know type
Has your mother had any Diabetes?	○	○	○	○	○
Has your father had any Diabetes?	○	○	○	○	○

High Blood Pressure and Cholesterol

	No	Yes	Don't know
Has your mother had high blood pressure?	○	○	○
Has your father had high blood pressure?	○	○	○
Has your mother had high cholesterol?	○	○	○
Has your father had high cholesterol?	○	○	○

HIV

Are you HIV positive? ○ Yes ○ No

If positive, are you on ART? ○ Yes ○ No

Which line of ART are you on? ○ 1st ○ 2nd

What is the name of the ART?

_____

For how long have you been on ART?

_____
((weeks))

Date you were diagnosed with HIV?

_____

If date unknown, how long ago approximately were you diagnosed with HIV

○ In last year
○ 2 - 5 years ago
○ >5 years ago
○ >15 years ago

Family Planning

Do you currently have a baby younger than 3 months? ○ Yes ○ No

Are you currently pregnant? ○ Yes ○ No ○ Not applicable

Are you currently breast feeding? ○ Yes ○ No

Are you on family planning? ○ Yes ○ No

Please ask following question at month 18 follow-up event.	
When was your last menstrual period? (days)	_____ (number)

Medications	
Do you take any medications?	○ Yes ○ No
Which medications do you take?	☐ Beta blockers ☐ Statins ☐ Aspirin ☐ Calcium channel blockers ☐ ACE inhibitors ☐ Other ☐ Anti-inflammatory
If other, specify	_____
What dosage of aspirin do you take?	_____ (mg)

Lifestyle

Study ID	_____

Cigarette Smoking

Are you a smoker	◯ Yes currently ◯ In the past ◯ Never smoked
What type of cigarette do/did you smoke?	☐ Snuf ☐ Tobacco ☐ Dagga
On average, how many cigarettes do you smoke on the days that you smoke?	◯ More than 20 daily ◯ Less than 20 daily
If you have stopped, how long has it been since you last smoked (months)?	_____
How many cigarettes do you smoke in a day?	_____ (number)
For how long have you been smoking? (Years)	_____ (number)
For how long did you smoke before you stopped? (Years)	_____ (number)
Pack years	_____

Alcohol

Have you consumed an alcoholic drink within the past 12 months?	◯ Yes ◯ No
How often do you typically drink ?	◯ Daily ◯ 8 or more days a month ◯ Less than 8 days a month
At what age did you start drinking regularly (at least once a week)?	_____ ((answer in years))

What do you drink?		
	Yes	No
Beer	○	○
Spirits (brandy, vodka, cane etc.)	○	○
Red Wine	○	○
White Wine	○	○
Other	○	○

When you drink beer, how many standard units do you typically have on a single occassion? (see reference card).	_____
When you drink spirits, how many standard units do you typically have on a single occassion? (see reference card).	_____
When you drink red wine, how many standard units do you typically have on a single occassion? (see reference card).	_____
When you drink white wine, how many standard units do you typically have on a single occassion? (see reference card).	_____
When you drink other, how many standard units do you typically have on a single occassion? (see reference card) and describe.	_____

Standard unit reference card

1 standard drink=

1 standard bottle or can of regular beer (340ml)

1 single measure of spirits (30ml)

1 medium size glass of wine (120ml)

How many drinks to you have on a weekend?	◯ Less than 5 ◯ 5 - 10 ◯ More than 10 (Friday night to Sunday night)

Eating Habits

How many meals do you have on a typical day?	◯ One ◯ Two ◯ Three ◯ More than three
Do these meals include fruit and/or vegatables?	◯ Yes ◯ No

Exercise	
Are you physically active?	◯ Yes ◯ No
If yes, how many times a week?	◯ Once ◯ Twice ◯ Three times ◯ More than three times
How would you describe the type of exercise you do?	◯ Mild ◯ Moderate ◯ Intensive

Environment	
In what type of environment do you live?	◯ City ◯ Suburb ◯ Countryside ◯ Industrial ◯ Other
If other, please specify	_____
Do you live on a main road?	◯ Yes ◯ No
Where you live, do you	◯ Rent a room in a house ◯ Rent a house/ self contained flat ◯ Own house/ flat ◯ Live with family ◯ Not have a usual place to live ◯ Live in a shelter (homeless) ◯ Live separately (backyard/garden) on a property ◯ Informal settlement (shack)
What type of fuel do you mostly use?	◯ Electricity ◯ Wood ◯ Parafin ◯ Coal
What is your main source of water for drinking and cooking?	◯ Private connection to pipeline ◯ Private well ◯ Public taps/standpipe ◯ Public well ◯ Neighbours ◯ Water vendor ◯ Spring ◯ River, stream, lake, pond ◯ Rainwater ◯ Bottled water ◯ Other

Sleeping Habits

On average how many hours of sleep do you get in a 24hr period?

- ◯ 1-3 hours
- ◯ 3-6 hours
- ◯ 6-9 hours
- ◯ > 9 hours

Do you currently do any shift work affecting your sleeping hours?

◯ Yes ◯ No

How well do you sleep?

Bad — Very well

(Place a mark on the scale above)

Rooibos Tea

Do you drink Rooibos tea?

◯ Yes ◯ No

How many cups do you drink on average day?

- ◯ 1 cup
- ◯ 2-3 cups
- ◯ 3-4 cups
- ◯ >4 cups

How long have you drunk Rooibos regularly?

- ◯ Never
- ◯ Less than a year
- ◯ More than a year
- ◯ My whole life

How do you drink your Rooibos tea?

- ☐ Milk
- ☐ Honey
- ☐ Sugar
- ☐ Lemon

Education

What level of education have you completed?

- ☐ None
- ☐ Primary school
- ☐ High school
- ☐ ABET (Adult Basic Education Training)
- ☐ College/University/Other tertiary institution

(Tick all that apply)

Employment

Which of the following applies to your current employment situation?

- ◯ Unemployed
- ◯ Employed (full time)
- ◯ Employed (part time)
- ◯ Self-employed

As someone who is unemployed, which of the following applies to you?

- ◯ Looking for work
- ◯ Discourage job seeker - not looking for work
- ◯ Student
- ◯ Homemaker
- ◯ Illness/disability prevent me to work
- ◯ Too old to work
- ◯ Other

If other, specify?

_____

Income	
Do you or someone in your household receive a Government Social Grant?	◯ Yes ◯ No
What is the total of your household income per month?	◯ less than R1,000 ◯ R1,000 - R4,999 ◯ R5,000 - R9,999 ◯ R10,000 - R20,000 ◯ more than R20,000

Appendix B: Cross-sectional study: Gender analysis

Differences between HIV-infected and HIV-free groups in male female groups separately

Table B1: All study parameters compared between HIV-infected and HIV-free in the male population

Variable	HIV-free	HIV-infected	P value
N	32 (33.3%)	64 (66.7%)	-
Flow-mediated dilatation (%)	5.19 ± 3.46	5.98 ± 4.59	0.391
Mean IMT (µm)	595.08 ± 105.14	648.88 ± 133.92	0.053
BMI	20.15 (18.13- 27.73)	20.18 (18.10- 25.10)	0.542
Waist circumference	83.00 (68.00- 99.50)	81.50 (72.25- 91.00)	0.966
Waist to hip ratio	0.89 (0.85- 0.96)	0.93 (0.87- 0.98)	0.201
Systolic Blood Pressure (mmHg)	126.00 (108.75- 137.50)	120.00 (108.25- 130.50)	0.451
Diastolic Blood pressure (mmHg)	84.50 (71.25- 96.00)	80.00 (73.25- 90.00)	0.408
Heart Rate (bpm)	64.85 (59.75- 77.75)	70.50 (64.00- 83.00)	0.125
Total cholesterol	4.31 (3.77- 5.05)	4.32 (3.80- 5.01)	0.852
High-Density Lipoprotein Cholesterol (HDL) (mmol/L)	1.32 (1.07- 2.01)	1.27 (0.99- 1.59)	0.129
Low-Density Lipoprotein Cholesterol (LDL) (mmol/L)	2.29 (1.86- 2.77)	2.37 (1.92- 3.01)	0.547
Fasting glucose (mmol/L)	4.60 (4.30- 5.20)	5.05 (4.63- 5.48)	0.061
Serum creatinine (µmol/L)	77.00 (69.00- 81.75)	72.00 (65.50- 82.75)	0.310
Baseline brachial artery diameter (mm)	3.76 ± 0.63	3.88 ± 0.64	0.385
Triglycerides	1.13 (0.74- 1.40)	1.26 (0.90- 1.73)	0.309
Glycated Haemoglobin (HbA1c) %	5.40 (5.23- 5.68)	5.40 (5.13- 5.60)	0.598
C-reactive protein	3.50 (1.30- 9.10)	4.35 (1.68- 13.18)	0.318
GGT (U/L)	32.00 (18.50- 96.50) [a]	56.00 (31.75- 102.75) [a]	0.012
Urine albumin (mg/L)	11.25 (1.83- 37.78)	8.10 (3.68- 37.58)	0.724
Albumin-to-creatinine ratio	0.68 (0.17- 2.08)	0.60 (0.40- 2.50)	0.581
CD4	-	463.86 ± 195.47	-
Tnf-α (pg/ml)	22.28 (20.04- 24.20)	24.32 (19.71- 27.39)	0.354
VCAM-1 (ng/ml)	688.53 (580.04- 1253.60)	880.54 (610.63- 1253.10)	0.531
ICAM-1 (ng/ml)	399.37 (241.17- 611.98)	378.47 (230.78- 834.19)	0.728
E-selectin (ng/ml)	32.40 (25.69- 51.87)	36.58 (23.47- 46.64)	0.841
P-selectin (ng/ml)	30.55 (25.36- 50.39)	35.48 (26.55- 47.99)	0.980
VEGF (pg/ml)	79.21 (37.36- 135.34)	71.07 (41.22- 163.55)	0.900

PAI-1 (ng/ml)	102.09 (78.66- 128.35)	80.19 (47.21- 99.62)	0.065

Data presented as mean ± SD or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). Means with same superscript letter: differ significantly ($p < 0.05$).

Table B2: All study parameters compared between HIV-infected and HIV-free in the female population

Variable	HIV-free	HIV-infected	P value
N	111 (44.2%)	140 (55.8%)	-
Flow-mediated dilatation (%)	7.18 ± 5.52	7.19 ± 6.00	0.992
Mean IMT (µm)	641.30 ± 142.95	614.57 ± 134.28	0.129
BMI	26.60 (21.40- 33.80)	22.98 (19.30- 28.48)	0.001
Waist circumference	94.00 (80.00- 105.00)	84.00 (75.00- 94.00)	0.000
Waist to hip ratio	0.89 (0.83- 0.94)	0.86 (0.83- 0.94)	0.254
Systolic Blood Pressure (mmHg)	125.00 (114.00- 139.00) [a]	118.50 (109.25- 133.50) [a]	0.021
Diastolic Blood pressure (mmHg)	86.67 (78.00- 96.00)	85.00 (77.00- 91.00)	0.076
Heart Rate (bpm)	70.00 (61.00- 77.00)	71.00 (65.00- 80.00)	0.098
Total cholesterol	4.30 (3.80- 4.88)	4.41 (3.87- 5.08)	0.388
High-Density Lipoprotein Cholesterol (HDL) (mmol/L)	1.32 (1.10- 1.52) [b]	1.49 (1.13- 1.86) [b]	0.007
Low-Density Lipoprotein Cholesterol (LDL) (mmol/L)	2.46 (1.91- 3.04)	2.33 (1.80- 2.91)	0.134
Fasting glucose (mmol/L)	4.60 (4.20- 5.00)	4.70 (4.30- 5.10)	0.178
Serum creatinine (µmol/L)	58.00 (52.00- 65.00)	57.00 (50.00- 63.00)	
Baseline brachial artery diameter (mm)	3.25 ± 0.55	3.18 ± 0.47	0.283
Triglycerides	0.93 (0.71- 1.33)	0.94 (0.76- 1.24)	0.950
Glycated Haemoglobin (HbA1c) %	5.40 (5.00- 5.60) [c]	5.20 (4.90- 5.40) [c]	0.004
C-reactive protein	5.25 (1.93- 8.98)	5.00 (1.58- 11.50)	0.665
GGT (U/L)	25.00 (17.00- 36.00) [d]	39.00 (27.00- 67.75) [d]	0.000
Urine albumin (mg/L)	8.40 (3.28- 19.13)	9.50 (4.70- 30.80)	0.065
Albumin-to-creatinine ratio	0.70 (0.40- 1.43) [e]	0.90 (0.50- 2.35) [e]	0.020
CD4	-	529.95 ± 254.93	-
Tnf-α (pg/ml)	22.25 (19.43- 27.57)	20.85 (17.67- 24.79)	0.130
VCAM-1 (ng/ml)	754.13 (546.42- 964.16) [f]	1018.70 (703.02- 1341.90) [f]	0.004
ICAM-1 (ng/ml)	363.48 (221.32- 635.43)	297.74 (163.69- 442.78)	0.118
E-selectin (ng/ml)	34.15 (28.12- 43.94)	32.31 (25.12- 43.65)	0.436
P-selectin (ng/ml)	36.60 (27.45- 40.18)	31.99 (23.64- 39.92)	0.178
VEGF (pg/ml)	99.64 (70.29- 142.58)	77.99 (53.23- 146.05)	0.301

PAI-1 (ng/ml)	97.09 (78.14- 120.70)	78.73 (62.50- 115.50)	0.115

Data presented as mean ± SD or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). Means with same superscript letter: differ significantly ($p < 0.05$).

Differences between male and female groups were analyzed in HIV-infected and HIV-free groups separately

Table B3: All study parameters compared between male and female groups in the HIV-free population

Variable	Male	Female	P value
N	32 (22.4%)	111 (77.6%)	-
Flow-mediated dilatation (%)	5.19 ± 3.46	7.18 ± 5.52	0.055
Mean IMT (µm)	595.08 ± 105.14	641.30 ± 142.95	0.096
BMI	20.15 (18.13- 27.73) [a]	26.60 (21.40- 33.80) [a]	0.000
Waist circumference	83.00 (68.00- 99.50) [b]	94.00 (80.00- 105.00) [b]	0.006
Waist to hip ratio	0.89 (0.85- 0.96)	0.89 (0.83- 0.94)	0.304
Systolic Blood Pressure (mmHg)	126.00 (108.75- 137.50)	125.00 (114.00- 139.00)	0.550
Diastolic Blood pressure (mmHg)	84.50 (71.25- 96.00)	86.67 (78.00- 96.00)	0.301
Heart Rate (bpm)	64.85 (59.75- 77.75)	70.00 (61.00- 77.00)	0.377
Total cholesterol	4.31 (3.77- 5.05)	4.30 (3.80- 4.88)	0.618
High-Density Lipoprotein Cholesterol (HDL) (mmol/L)	1.32 (1.07- 2.01)	1.32 (1.10- 1.52)	0.259
Low-Density Lipoprotein Cholesterol (LDL) (mmol/L)	2.29 (1.86- 2.77)	2.46 (1.91- 3.04)	0.310
Fasting glucose (mmol/L)	4.60 (4.30- 5.20)	4.60 (4.20- 5.00)	0.381
Serum creatinine (µmol/L)	77.00 (69.00- 81.75)	58.00 (52.00- 65.00)	
Baseline brachial artery diameter (mm)	3.76 ± 0.63 [c]	3.25 ± 0.55 [c]	0.000
Triglycerides	1.13 (0.74- 1.40)	0.93 (0.71- 1.33)	0.347
Glycated Haemoglobin (HbA1c) %	5.40 (5.23- 5.68)	5.40 (5.00- 5.60)	0.322
C-reactive protein	3.50 (1.30- 9.10)	5.25 (1.93- 8.98)	0.475
GGT (U/L)	32.00 (18.50- 96.50)	25.00 (17.00- 36.00)	0.010
Urine albumin (mg/L)	11.25 (1.83- 37.78)	8.40 (3.28- 19.13)	0.707
Albumin-to-creatinine ratio	0.68 (0.17- 2.08)	0.70 (0.40- 1.43)	0.883
CD4	-	-	-
Tnf-α (pg/ml)	22.28 (20.04- 24.20)	22.25 (19.43- 27.57)	0.982
VCAM-1 (ng/ml)	688.53 (580.04- 1253.60)	754.13 (546.42- 964.16)	0.799
ICAM-1 (ng/ml)	399.37 (241.17- 611.98)	363.48 (221.32- 635.43)	0.751
E-selectin (ng/ml)	32.40 (25.69- 51.87)	34.15 (28.12- 43.94)	0.834
P-selectin (ng/ml)	30.55 (25.36- 50.39)	36.60 (27.45- 40.18)	0.974
VEGF (pg/ml)	79.21 (37.36- 135.34)	99.64 (70.29- 142.58)	0.298
PAI-1 (ng/ml)	102.09 (78.66- 128.35)	97.09 (78.14- 120.70)	0.727

Data presented as mean ± SD or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). Means with same superscript letter: differ significantly ($p < 0.05$).

Table B4: All study parameters compared between male and female groups in the HIV-infected population

Variable	Male	Female	P value
N	64 (31.4%)	140 (68.6%)	-
Flow-mediated dilatation (%)	5.98 ± 4.59	7.19 ± 6.00	0.155
Mean IMT (µm)	648.88 ± 133.92	614.57 ± 134.28	0.092
BMI	20.18 (18.10- 25.10) [a]	22.98 (19.30- 28.48) [a]	0.004
Waist circumference	81.50 (72.25- 91.00)	84.00 (75.00- 94.00)	0.120
Waist to hip ratio	0.93 (0.87- 0.98) [b]	0.86 (0.83- 0.94) [b]	0.000
Systolic Blood Pressure (mmHg)	120.00 (108.25- 130.50)	118.50 (109.25- 133.50)	0.987
Diastolic Blood pressure (mmHg)	80.00 (73.25- 90.00)	85.00 (77.00- 91.00)	0.067
Heart Rate (bpm)	70.50 (64.00- 83.00)	71.00 (65.00- 80.00)	0.863
Total cholesterol	4.32 (3.80- 5.01)	4.41 (3.87- 5.08)	0.857
High-Density Lipoprotein Cholesterol (HDL) (mmol/L)	1.27 (0.99- 1.59) [c]	1.49 (1.13- 1.86) [c]	0.009
Low-Density Lipoprotein Cholesterol (LDL) (mmol/L)	2.37 (1.92- 3.01)	2.33 (1.80- 2.91)	0.361
Fasting glucose (mmol/L)	5.05 (4.63- 5.48) [d]	4.70 (4.30- 5.10) [d]	0.002
Serum creatinine (µmol/L)	72.00 (65.50- 82.75)	57.00 (50.00- 63.00)	
Baseline brachial artery diameter (mm)	3.88 ± 0.64 [e]	3.18 ± 0.47 [e]	0.000
Triglycerides	1.26 (0.90- 1.73) [f]	0.94 (0.76- 1.24) [f]	0.003
Glycated Haemoglobin (HbA1c) %	5.40 (5.13- 5.60) [g]	5.20 (4.90- 5.40) [g]	0.000
C-reactive protein	4.35 (1.68- 13.18)	5.00 (1.58- 11.50)	0.807
GGT (U/L)	56.00 (31.75- 102.75) [h]	39.00 (27.00- 67.75) [h]	0.004
Urine albumin (mg/L)	8.10 (3.68- 37.58)	9.50 (4.70- 30.80)	0.546
Albumin-to-creatinine ratio	0.60 (0.40- 2.50)	0.90 (0.50- 2.35)	0.158
CD4	463.86 ± 195.47	529.95 ± 254.93	0.070
Tnf-α (pg/ml)	24.32 (19.71- 27.39) [i]	20.85 (17.67- 24.79) [i]	0.025
VCAM-1 (ng/ml)	880.54 (610.63- 1253.10)	1018.70 (703.02- 1341.90)	0.282
ICAM-1 (ng/ml)	378.47 (230.78- 834.19)	297.74 (163.69- 442.78)	0.174
E-selectin (ng/ml)	36.58 (23.47- 46.64)	32.31 (25.12- 43.65)	0.555
P-selectin (ng/ml)	35.48 (26.55- 47.99)	31.99 (23.64- 39.92)	0.145
VEGF (pg/ml)	71.07 (41.22- 163.55)	77.99 (53.23- 146.05)	0.675
PAI-1 (ng/ml)	80.19 (47.21- 99.62)	78.73 (62.50- 115.50)	0.295

Data presented as mean ± SD or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). Means with same superscript letter: differ significantly ($p < 0.05$).

Appendix C: Longitudinal sub-study: male and female population analysis

Table C1: Differences in all study parameter measurements between baseline and 12-month follow up in the male population

Variable	Baseline (time point 1)	12-month follow up (time point 2)	N	Difference (Time point 1- Time point 2) [a]	P value
Flow-mediated dilatation (%)	2.24 ± 5.10	3.96 ± 3.95	7	-1.72	0.595
Mean IMT (µm)	668.50 ± 114.88	525.43 ± 240.64	7	143.07	0.212
BMI	21.51 ± 6.09	23.06 ± 7.03	7	-1.54	0.160
Waist circumference	75.00 ± 13.18	77.14 ± 17.16	7	-2.14	0.332
Waist to hip ratio	0.85 ± 0.09	0.85 ± 0.08	7	0	0.821
Systolic Blood Pressure (mmHg)	129.29 ± 16.12	140.43 ± 17.32	7	-11.14	0.064
Diastolic Blood pressure (mmHg)	88.57 ± 11.00	93.14 ± 8.69	7	-4.57	0.219
Total cholesterol	4.20 ± 0.67	4.27 ± 1.00	7	-0.07	0.749
High-Density Lipoprotein Cholesterol (HDL) (mmol/L)	1.50 ± 0.41	1.55 ± 0.70	7	-0.05	0.741
Low-Density Lipoprotein Cholesterol (LDL) (mmol/L)	2.20 ± 0.61	2.21 ± 0.77	7	-0.01	0.946
Fasting glucose (mmol/L)	5.20 (4.40- 6.00)	4.60 (4.30- 5.20)	7	-1.99	0.046
Serum creatinine (µmol/L)	83.86 ± 14.23	83.43 ± 18.14		0.43	0.922
Baseline brachial artery diameter (mm)	4.03 ± 0.69	3.85 ± 0.64		0.18	0.460
Triglycerides	0.74 (0.59- 1.96)	1.14 (0.68- 1.33)	7	-0.85	0.398
Glycated Haemoglobin (HbA1c)	5.47 ± 0.34	5.61 ± 0.25	7	-1.27	0.206
C-reactive protein	4.30 (1.00- 21.80)	3.50 (0.60- 19.40)	0	-0.51	0.612
GGT (U/L)			0	-1.52	0.128
Urine albumin (mg/L)	8.00 (5.60- 19.50)	11.30 (11.20- 120.10)	7	-1.35	0.176
Albumin-to-creatinine ratio	0.50 (0.30- 0.80)	1.30 (0.40- 6.80)	7	-1.78	0.075
Tnf-α (pg/ml)	22.19 (19.44- 36.65)	24.35 (19.56- 38.56)	3	-0.54	0.593
VCAM-1 (ng/ml)	1061.9 (602.01- 1176.90)	676.75 (609.83- 1139.50)	3	-1.60	0.109
ICAM-1 (ng/ml)	630.95 (385.95- 1146.50)	740.35 (412.58- 1290.40)	3	-1.60	0.109
E-selectin (ng/ml)	35.65 (33.97- 50.53)	29.15 (23.49- 52.55)	3	-0.54	0.593

P-selectin (ng/ml)	29.88 (27.31-84.53)	30.55 (27.68-92.33)	3	-1.60	0.109
VEGF (pg/ml)	109.09 (96.83-155.68)	154.00 (100.07-224.94)	3	0	1.000
PAI-1 (ng/ml)	114.13 (56.09-145.23)	153.35 (100.07-224.94)	3	-0.54	0.593

Data presented as mean ± SD or n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. [a] **Negative difference indicates an increase in the variable at 12-month follow up**

Table C2: Differences in all study parameter measurements between baseline and 12-month follow up in the female population

Variable	Baseline (time point 1)	12-month follow up (time point 2)	N	Difference (Time point 1- Time point 2) [a]	P value
Flow-mediated dilatation (%)	6.22 ± 4.37	7.05 ± 6.40	47	-0.64	0.463
Mean IMT (µm)	648.66 ± 126.90	655.53 ± 153.18	49	-6.87	0.717
BMI	29.28 ± 8.06	29.28 ± 7.86	49	0.01	0.974
Waist circumference	92.02 ± 16.32	93.74 ± 16.64	49	-1.72	0.053
Waist to hip ratio	0.88 ± 0.09	0.88 ± 0.09	49	0.01	0.325
Systolic Blood Pressure (mmHg)	125.52 ± 20.34	131.12 ± 21.71	49	-5.60	0.013
Diastolic Blood pressure (mmHg)	84.09 ± 11.83	88.66 ± 14.12	49	-4.57	0.002
Total cholesterol	4.34 ± 0.70	4.30 ± 0.88	49	0.04	0.573
High-Density Lipoprotein Cholesterol (HDL) (mmol/L)	1.36 ± 0.28	1.32 ± 0.28	49	0.03	0.204
Low-Density Lipoprotein Cholesterol (LDL) (mmol/L)	2.46 ± 0.69	2.47 ± 0.82	48	0.02	0.726
Fasting glucose (mmol/L)	4.85 (4.30- 5.60)	4.65 (4.30- 5.10)	49	-1.73	0.084
Serum creatinine (µmol/L)	61.16 ± 12.66	60.16 ± 13.38	49	1.00	0.348
Baseline brachial artery diameter (mm)	3.33 ± 0.55	3.34 ± 0.63	48	-0.02	0.747
Triglycerides	1.00 (0.67- 1.41)	1.04 (0.74- 1.45)	50	-0.15	0.882
Glycated Haemoglobin (HbA1c)	5.55 ± 1.22	5.58 ± 1.04	50	-1.50	0.133
C-reactive protein	5.10 (1.25- 10.20)	2.80 (0.80- 9.40)	0	-1.16	0.245
GGT (U/L)	26.00 (16.00- 42.50)	27.00 (16.00- 38.50)	0	-1.31	0.189
Urine albumin (mg/L)	6.75 (2.88- 15.75)	7.40 (3.00- 15.40)	50	-0.40	0.692
Albumin-to-creatinine ratio	0.60 (0.31- 1.33)	0.60 (0.38- 1.03)	50	-0.02	0.983
Tnf-α (pg/ml)	22.95 (20.27- 26.86)	22.49 (19.44- 27.83)	33	-0.84	0.404
VCAM-1 (ng/ml)	721.31 (552.17- 1025.36)	725.84 (542.13- 968.51)	33	-0.35	0.730
ICAM-1 (ng/ml)	411.01 (237.62- 616.40)	346.77 (205.00- 624.73)	33	-0.43	0.670
E-selectin (ng/ml)	33.83 (27.4- 45.05)	34.82 (28.36- 44.35)	33	-0.30	0.765
P-selectin (ng/ml)	36.91 (31.54- 45.90)	36.88 (27.98- 40.60)	33	-0.86	0.388

VEGF (pg/ml)	95.31 (78.74-147.44)	102.12 (69.91-142.39)	33	-1.94	0.052
PAI-1 (ng/ml)	90.75 (77.58-111.45)	97.00 (77.97-119.70)	33	-0.25	0.802

Data presented as mean ± SD or n (% of group) or median with 25th and 75th percentiles for interquartile range (for non-normally distributed data). n, number of participants; SD, standard deviation. a Negative difference indicates an increase in the variable at 12-month follow-up.

Table C3: Cross tabulation of BMI categories using McNemar-Bowker Test

		BMI categories Time point 2				Total
		Underweight	Normal weight	Overweight	Obesity	
BMI categories Time point 1	Underweight	4	2	0	0	6
	Normal weight	0	14	3	0	17
	Overweight	0	1	10	2	13
	Obesity	0	0	1	20	21
Total		4	17	14	22	57

p value= 0.343.

Table C4: Cross tabulation of Waist to hip ratio (WHR) categories using McNemar-Bowker Test

		WHR categories Time point 2			Total
		Low	Moderate	High	
WHR categories Time point 1	Low	13	0	0	13
	Moderate	3	6	5	14
	High	1	7	22	30
Total		17	13	27	57

p value= 0.228

Table C5: Cross tabulation of systolic blood pressure categories using McNemar-Bowker Test

		SBP categories Time point 2				Total
		low	normal	elevated	high	
SBP categories Time point 1	low	13	6	7	3	29
	normal	3	2	3	1	9
	elevated	0	2	1	1	4
	high	0	2	2	11	15
Total		16	12	13	16	57

p= 0.065

Table C6: Cross tabulation of diastolic blood pressure categories using McNemar-Bowker Test

		DBP categories Time point 2				Total
		low	normal	elevated	high	
DBP categories Time point 1	low	11	2	2	5	20
	normal	3	3	2	4	12
	elevated	0	2	1	6	9
	high	0	0	4	12	16
Total		14	7	9	27	57

p= 0.072

Appendix D: Effect size categories

Table D1: Effect size categories

ANALYSIS	EFFECT SIZE	SMALL	MEDIUM	LARGE
Chi-Square Independence Test	Cohen's W	0.1	0.3	0.5
Chi-Square Independence Test	Contingency Coefficient	0.1	0.29	0.45
Chi-Square Independence Test	Cramér's V	-	-	-
Chi-Square Goodness-of-Fit Test	Cohen's W	0.1	0.3	0.5
Independent Samples T-Test	Cohen's D	0.2	0.5	0.8
Independent Samples T-Test	Rpb - Point-Biserial Correlation	0.1	0.24	0.37
Paired Samples T-Test	Cohen's D	0.2	0.5	0.8
One-Sample T-Test	Cohen's D	0.2	0.5	0.8
Pearson Correlation	R - Correlation	0.1	0.3	0.5
ANOVA	ω^2 - Omega Squared	0.01	0.06	0.14
ANOVA	η^2 - (Partial) Eta Squared	0.01	0.06	0.14
ANOVA	Cohen's F	0.1	0.25	0.4
Linear Regression - Entire Model	Model R^2 - R Squared	0.02	0.13	0.26
Linear Regression - Entire Model	Model F^2 - F Squared	0.02	0.15	0.35
Linear Regression - Individual Predictor	Predictor R^2sp - Squared Semipartial ("Part") Correlation	0.02	0.13	0.26
Linear Regression - Individual Predictor	Predictor F^2 - F Squared	0.02	0.15	0.35

Highlighted effect size used in the current PhD study to measure effect sizes.

Appendix E: New IMT cut off

Table E1: Carotid IMT and subclinical atherosclerosis measurements of the total, HIV-free and HIV-infected study groups with new cut off values

Variable	Total cohort	HIV-free	HIV-infected	P value
Mean IMT (µm)	627.74 ± 135.37	631.21 ± 136.61	625.33 ± 134.79	0.69
IMT categories				0.98
Subclinical atherosclerosis [a] (> 780 µm), n (%)	33 (9.5%)	14 (9.8%)	19 (9.3%)	
Plaque [b] (> 1000 µm), n (%)	7 (2.0%)	3 (2.1%)	4 (2%)	

Data presented as mean ± SD or n (% of group). n, number of participants; SD, standard deviation. Means or medians with same superscript letter: differ significantly ($p < 0.05$). [a] Subclinical atherosclerosis cut off was based on guidelines by Ssinabulya *et al.*, 2014; Sarfo *et al.*, 2019; Schoffelen *et al.*, 2015; de Groot *et al.*, 2004 [b] Plaque was defined as an average IMT of more than 1000 µm, as suggested by Roozen et al., 2020.

Table E2: Carotid IMT and subclinical atherosclerosis measurements of the HIV/ no ART and HIV with ART study groups with new cut off values

Variable	HIV-infected/ no ART	HIV-infected + ART	P value
Mean IMT (µm)	530.33 ± 92.79 [a]	633.24 ± 135.17 [a]	0.004
IMT categories			0.33
Subclinical atherosclerosis [b] (> 780 µm), n (%)	0 (0%)	19 (10.1%)	
Plaque [c] (> 1000 µm), n (%)	0 (0%)	4 (2.1%)	

Data presented as mean ± SD or n (% of group). n, number of participants; SD, standard deviation. Means or medians with same superscript letter: differ significantly ($p < 0.05$). [b] Subclinical atherosclerosis cut off was based on guidelines by Ssinabulya *et al.*, 2014; Sarfo *et al.*, 2019; Schoffelen *et al.*, 2015; de Groot *et al.*, 2004. [c] Plaque was defined as an average IMT of more than 1000 µm, as suggested by Roozen et al., 2020

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