INVESTIGATING THE EFFECTS OF HAART ON EARLY MARKERS OF CARDIOVASCULAR DISEASE AMONG HIV-POSITIVE PATIENTS IN THE MANKWENG DISTRICT, LIMPOPO PROVINCE

by

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DECLARATION

I declare that the thesis hereby submitted to the University of Limpopo, for the degree of Doctor of Philosophy & Cardiovascular and HIV Research has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

Hanser, S (Mr) 21 April 2021

Surname, Initials, (Title) **Date**

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ABSTRACT

Background: Human immunodeficiency virus (HIV)-infection remains a major public health burden where approximately 38 million people are affected globally. Human immunodeficiency virus infection is associated with chronic inflammation which can lead to endothelial dysfunction and thrombosis, which are precursor events for cardio-metabolic abnormalities such as dysglycaemia and dyslipidaemia. The degree of chronic inflammation, endothelial dysfunction, and hypercoagulation among HIV-positive adults on highly active antiretroviral therapy are not well understood in Sub-Saharan Africa. The objective of this study was to determine the effect of highly active antiretroviral therapy (HAART) on chronic inflammation, endothelial dysfunction, and hypercoagulation among HAART-exposed adult South African participants in a rural setting.

Aim: The study aimed to determine the effects of HAART on early biomarkers of cardiovascular disease in the HIV-positive subjects.

Methods: The study was cross-sectional, descriptive, and quantitative in design. The research population consisted of 158 participants of males and females within the age range of 18 – 81 years from Mankweng Hospital and surrounding clinics. The study population comprised of three groups, HIV-negative (control group), HIV-positive treatment naïve (HAART-naïve group), and HIV-positive participants on HAART (HAART-exposed group). Weight and height were measured using Omron BF 400 and a portable stadiometer respectively, to calculate the body mass index. Glucose and lipid levels were determined on Cobas® Integra 400 plus auto-analyser. The $CD4^+$ T cell count was determined on the Cytomics FC500 Flow Cytometer Multi-Platform loader. The concentration of fibrinogen, c-reactive protein (CRP), L-selectin, D-dimers, P-selectin, von Willebrand factor (VWF), soluble intercellular adhesion molecule (sICAM-1), and soluble vascular cell adhesion molecule (sVCAM-1) in serum samples were determined on the Luminex 200^{TM}. Data were analysed using SPSS version 25.0. Descriptive statistics were performed on all variables and analysis of covariance was used to determine differences across all groups. Correlation coefficients and multiple regression analyses were used to determine associations.

Results: Body mass index (BMI) and glucose metabolism were not significantly affected by HAART exposure. However, the HAART-exposed group had significantly increased LDL-C ($F(2, 154) = 7.501$, $p = 0.001$) and TC ($F(2, 154) = 9.174$, $p =$

0.0002) levels. The prevalence of high LDL-C levels was significantly elevated in the HAART-exposed group (29.6%) ($p = 0.041$). The prevalence of pre-diabetes (11.3%) was the highest among the HAART-exposed group (non-significant), although, no significant difference was observed. While P-selectin was significantly reduced in the HAART-exposed group ($F (2, 154 = 7.253$, $p = 0.001$). On the other hand, the HAART-exposed group also significantly increased VWF ($F (2, 154 = 4.556$, $p = 0.011$). The HAART-exposed group showed no significant effect on L-selectin, sICAM-1, sVCAM-1, CRP, fibrinogen and D-dimer levels. However, D-dimer was negatively associated with HAART ($r = -0.249$, $p = 0.011$). There were significant independent association between the combined HAART regimens and P-selectin (Std $\beta = 0.219$, $p = 0.032$), first-line regimen with both P-selectin (Std $\beta = 0.434$, $p = 0.004$) and sVCAM-1 Std $\beta = 0.328$, $p = 0.031$), second-line regimens with L-selectin (Std $\beta = 1.032$, $p = 0.005$) and, a positive independent association between first-line regimen and D-dimer ($\beta = 0.741$, $p = 0.0001$). Although BMI and glucose metabolism were not significantly affected in both the HAART-exposed and HAART-naïve groups, dyslipidaemia was present across the three groups (HAART-exposed, HAART-naïve and control). HAART-exposure showed a protective effect by reducing endothelial dysfunction (ED) and hypercoagulation. Yet, ED was still present among this rural South African HAART-exposed population. The HAART-exposed group may be at increased risk for CVD. Therefore, CVD should be regularly monitored in the HAART-exposed population.

TABLE OF CONTENT

PAGE

LIST OF TABLES

PAGES

LIST OF FIGURES

PAGES

LIST OF ABBREVIATIONS

3TC:	Lamivudine
ABC:	Abacivir
ADMA:	Asymmetric dimethyl arginine
ADP:	Adenosine diphosphate
AIDS:	Aquired immunodefiency syndrome
ARV:	Antiretroviral
ATP:	Adenosine triphosphate
AZT:	Zidovudine
CAMs:	Cell-adhesion molecules
CCMDD:	Centralised chronic medicine dispensing and distribution
CCR5:	Chemokine co-receptor type 5
$CD4^+$:	Cluster of differentiation 4
CAD:	Coronary artery disease
CHD:	Coronary heart disease
CI:	Confidence interval
CIMT:	Carotid intima-media thickness
CRP:	C-reactive protein
CVD:	Cardiovascular disease
CXCR4:	Chemokine co-receptor type 4
D:A:D:	Data Collection on Adverse events of Anti-HIV Drugs
DHAP:	Dihydroxyacetone phosphate
DM:	Diabetes mellitus
DNA:	Deoxyribonucleic acid
DTG:	Dolutegravir
ED:	Endothelial dysfunction
EDHF:	Endothelial-derived hyperpolarizing factor
EDTA:	Ethylenediaminetetra acetic acid
EFV:	Efavirenz
eNOS:	Endothelial nitric oxide synthase
ER:	Endoplasmic reticulum
FDA:	Food and Drug Administration
FDPs:	Fibrin degradation products

FRAM:	Fat Redistribution and Metabolic Change in HIV Infection
FTC:	Emtricitabine
G6P:	Glucose-6-phosphate
G6PD:	Glucose-6-phosphate dehydrogenase
gp120:	Glycoprotein 120
gp41:	Gluco-protein 41
$H_2O_{2:}$	Hydrogen peroxide
HAART:	Highly active antiretroviral therapy
HDL-C:	High-density lipoprotein
HIV:	Human immunodeficiency virus
HIV-1:	HIV type 1
HIV-2:	HIV type 2
HK:	Hexokinase
hsCRP:	High sensitivity C-reactive protein
FVII:	factor VII
IGT:	Impaired glucose tolerance
IL-6:	Interleukin-6
iNOS:	Inducible nitric oxide synthase
IR:	Insulin resistance
IQRs:	Interquartile ranges
LDL-C:	Low-density lipoprotein cholesterol
LPV/r:	Liponavir/ritonavir
MFI:	Mean fluorescence intensity
Mg^{2+}:	Magnesium
MI:	Myocardial infarction
Mks:	Megakaryocytes
MMP:	Matrix-metalloproteinase
MPL:	Multi-Platform loader
n:	Sample size
$NADP^{+}$:	Nicotinamide adenine dinucleotide phosphate
NADPH:	Nicotinamide adenine dinucleotide phosphate
NCDs:	Non-communicable diseases
Nef:	Negative regulatory factor
nNOS:	Neuronal nitric oxide synthase

NNRTIs:	Non-nucleoside reverse transcriptase inhibitors
NO:	Nitric oxide
NOS:	Nitric Oxide Synthase
NRTIs:	Nucleoside reverse transcriptase inhibitors
NVP:	Nevirapine
$ONOO^-$:	Peroxynitrite
OS:	Oxidative stress
ANOVA:	One-way analysis of variance
ANCOVA:	One-way analysis of covariance
PAI-1:	Plasminogen activator inhibitor-1
PGI2:	prostacyclin
PIs:	Protease inhibitors
PLG:	Panleucogating
PLWH:	People living with HIV
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
RCT:	Reverse cholesterol transport
RPV:	Rilpivirine
RTV:	Ritonavir
sICAM-1:	soluble Intercellular adhesion molecule-1
sVCAM-1:	soluble Vascular cell adhesion molecule-1
SSA:	Sub-Saharan Africa
STD:	Standard deviation
SPSS:	Statistical Package for Social Sciences
T2DM:	Type 2 diabetes mellitus
Tat:	Trans-activator of transcription
TC:	Total cholesterol
TDF:	Tenofovir
TG:	Triglycerides
TNF-α:	Tumor necrosis factor
tPA-1:	tissue plasminogen activator-1
TM:	Trademark
TREC:	Turfloop Campus Research Ethics Committee
UL:	University of Limpopo

uPA-1:	Urokinase plasminogen-1
UTT:	Universal test and treat
UNAIDS:	Joint United Nations Programme on HIV/AIDS
USA:	United States of America
VL:	Viral load
VSMCs:	Vascular smooth muscle cells
VWF:	Von Willebrand factor
WCC:	White cell count
WHO:	World Health Organisation

CHAPTER 1

BACKGROUND

1.1 INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is a complicated condition that develops with time when an individual has been infected with HIV (Human Immunodeficiency Virus). This virus is a member of the lentivirus group within the family Retroviridae (Eberlé and Gütler, 2012; Zhang *et al.,* 2015). With its high transmission rate, this debilitating condition presents with a variety of clinical features such as unexplained diarrhoea, anaemia, fatigue, and skin discoloration (Bhusal *et al.,* 2016; Hoffbrand and Moss, 2016).

Human immunodeficiency virus and AIDS have caused approximately 35 million deaths and has infected over 70 million individuals globally since its discovery 35 years ago. HIV remains a major public health burden worldwide where approximately 690 000 (500 000 – 970 000) died of AIDS-related complications in the year 2019 (UNAIDS, 2020). The Joint United Nations Programme on HIV/AIDS (UNAIDS) reported that globally approximately 38 million people were living with HIV in 2019, of which approximately 36.2 million were adults (UNAIDS, 2020). The majority of people currently living with HIV and AIDS reside in Sub-Saharan Africa (SSA). This includes low-and-middle-income countries such as South Africa, Ghana, Angola, and Botswana (UNAIDS, 2017). At present South Africa is one of the countries with the highest infection rate (Vermund *et al.,* 2015; Blecher *et al.,* 2016).

HIV infection can lead to metabolic and thrombotic abnormalities that can cause cardiovascular complications, such as atherosclerosis and endothelial damage (Freiberg and So-Armah, 2016; Husain *et al.,* 2017). The metabolic abnormalities can manifest as hyperglycaemia, obesity, and dyslipidaemia (Husain and Ahmed, 2015; Anyabolu, 2016; Njuguna *et al.,* 2018). HIV infection is associated with chronic

inflammation which can cause factor VII hyperactivity and result in vascular tissue damage (Hijmans *et al.,* 2019).

Blood vessels, including those in the coronary circulation, consist, of among others, the endothelium, the innermost layer (Rajendran *et al.,* 2013). Damage to or activation of the endothelium, as typically observed during inflammation, can adversely affect its functionality and many of the endothelial biomarkers. Alterations to these intracellular biological molecules may, in time, induce cardiovascular damage (Rajendran *et al.,* 2013).

The vascular endothelial cells are constantly exposed to high concentrations of highly active antiretroviral therapy (HAART) and its direct side effects may lead to endothelial dysfunction (ED). Endothelial dysfunction is found in all forms of cardiovascular disease (CVD) where several studies have demonstrated a strong link between HAART and ED. The role of inflammation, endothelial activation, and ED in the development of atherosclerosis are important and several biomarkers such as VCAM-1, ICAM-1, and VWF has been reported to be reliable indicators of ED in atherosclerosis (Castellon and Bogdanova, 2015; Gimbrone and García-Cardeña, 2016).

The original predetermined sample size was 155; however, a total number of 158 participants were recruited in this study. The sample size (n = 155) was determined based on the prevalence of people living with HIV in the Limpopo Province, which is 11.4% with a confidence interval (CI) of 95% and margin error of 5% (Day and Gray, 2012). The huge disparity in the present study in terms of the number of participants per group were due to the availability of participants

Highly active antiretroviral therapy, a potent combination of antiretroviral (ARV) drugs, is specifically used to treat HIV infection by eradicating the virus and control its replication (Insight Start Study Group., 2015). The introduction of HAART directly after diagnosis of HIV infection has effectively reduced AIDS-related deaths and it further improved the health and life expectancy among HIV-positive patients (Mills *et al.,* 2011; Deeks *et al.,* 2013). With the introduction of HAART, HIV infection is no longer considered a “death sentence”, but a chronic disease. However, there is evidence that

HAART induces metabolic abnormalities such as dyslipidaemia, insulin resistance (IR), and dysglycaemia, all of which are considered risk factors for cardiovascular disease (CVD) (Gutierrez and Balasubramanyam, 2012; Abrahams *et al.,* 2015). These can complicate therapeutic interventions and the management of the disease and other co-morbidities.

The need for effective and comprehensive health care management systems and safe treatment of HIV-infected patients is required. Managing both HIV and its treatment in conjunction with the cardiovascular risk profile of all HIV-positive individuals is important. It has the potential to reduce the high co-morbidities and mortalities caused by the CVD risk factors observed currently in the HIV-positive population. The incidence of CVD in the HIV-positive population can be reduced through informed population-based intervention programs that specifically focus on these modifiable CVD risk factors. The advent of HAART, as part of the management program, significantly reduced the incidence of opportunistic infections and improved the quality of the HIV-positive subjects' lives (Hemkens and Bucher, 2014). Yet, it is important to understand the mode of action of HAART and its possible side-effects for safe treatment of CVD, an area that still needs extensive elucidation. The present study planned to determine the potential effects of HAART on the early cardiovascular markers among HIV-positive individuals in South Africa, specifically in the rural regions of Limpopo Province, where such information has been scarce. Most studies assessing ED make use of one or two ED biomarkers. In the present study we elucidated on the interplay of the role of HAART on endothelial function with the use of five different biomarkers of ED in a HIV-positive rural black South African population.

1.2 PROBLEM STATEMENT

South Africa is regarded as one of the countries with a high HIV transmission rate. Cardiovascular disease-related deaths are 1.5 – 2.0 times higher in HIV-positive patients compared to the general population (D:A:D Study Group *et al.,* 2010). Furthermore, these mortalities have also been linked to HIV infection and HAART (Wada *et al.,* 2014). Since metabolic disorders, endothelial biomarker abnormalities,

coagulation and inflammatory disorders, are early CVD risk factors in HIV-infected patients, improved HIV infection status following HAART treatment would reflect changes in the patients. However, the effect of HAART on early CVD markers is poorly understood, especially regarding rural populations in South Africa. Therefore, the focus of this study was to investigate whether endothelial function, inflammation and coagulation activity of HIV-positive rural South Africans will be affected by HAART.

1.3 RESEARCH QUESTION

What is the effect of HIV and subsequent effect of HAART on early markers of CVD among HIV positive subjects in the Mankweng District, Limpopo Province?

1.4 AIM AND OBJECTIVES

The aim and objectives of the study were to investigate the impact of HAART on endothelial dysfunction (ED) and coagulating factors as early markers of CVD among HIV-positive subjects in the Mankweng District, Limpopo Province.

1.4.1 Aim of the study

To determine the effect of HAART therapy on early markers of CVD among the HIV-positive population in the Mankweng District.

1.4.2 Specific objectives

The objectives of this study are to:

I. Determine BMI as an indicator of body fatness.
II. Determine the glucose levels (for hyperglycemia screening).

III. Determine the lipid profile (TC, LDL-C, HDL-C, and TG) as cardiovascular markers.
IV. Measure the endothelial biomarkers L-selectin, P-selectin, sICAM, sVCAM, and VWF as early CVD risk markers.
V. Measure of CRP levels as an inflammatory risk marker for CVD.
VI. Measure the fibrinogen and D-dimers as CVD risk markers.
VII. Determine the strength of the association between HAART and CVD risk factors.
VIII. Determine the strength of the association between HAART and endothelial biomarkers.
IX. Determine the strength of the association between HAART, inflammatory, and coagulation markers.

1.4.3 Null Hypotheses (H_0)

In the current study the following hypotheses were investigated:

I. H_0: HAART regimen does not increase dysglycaemia and dyslipidaemia
II. H_0: HAART regimen does not increase VWF, L-selectin, and P-selectin levels, sICAM, and sVCAM.
III. H_0: HAART regimen does not increase CRP levels.
IV. H_0: HAART regimen does not increase D-dimer and fibrinogen levels.

1.5 SIGNIFICANCE OF THE STUDY

The study intended to identify the potential side effects of HAART so that prompt actions are taken to reduce the risk of CVDs. The present study made use of biomarkers of endothelial function, inflammation and coagulation activity to determine future predictor variables of CVD among HAART-exposed individuals which may be useful in a clinical setting. The significance of the study is that it further provides relevant information on the effects of HAART treatment on the vascular endothelium. Based on these findings, alternative treatment could be considered.

This study may empower the Department of Health and various stakeholders such as local clinics, HIV-positive groups, and field workers to make more informed decisions in regards to the HAART treatment. This study may potentially enhance the existing strategies or interventions by empowering stakeholders to design more effective treatments to reduce the prevalence of CVD in the HIV population. These intervention programs could minimize the risk for CVD and reduce the medical expenditure for both the HIV-positive populations and the Department of Health. This will also promote positive social change by improving the quality of life by reducing the CVD risk among HIV-positive individuals.

1.6 COMPOSITION OF THE THESIS

This thesis consists of eight chapters demarcated as follows: introduction, literature review, methodology, four results and discussion chapters, conclusion which include sections on the limitation and recommendation of the study, and a proposed model to assess the predisposition of a patient for CVD development in HAART. Chapter one provides an introduction and the motivation of the study. It further addresses the problem of the study, its aims, objectives, and hypotheses. Chapter two provides an extensive literature review with relevant information about the focus of this study; the contribution of HAART to metabolic complications (dysglycaemia and dyslipidaemia) among the HIV-positive population in the high-income, and low and middle-income countries. It further provides information on the effects of HAART on early markers of CVD such as inflammation, ED, and atherosclerosis.

Chapter three, the methodology chapter represents the study design, sample techniques, and instruments used to generate information on all parameters to compare the three groups of interest. The statistical tests (one-way analysis of variance (ANOVA), Bonferroni correction, Bonferroni Post-hoc test, Kruskal-Wallis test, Kruskal-Wallis Post-hoc test, Chi-square test, Chi-square Bonferroni and Post Hoc-test were used to obtain differences and the prevalence of the cardiovascular risk factors which were investigated in this study. It further provides the statistical tests

used to examine relationships between either HAART regimens and cardiovascular risk factors under investigation.

Chapters four through seven are the results and discussion sections of the stipulated objectives of this study. The results and discussion chapters address the research findings and the analysis and interpretation thereof. Chapter four focuses specifically on the socio-demographic characteristics of all the groups (HAART-exposed, HAART-naïve, and control). Chapter five further evaluates the effect of HAART on BMI, the lipogram, and fasting blood glucose concentration to identify metabolic abnormalities. Chapter six assesses the effect of HAART on the endothelial function. Chapter seven focuses on the effect of HAART on selected inflammatory biomarkers and it further also evaluates the effect of HAART on coagulation (atherosclerotic plaque formation). Chapter eight, the concluding chapter, summarises the key research findings. It further highlights the proposed criterion model to evaluate the predisposition of a patient for CVD development whilst on HAART treatment. The concluding chapter ends with the recommendations and the limitations to be considered for future studies.

1.7 SCOPE OF THE STUDY

This is a cross-sectional, quantitative study that specifically reflects on the roll-out of HAART among an HIV-positive population in the Mankweng District, Limpopo Province, South Africa. This study evaluated the impact of HAART on the cardiovascular risk factors which included dyslipidaemia and dysglycaemia. Furthermore, the effects of HAART on early markers of CVD which included endothelial function, inflammation and coagulation were investigated.

The target population was divided into three groups which included HAART-exposed, HAART-naïve and control. Standardised techniques were used to achieve all the objectives of the study. This study provides exploratory findings in the rural South African context where it addressed an area of research that focuses on an additional burden of disease where the HIV-positive population are disproportionally affected by CVD. Cardiovascular disease may pose serious complications in the

treatment process and it can become an enormous financial burden for the individual and the government. This study aimed to achieve insight into the contribution of HAART in the development of CVD among the HIV-positive population in the Mankweng District, Limpopo Province.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

The Human immunodeficiency virus is a lentivirus within the family of Retroviridae. The family consists of two distinct virus types, HIV type 1 (HIV-1) and HIV type 2 (HIV-2). The HIV-1 is more prominent and causes most HIV infections globally (Eberlé and Gürtler, 2012; Zhang *et al.,* 2015). HIV type 2 is less common and predominantly found in West Africa, Europe, India, and the United States of America (USA) (Campbell-Yesufu and Gandhi, 2011; Esbjörnsson *et al.,* 2018). The genetically distinct sub-groups observed in HIV are specifically due to its ability to mutate and change over time. HIV-1 can be classified into four groups, namely the M group, N group, O group, and P group (Robertson *et al.,* 2000). The M group is the major HIV-1 subtype which causes most HIV infections. Within the M group, there are at least nine genetically distinct subtypes and these are A, B, C, D, F, G, H, J, and K (Avert, 2015).

Although the infection with subtype C is more prevalent globally, most clinical research is based on subtype B (Lessells *et al.,* 2012; Günthard and Scherrer, 2016). This is because subtype B predominates among the Americans, Western European, and Australian populations, although subtype B represents only 12% of global infections (Fourie *et al.,* 2011). HIV type 1 progresses at a very rapid rate into AIDS, especially when ARV therapy is not administered, while only 30% of the HIV patients infected with HIV-2 progresses to AIDS (Chu *et al.,* 2017). HIV type 2 is significantly less pathogenic compared to HIV-1. While many commonly used ARV drugs are effective in controlling the replication of HIV-2, non-nucleoside reverse transcriptase inhibitors (NNRTI) such as nevirapine and efavirenz, which are standard therapy in West-Africa, are not effective against HIV-1 (Witvrouw *et al.,* 2004).

Several advances have been made in implementing comprehensive intervention programs that specifically focussed on HIV awareness and prevention to curb the

spread of HIV. However, globally approximately 1.8 million people still became newly infected with HIV in 2016 (UNAIDS, 2018). It is important to note that the majority of people currently living with HIV/AIDS reside in SSA, which includes low-and-middle-income countries such as South Africa, Botswana and Ghana (UNAIDS, 2018).

2.2 HIV INFECTION IN SUB-SAHARAN AFRICA AND SOUTH AFRICA

Although SSA consists of only 12% of the global population, it is currently considered the most affected region in the entire world, with an estimated 25.6 million people living with HIV/AIDS. In SSA approximately 5% of people, from the total population, are living with HIV/AIDS and it contributes to nearly 71% of the total global HIV population (Deeks *et al.,* 2015; Kharsany and Karim, 2016). The increase in the number of people living with HIV/AIDS in SSA can be attributed to the successful roll-out of ARV drug therapy, which increased the life expectancy and quality of life (Mutevedzi and Newell, 2014).

The decrease in mortality rates as HAART became widely available in SSA created an opportunity for some in the HIV-positive population to indulge in riskier sexual behaviours, which caused the increase in the spread of HIV/AIDS (Temah, 2009). Socioeconomic status, social and cultural practices, drug and alcohol abuse were reported to be contributing factors to the high incidence rate observed in SSA (Inungu and Karl, 2006). Although a higher number in the incidence of HIV/AIDS exist among low socioeconomic status individuals, it was observed that HIV/AIDS was also the leading cause of death among the wealthier in SSA (Hajizadeh *et al.,* 2014).

Despite the South African Government spending billions trying to curb the spread of HIV/AIDS, South Africa is still globally regarded as the country with the highest HIV infection prevalence rate (Blecher *et al.,* 2016; Vandormael *et al.,* 2020). Regardless of HIV awareness campaigns, the rate of HIV infection keeps on increasing every year in this country (UNAIDS, 2018). Currently, the prevalence rate of HIV infection amongst South African adults is 18.9%. It is further estimated that approximately 7 million South Africans are currently living with HIV (UNAIDS, 2018). Most of the affected are females in the age group 30 – 34 years, while most of the infected males

are in the age group 35 – 39 years (Stats SA, 2017). Cardiovascular disease-related deaths are currently at 1.5 – 2.0 fold higher among HIV-positive patients compared to uninfected individuals (D:A:D Study Group *et al.,* 2010). These mortalities have been related both to HIV infection and HAART (Wada *et al.,* 2014).

2.3 CURRENT PROSPECTS OF ARV THERAPY IN SOUTH AFRICA

2.3.1 Antiretroviral composition and the goals for the treatment

South Africa initiated the HAART roll-out program in July 2004 with the primary objective to reduce the high mortality and morbidity rates among the HIV-infected population (Nattrass, 2006). General goals of ARV therapy are to:

- maximise viral load (VL) suppression.
- revive and maintain immune function which will result in the increase of cluster of differentiation T4 Lymphocyte count or $CD4^+$ (Cluster of differentiation 4).
- reduce HIV related infections and non-infectious morbidity.
- improve life expectancy.
- reduce the transmission of HIV.
- minimise the adverse side effects of treatment (Meintjes *et al.,* 2017).

Currently, South Africa has the largest HAART roll-out program in the world, where it is estimated that more than 3.8 million people which includes an estimated 171 thousand children (<15 years old) and 3.6 million adults (1.2 million males and 2.4 million females, >15 years old) are on ARV therapy (UNAIDS, 2018). The HAART roll-out program of South Africa in both the public and private sectors was initially based on the following eligibility criteria: A $CD4^+$ count of ≤200 cells/mm^3 or symptoms of HIV or with the World Health Organisation (WHO) stage IV of HIV (progression of HIV to AIDS) (Bassett *et al.,* 2010). Prior to the implementation of the universal test and treat (UTT) policy, HAART was only initiated on HIV-positive patients who displayed a $CD4^+$ count of ≤ 500 cells/mm^3 and were at a clinical-stage of 3 or 4 (Ying *et al.,* 2016). In

September 2016 the UTT, same-day testing and initiation of HAART policy, differentiated service delivery, the establishment of centralised chronic medicines dispensing and distribution (CCMDD) models were launched as vehicles of universal access to HAART and multi-month ARV (ARV) therapy supply (Perriat *et al.,* 2018). Despite all these initiatives from the South African Government and other private stakeholders, the number of new HIV-infections remains staggeringly high (Hopkins *et al.,* 2018).

Human immunodeficiency virus enters human cells through its envelope proteins, glycoprotein 120 (gp120) that binds to the CD4 receptors located within the cell membrane (Zimmermann *et al.,* 2015) (Figure 2.1). This in turn leads to a conformational change in the viral envelope which causes the HIV genetic material (HIV-RNA) to bind to either chemokine co-receptor type 5 (CCR5) or chemokine co-receptor type 4 (CXCR4) of the target cell. This consequently leads to a conformational change in another viral envelope protein, gluco-protein 41 (gp41), which causes the fusion of the HIV-RNA with the cell membrane of the target cell and entry of the HIV-RNA into the cytoplasm (Barmania and Pepper, 2013). Through the process of reverse transcription, the HIV-RNA is converted into HIV-DNA by reverse transcriptase. The HIV-DNA crosses the nuclear membrane and HIV integrase mediates the integration of HIV-DNA into the host. The host cell machinery is then used to synthesize several chains of HIV-proteins, such as negative regulatory factor (Nef), Trans-Activator of transcription (Tat), and Rev, which support viral replication and the formation of new HIV genetic material (Lata *et al.,* 2018). The newly formed HIV genetic material bud from the host cell and are ready to infect new cells (Azevedo-Pereira *et al.,* 2015). The ARV drugs were developed to attack different target sites in the HIV life cycle to reduce the VL and increase the $CD4^+$ count (Ognenovska *et al.,* 2018). Antiretroviral drugs are divided into different classes according to their inhibitory effects on the different stages of the HIV life cycle (Wilen *et al.,* 2012).

Since the discovery of HIV, a large number of ARV drugs have been developed (Broder*,* 2010). Currently, more than thirty Food and Drug Administration (FDA) approved ARV drugs are used globally to therapeutically manage HIV-infection (Zhang*,* 2018). In South Africa, the three-drug combination (three different ARV drug

classes) in one capsule is used to treat HIV-infected people. The three ARV drug combinations in a capsule consist of either two nucleoside reverse transcriptase inhibitors (NRTIs) with one (non-nucleoside reverse transcriptase inhibitors (NNRTIs) or two NRTIs in combination with protease inhibitors (PIs) (Pau and George, 2014). In addition, the HAART regimens consist mainly of three ARV drugs from the three main ARV drug classes (Table 2.1). These ARV drugs have specific individual doses recommended by WHO which demands strict adherence (Table 2.2).

The introduction of the three ARV drug combination reduced AIDS-related morbidity and mortality significantly (Tseng *et al.,* 2015). Three different ARV treatment approaches are applied and these include the so-called first, second, and third-line treatments (Meintjies *et al.,* 2017).

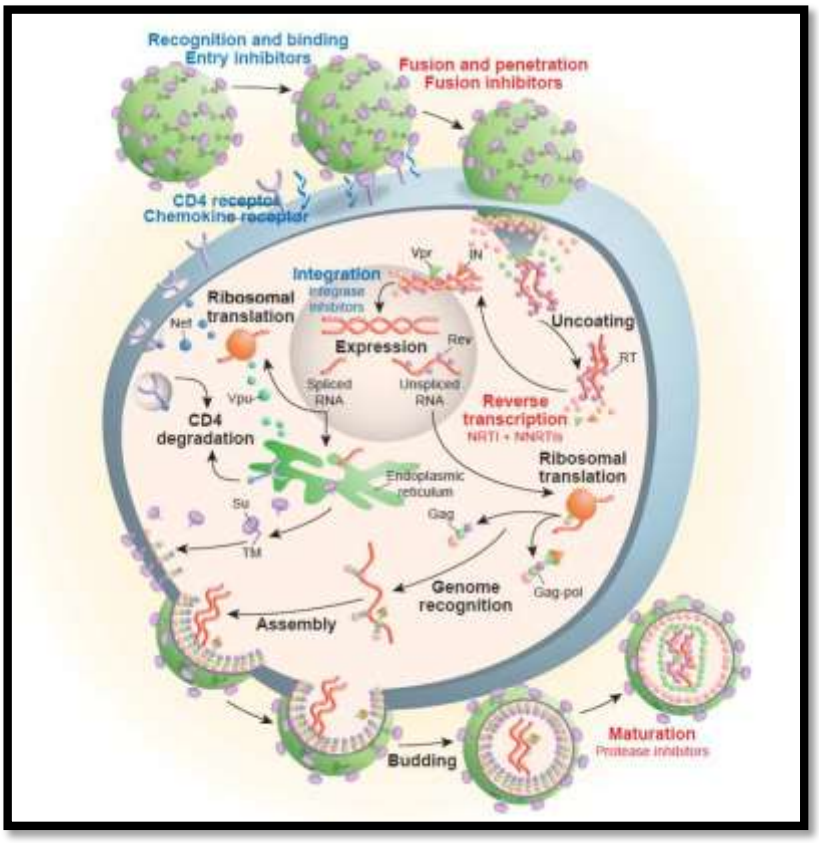

Figure 2.1: Schematic representation of the HIV life cycle and inhibitory actions of ARVs (Pomerantz and Horn, 2003).

Table 2.1: ARV drug classes used in South Africa (Adopted from Mentjies *et al*., 2017).

NRTIs	NNRTIs	PIs
Abacavir (ABC)	Etravirine (ETV)	Amprenavir (APV)
Didanosine (ddi)	Efavirenz (EFV, EFZ)	Atazanavir (ATV)
Emtricitabine (FTC)	Delavirdine (DLV)	fos-Amprenavir (fos-APV)
Lamivudine® (3TC)	Nevirapine (NVP)	Indinavir (IDV)
Stavudine® (d4T)		Lopinavir/ritonavir (LPV/r)
Tenofovir (TDF)		Nelfinavir (NFV)
Zalcitabine (ddC)		Ritonavir (RTV)
Zidovudine® (ZDV, AZT)		Saquinavir (SQV)

2.3.2 Different lines of antiretroviral therapy in South Africa

Antiretroviral therapy regimens should be used on HIV-infected individuals to maximally reduce the VL and prevent resistance (Meintjies *et al.,* 2017). The eligibility criteria to initiate HAART on HIV-infected patients have since advanced to a point where current ARV therapy is initiated to all HIV-infected patients regardless of $CD4^+$ count or clinical diagnoses (WHO, 2015). The immediate UTT policy has benefited all HIV-infected individuals; it has specifically reduced the risk of disease progression and HIV transmission (Koenig *et al.,* 2017).

The majority of HIV-infected people in both private and public health sectors receive the recommended first-line regimen (Meintjies *et al.,* 2017). The first-line regimen for adults is the ARV drug combination which is routinely used when patients first start ARV treatment. It is a course of therapy consisting of two NRTIs and one NNRTIs in the following possible preferred combinations (Gilks *et al.,* 2006; Boender *et al.,* 2016):

- Tenofovir (TDF) + Emtricitabine (FTC) or Lamivudine (3TC) + Efavirenz (EFV)
- TDF + FTC (or 3TC) + Dolutegravir (DTG)
- TDF + FTC (or 3TC) + Rilpivirine (RPV)

When VL>100,000 copies/ml, RPV cannot be used with rifampicin and DTG will require dose adjustment with rifampicin. It is important to note that rifampicin is administered to prevent HIV-associated TB. Stavudine was phased out and is currently replaced with TDF, or EFV if TDF is contraindicated. *

Table 2.2: The South African recommended doses for first-line ARV treatment. (Adapted from Mentjies *et al*., 2017)

Drug	Dose
EFV	600mg at night
FTC	200mg daily
3TC	300mg daily
NVP	200mg daily (14 days, then 200mg twice daily)
TDF	300mg daily
AZT	300mg twice daily

Switching from the first-line to the second-line regimen is often challenging. The second-line regime is initiated when either side-effect is observed or when resistance develops and VL failure persists (VL>1,000 copies/ml) over a long period. Even when good adherence is observed, if the HIV-infected patients fail to respond to the first-line combination, the second-line combination of ARV drugs is initiated. The preferred second-line regimen is a combination of two NRTIs and ritonavir (RTV)-boosted PIs in the following possible preferred combinations:

- Zidovudine (AZT) + 3TC + Lopinavir/ritonavir (LPV/r)
- TDF+ 3TC or FTC + LPV/r

Lopinavir is available in South Africa as a combination tablet with RTV. This combination is known as Aluvia (LPV/r) and is a tablet consisting of 400mg of LPV and 100mg RTV. Two tablets of Aluvia are taken twice daily, that is, every 12 hours.

The third-line regimen is usually recommended when virological failure persists ARV drug classes (NNRTIs, NRTIs, and PIs). Protease inhibitor resistance is identified and when the HIV-infected patient was on a PI-based regimen for longer than one year. The third-line regimen combination consists of Darunavir/ritonavir (DRV/r) and two NRTIs, 3TC and FTC with either TDF or AZT. The third-line ARV drug consists specifically of 600mg DRV and 100mg RTV. It is recommended that this treatment be administered twice daily (Meintjies *et al.,* 2017). Currently the two drug therapy for HIV treatment is also available. It has been reported that is comparable with the conventional three drug regimens. The clinical outcome and the life expectancy among the HIV-infected population were significantly improved since the introduction of the HAART regimens (Hemkens and Bucher, 2014). Despite the HAART associated positive outcomes, both *in vivo* and *in vitro* studies observed endothelial toxicity as a result of either HIV or HAART (Kline and Sutliff, 2008; Graham *et al.,* 2013; Beltrán *et al.,* 2015).

2.4 EFFECTS OF HAART ON CELLULAR FUNCTION

The vascular endothelial cells are constantly exposed to high concentrations of HIV-PIs which may lead to ED. Several studies have demonstrated a strong link between HIV-PIs treatment and ED (Stein *et al.,* 2001; Solages *et al.,* 2006; Wang *et al.,* 2007; Mondal *et al.,* 2013). The molecular mechanisms of HIV-PI toxicity in endothelial cells have been described, and many *in vitro* studies allude to oxidative stress (OS) in HIV-PI-induced ED (Conklin *et al.,* 2004; Jiang *et al.,* 2007; Wang *et al.,* 2007). The administration of HIV-PI drugs causes serious mitochondrial damage which is the main source for reactive oxygen species (ROS) production, which often causes a reduction in cellular respiration, adenosine triphosphate (ATP) production, as well as reduced mitochondrial membrane potential and mitochondrial DNA damage (Fiala *et al.,* 2004; Mondal *et al.,* 2004; Chai *et al.,* 2005; Touzet and Philips, 2010).

Previous *in vitro* studies have shown that HIV-PI drugs activate endoplasmic reticulum (ER) stress and disrupt lipid metabolism in adipocytes, hepatocytes, and macrophages (Chen *et al.,* 2009; Djedaini *et al.,* 2009; Banerjee *et al.,* 2013). Activation of ER stress was also found to be involved in HIV-PI induced inflammation and foam cell formation in macrophages (Zhang *et al.,* 2014). Little is known on how HIV PI drugs can cause ED, although Saquinavir (PI drug) was found to induce autophagy by the breakdown of lipids deposited in adipose tissue causing lipodystrophy (Polus *et al.,* 2017). Several mechanisms have been proposed in HIV and HAART induced OS and mitochondrial damage which may result in ED and adverse cardiovascular complications.

2.5 COMPLICATIONS OF HIV INFECTION AND ITS THERAPY

2.5.1 Human immunodeficiency virus and non-communicable diseases

The incidence of non-communicable diseases (NCDs) is rapidly growing on a global scale. It is estimated that more than 85% of NCD-associated deaths occurred in low- and middle-income countries. Annually CVDs account for most NCDs mortalities, followed by the various cancers (9.0 million), respiratory diseases (3.9 million), and diabetes mellitus (DM) (1.6 million) (WHO, 2015). The use of ARV therapy and screening for HIV has progressed over the past years to such an extent that fewer people living with HIV (PLWH) die of opportunistic infections, but more are currently suffering from age-related NCDs (Smit *et al.,* 2018; Juma *et al.,* 2019). The increased prevalence of NCDs among PLWH will necessitate these individuals to use multiple therapeutic interventions which may expose them to potential drug interactions. A study conducted in the Netherlands predicted that due to the rise in NCDs, 40% of PLWH may experience drug-interaction by 2030 with the currently recommended ARV therapy (Smit *et al.,* 2015). In SSA where the HIV epidemic is high, the prevalence of NCDs is progressively growing among PLWH (Smit *et al.,* 2018; Coetzee *et al.,* 2019). Studies regarding the prevalence of NCDs among PLWH are currently still lacking, and similar outcomes as projected in Smit *et al.* (2015) might occur in SSA due to the

increase in NCDs. These drug interactions, currently largely unexplored, have the potential to render ARV therapy ineffective which may result in increased mortality among PLWH.

The successful roll-out of the ART programs, as observed in South Africa since 2004, extends the life expectancy of HIV-infected individuals (Evans, 2013). An observational, model-based study estimated that 40% of the HIV population may experience complications due to drug interactions with current HAART regimens by 2030 (Smit *et al.,* 2018). Among the HIV-positive population it was previously indicated that CVD conditions such as metabolic disorders and vascular conditions are major concerns (Willig and Overton, 2016; Laurence *et al.,* 2018).

2.5.2 Human immunodeficiency virus and cardiovascular disease

The risk for CVD among HIV infected people has increased dramatically (Zanetti *et al.,* 2018). Observational studies conducted in Europe (Lang *et al.,* 2010) and the USA (Triant, 2013) showed that overall risk for coronary heart disease (CHD), defined as myocardial infarction (MI), is 1.5 – 3 times higher in the HIV-positive population compared to the general population, independent of age. Some studies also pointed out that other cardiovascular risk factors such as hypertension, DM, and dyslipidaemia were more prevalent among the HIV-positive population (Hemkens and Bucher, 2014; Divala *et al.,* 2016). The Fat Redistribution and Metabolic Change in HIV Infection (FRAM) study specifically focussed on HIV-positive men. They found that the impact of HIV itself on the carotid intima-media thickness (CIMT), one of the surrogate markers of atherosclerosis, was similar to that of smoking (Grunfeld *et al.,* 2009). It is well known that smoking is a major risk factor of CVD and it is becoming a major health concern among HIV-infected individuals (Rigotti and Clair, 2013; Hemkens and Bucher, 2014). The HIV-infected population is at increased risk for developing CVD due to the high prevalence of CVD risk factors.

A high frequency of risk behaviours which include smoking, alcohol, sedentary lifestyle, and poor diets have been attributed to the increase in risk for CVD among the HIV-infected population (Hyle *et al.,* 2017). There are speculations that there might be

other driving forces contrary to just the well-known traditional risk factors which rapidly increased the risk for developing CVD among the HIV-positive population. Some suggested that HIV itself might aggravate inflammation by disturbing the endothelium which might lead to ED (Fourie *et al.,* 2011). Furthermore, there is evidence that HIV infection causes a dysregulation of coagulation (Santos *et al.,* 2004), which might initiate atherosclerosis and can cause MI.

The human immunodeficiency virus and the potential side effects of some HAART regimens are associated with metabolic abnormalities and might increase the risk for CVD (Boccara and Cohen, 2016). The Data collection on Adverse events of Anti-HIV Drugs (D:A:D) Group Study showed, even for a particular age, the longer you are exposed to HAART the higher the likelihood of developing metabolic disorders. It further also pointed out that it is not all HAART regimens but specifically the PI-based regimens that are associated with metabolic abnormalities (D:A:D Study Group *et al.,* 2010). Although there were a number of advances made in developing HAART regimens with fewer side effects, the reality remains that many low and middle-income countries still cannot afford the less toxic HAART regimens which are currently available in high-income countries (Sagaon-Teyssier *et al.,* 2016). This leaves the low and middle-income countries with no alternative option(s) but to continue with ARV drugs accompanied by multiple side effects. Specific HAART regimens have already been pointed out as detrimental in causing metabolic abnormalities such as dyslipidaemia and dysglycaemia.

2.5.3 HAART and metabolic abnormalities

Highly active antiretroviral therapy has been increasingly associated with CVD and several mechanisms have been proposed where HAART is implicated in dyslipidaemia and dysglycaemia (Jin *et al.,* 2016). HAART associated dyslipidaemia and dysglycaemia are not well-understood; however, in *in vitro* studies mitochondrial toxicity is often implicated (Kakuda, 2000; Gnanasekaran, 2020). Dyslipidaemia and dysglycaemia may increase the risk for CVD which may cause serious health complications for the HIV-positive populations.

The low-and middle-income countries are still markedly affected by HIV/AIDS, despite the improved access to ARV treatment. There is still limited information in these countries regarding HAART induced metabolic abnormalities. High-income countries are still in a better position to manage metabolic abnormalities in their HIV-positive populations (Husain *et al.,* 2017). Metabolic abnormalities are common risk factors of CVD which are commonly found in the general population.

Besides the traditional CVD risk factors and HIV itself, specific ARV drug classes were also found to be responsible for metabolic abnormalities (Dimala *et al.,* 2018). Metabolic abnormalities have become a huge health concern in the last two decades among the HIV-infected population on HAART (Hejazi *et al.,* 2013). The adverse effects of the PI-based regimens, have been frequently associated with a variety of metabolic abnormalities that include dyslipidaemia, IR, type 2 diabetes mellitus (T2DM), and changes in body fat distribution (Domingos *et al.,* 2009; Dimala *et al.,* 2018; Khoza *et al.,* 2018). Dyslipidaemia and increased blood glucose as metabolic abnormalities are the most common health threats among the HIV-positive population on HAART (Husain *et al.,* 2017). Information on dyslipidaemia and dysglycaemia among HIV-infected individuals especially in rural communities are inadequate, especially in the low-and middle-income countries, such as South Africa.

2.5.3.1 HAART and dyslipidaemia

Dyslipidaemia is characterized by elevated triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), and a decline in high-density lipoprotein cholesterol (HDL-C) (Nelson, 2013). As previously mentioned, cardiovascular disease-related deaths are 1.5 – 2 times higher among the HIV-positive population compared to the general population. Dyslipidaemia is a key factor associated with CVD. It has been commonly reported in HIV-infected subjects on HAART (Lee *et al.,* 2017). Elevated LDL-C and reduced HDL-C levels are associated with increased risk for CVD among the HIV-positive population (Ahmed *et al.,* 2016). Some studies found that elevated levels of TG were also associated with CVD among the HIV-positive

population, even after adjusting for the well-known risk factors of CVD such as reduced HDL-C and elevated LDL-C levels (Hejazi *et al.,* 2013; Toth*,* 2016).

Prior to the implementation of HAART, some studies reported that the lipid profile of HIV-infected populations differed from that of the general population (Rose *et al.,* 2006; Shen *et al.,* 2015). One of the earliest studies which reported this phenomenon observed a decline in TC, HDL-C, and LDL-C over time, elevated levels of TG developed in most of these HIV-infected patients (Grunfeld *et al.,* 1992). It was observed that, after HAART initiation, TC and LDL-C would increase with time. Even though HDL-C increased after HAART administration, HDL-C was noted to decrease with time and remain low. Many HIV-positive patients also develop high TG levels (Riddler *et al.,* 2003). The observed lipid pattern of the HIV-positive population is therefore quite different from what is usually observed among the general population.

Each ARV drug composition within the ARV classes determines a different lipid profile pattern. For example, PIs elevates the LDL-C, TG, and TC levels and it is considered one of the major ARV drug classes causing dyslipidaemia among the HIV infected individuals (Hejazi *et al.,* 2013). Although Atazanavir and Darunavir are also PIs, they have a smaller impact on the development of dyslipidaemia (Laurence *et al.,* 2018). Some studies have shown NNRTIs are associated with a slight increase in HDL-C, as compared to the PIs (Non *et al.,* 2017; Laurence *et al.,* 2018). The different types of NRTIs are not equal, specifically in their effects on the lipid profile. Stavudine, a commonly used NRTIs, was found to have the worst impact on the lipid profile demonstrated by elevated triglyceride levels (Crane *et al.,* 2011; Dave *et al.,* 2016). Chronic use of Stavudine has been associated with the reduction in the expression of ɣ-DNA Polymerase, a key enzyme responsible for mitochondrial DNA replication (Lewis *et al.,* 2003). The inhibition of ɣ-DNA Polymerase is associated with a reduction in cellular oxidative phosphorylation which resulted in the build-up of intracellular lipids and lactic acid (Dave *et al.,* 2016). Besides dyslipidaemia, Stavudine has been phased-out for several other clinical manifestations which include peripheral neuropathy, lipoatrophy hyperlactatemia, and pancreatitis (Dave *et al.,* 2016).

It was also reported that TDF, which is also an NRTIs, has the smallest impact on the lipid profile with a slight lipid-lowering effect on HDL-C, LDL-C, and TC

(Tungsiripat *et al.,* 2010; Chen *et al.,* 2017). Tenofovir exhibited a protective effect compared the other ARV drug classes (PIs and NNRTIs). Tenofovir has been associated with a reduction in atherosclerosis and a decrease in coronary artery disease (CAD) (Chen *et al.,* 2017). Currently, in SA the standard treatment regimen offered to people newly diagnosed with HIV consist of TDF despite its higher risks of chronic kidney disease. Any HIV-positive patients who have a pre-existing renal disease or advance in age are at greater risk of chronic kidney disease (Mtisi *et al.,* 2019). In cases of nephrotoxicity, TDF is replaced with a lower TDF dose or Tenofovir alafenamide, a prodrug of TDF that presents with less renal toxicity (Venter *et al.,* 2018).

2.5.3.2 HIV and antiretroviral drug-associated glucose abnormalities

Glucose is the energy source of the various physiological processes in the human body. Insulin plays a major role in regulating glucose concentrations in the body. Insulin resistance is a condition where normal or elevated insulin levels produces an weakened biological response which is classically refers to impaired sensitivity to insulin mediated glucose disposal (Wilcox *et al.,* 2005). This often leads to elevated blood glucose levels and results in DM, specifically T2DM (Husain *et al.,* 2017). Therefore, hyperglycaemia is a well establish marker for IR.

Since the inception of HAART, the prevalence of HAART-associated glucose abnormalities, which include IR, impaired glucose tolerance (IGT), and DM, have increased significantly among the HIV infected population (Kramer *et al.,* 2009; Husain *et al.,* 2017). A cohort study conducted among South African HIV-infected participants which included patients who are HIV-positive treatment naïve, on the first-line regimen, and second-line regimen with Lopinavir/Ritonavir-boosted PIs, reported that the prevalence rates of dysglycaemia were 18%, 21%, and 37%, respectively. They further reported that DM was up to nine-fold higher among the HIV-positive patients who were on the PI-based regimen, compared to HAART-naïve and on the first-line groups (Levitt *et al.,* 2016). PI-based regimens exhibited the highest relative risk for developing glucose abnormalities such as IR (Gutierrez and Balasubramanyam,

2012). The increase in metabolic abnormalities is of great concern with the use of both TDF and Stavudine based first-line regimes (Husain *et al.,* 2017; Njuguna *et al.,* 2018).

2.5.3.3 HIV and HAART as risk factors for atherosclerosis

After the inception of the ARV therapy, atherosclerosis and atherosclerosis-associated complications were considered the leading causes of the increased morbidity and mortality observed among the HIV positive population (Kearns *et al.,* 2017). It was also noted that HIV itself independently accelerates the pathogenesis of atherosclerosis (Shrestha *et al.,* 2014). Antiretroviral therapy toxicity, drug abuse, opportunistic infections, lifestyle, dyslipidaemia, and dysglycaemia are described as risk factors for HIV-associated atherosclerosis (Kearns *et al.,* 2017). Although various risk factors were linked to HIV-associated atherosclerosis, the pathogenesis remains elusive and the condition a significant health burden.

The mechanism of HIV-induced atherogenesis remains unclear and it is further complicated by the introduction of ARV therapy (Kearns *et al.,* 2017; Currier and Hsue, 2020). Evidence regarding the role of HIV-infection, HAART, immune cell activation, cellular and molecular mechanisms of atherosclerosis still needs to be extensively investigated (Kearns *et al.,* 2017, Sokoya *et al.,* 2017). Several mechanisms have been proposed in HIV and HAART induced atherosclerosis which includes a list of cellular pathways, immune cell activation, formation/caspase-1 activation, autophagy, OS, ER stress, and ED (Shrestha *et al.,* 2014; Kearns *et al.,* 2017; Seecheran *et al.,* 2017; Sokoya *et al.,* 2017).

2.6 ENDOTHELIUM AND VASCULAR HOMEOSTASIS

The endothelium is a multi-functional organ that actively controls several vital physiological processes (Sena *et al.,* 2013; Fernández-Hernando and Suárez, 2018). It is essentially involved in regulating vascular tone, mediation of coagulation, platelet adhesion, and immune function (Cahill and Redmond, 2016). The endothelial cells produce a wide range of substances that regulate cellular adhesion, thrombo-

resistance, vessel wall inflammation, and smooth muscle cell proliferation (Čejková *et al.,* 2016).

The endothelium regulates both sensory and effector functions, housing receptors for a variety of cytokines, cell-adhesion molecules (CAMs), chemotactic and vasoactive peptides, and lipids (Rajendran *et al.,* 2013). It regulates both the inflammatory and thrombotic processes through selective expression of the specific factors such as endothelial nitric oxide synthase (eNOS), soluble vascular cell adhesion molecule (sVCAM), and von Willebrand factor (VWF) (Yau *et al.,* 2015; Yang *et al.,* 2016). It plays a critical role in sensing the chemical, physical and biochemical changes in its environment and converts them into physiological responses. Under physiological conditions, the endothelial cells secrete various endothelial-dependent substances which include nitric oxide (NO), prostacyclin (PGI2), endothelial-derived hyperpolarizing factor (EDHF), thrombomodulin, and tissue plasminogen activator-1 (tPA-1) to maintain blood fluidity. These substances facilitate vasodilation, fibrinolysis, coagulation, and platelet aggregation (Constans and Conri, 2006). The endothelium is important for several homeostatic functions in the body and its dysfunction is associated with several patho-physiological functions (Chien, 2007; Camaré *et al.,* 2017).

Endothelial dysfunction is characterised by vasoconstrictive, procoagulant, platelet-activating, and antifibrinolytic phenotypes. The dysfunction, described as endothelial damage, is also associated with increased levels of endothelin-1, angiotensin II, tPAI-1, and VWF (Constans and Conri, 2006). Endothelial dysfunction is a common event in several CVD and is a key pathogenic factor in atherosclerosis-related diseases (Constans and Conri, 2006). It is generally defined as a reduction in NO bioavailability and the inability of the endothelium to maintain vascular homeostasis (Sandoo *et al.,* 2010; Rajendran *et al.,* 2013). The activation of eNOS regulates NO production in the endothelial cells (Figure 2.2). The activation of eNOS leads to the downstream activation of various signalling molecules which ultimately results in the relaxation of the vascular smooth muscle cells (VSMCs) surrounding the blood vessels (Constans and Conri, 2006; Zhao *et al.,* 2015). The nitric oxide synthase (NOS) family, which consists of three isoforms, eNOS, neuronal nitric oxide synthase

(nNOS), and inducible nitric oxide synthase (iNOS), synthesise NO from the substrate L-Arginine. The major isoform, eNOS, is responsible for NO production under physiological conditions; and will eventually cause the relaxation of VSMC (Park and Park, 2015; Zhao *et al.,* 2015).

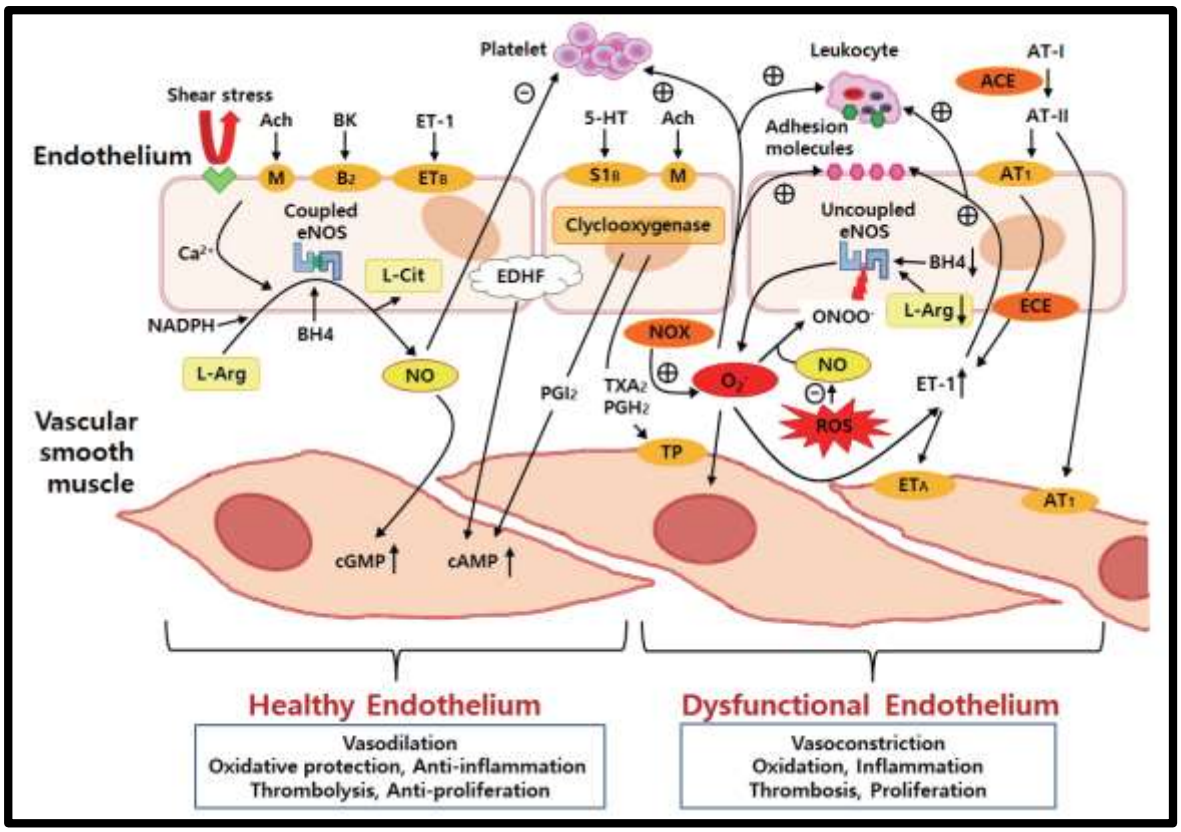

Figure 2.2: The mechanistic pathways of eNOS (Park and Park, 2015).

In the healthy endothelium, eNOS is responsible for NO production to induce vasodilation. However, during ED the intact eNOS becomes uncoupled eNOS, a condition associated with the excessive generation of potential ROS (Rajendran *et al.,* 2013; Park and Park, 2015). During this pathological state, the uncoupled eNOS generates ROS, such as peroxynitrite ($ONOO^-$) instead of NO. The remaining NO further combines with ROS to form more $ONOO^-$ which increases OS and results in the reduction of NO bioavailability (Clapp *et al.,* 2004; Park and Park, 2015). Endothelial damage is also associated with other phenotypic changes in the vascular

endothelium that promotes pro-inflammatory activity, OS, pro-thrombotic activity, dysregulation of vascular development, and metabolism (Polovina and Potpara, 2014). The endothelium plays a pivotal role in the regulation of vascular and metabolic homeostasis, and endothelial damage is often associated with cardiovascular complications.

Endothelial damage is an early event in atherosclerosis progression, and it is found in many traditional risk factors which include T2DM, IR, hypercholesterolemia, and smoking. It is also associated with adverse outcomes in patients with various established CVD such as MI (Lang *et al.,* 2010; Polovina and Potpara, 2014). These risk factors could also induce or enhance endothelial cell activation, leading to increased vasoconstriction, platelet aggregation, leukocyte adhesion, LDL oxidation, and matrix-metalloproteinase (MMP) activation (Fourie *et al.,* 2011; Park and Park, 2015).

Human Immunodeficiency Virus-infection promotes arterial inflammation and injury which may lead to endothelial activation and dysfunction and eventually atherosclerosis (Fourie *et al.,* 2011). Inflammatory biomarkers such as C-reactive protein (CRP), interleukin-6 (IL-6), and CAMs, such as sVCAM-1 and soluble intercellular cell adhesion molecule-1 (sICAM-1) were all elevated among the HIV-infected population (Fourie *et al.,* 2011). Elevation of these biomarkers reflects increased levels of vascular inflammation and endothelial activation among the HIV-infected population. HIV-infected individuals may experience accelerated vascular aging and rapid development of atherosclerosis which may result in devastating outcomes such as MI and strokes due to the increase in plaque formation.

2.7 ROLE OF ENDOTHELIUM IN REGULATING HAEMOSTASIS

The endothelium regulates prothrombotic and antithrombotic activity. Under normal circumstances, the healthy endothelium expresses antiplatelet and anticoagulant factors that would prevent platelet aggregation and fibrin formation. The endothelium forms a barrier between thrombogenic sub-endothelial matrix constituents, platelets, and coagulation proteins in the circulation (Yau *et al.,* 2015). The anticoagulant

proteins secreted by the endothelium include PGI2, NO, Protein S, and tissue plasminogen activator which inhibits platelet aggregation (Yau *et al.,* 2015; Koupenova *et al.,* 2017). Therefore, the endothelium is essential in maintaining haemostasis and preventing thrombosis.

During ED, endothelial cells trigger fibrin formation, as well as platelet adhesion and aggregation (Poggesi *et al.,* 2015). Endothelial cells release pro-fibrinolytic agents that initiate fibrinolysis to degrade the clot (Yau *et al.,* 2015). The essential role of the functional endothelium is to maintain haemostasis and prevent thrombosis (Verhamme and Hoylaerts, 2006). In the event of endothelium activation, the endothelial cells are usually pro-thrombotic. Endothelium dysfunction triggers the activation of a cascade of coagulation events which leads to the expression of tPAI-1, VWF, and thromboxane A_2 together with CAMs (sICAM-1 and sVCAM-1) and selectins (P-selectin, E-selectin, and L-selectin) which inhibit fibrinolysis to enhance coagulation (Widlansky *et al.,* 2003; Margetic, 2012). It was proposed that VWF may create an adhesive surface during endothelium activation to capture monocytes and leukocytes, which are similar to the CAMs (Smiljic, 2017).

The VWF is a large adhesive multimeric glycoprotein produced in the megakaryocytes (Mks) and the so-called Weibel-Palade bodies of the endothelial cells (Bryckaert *et al.,* 2015). The endothelial cells regulate the release of VWF under high shear stress in the bloodstream and upon stimulation by inflammation or vascular injury (Gogia and Neelamegham, 2015; Gragnano *et al.,* 2017). Vascular injury can rupture the inner lining of the blood vessel and may expose the sub-endothelial matrix components such as collagen which may trigger the release of VWF into the blood (Bryckaert *et al.,* 2015). In the blood, VWF rapidly unfolds and elongates into a long-chain conformation, changing its functional status from inactive to highly reactive where it interacts with the platelets. This interaction activates platelets to roll on the ECs to the site of injury to arrest bleeding.

Recently, VWF was implicated as an emerging mediator of vascular inflammation through macrophage and neutrophil recruitment in inflamed lesions (Wu *et al.,* 2017). Endothelial activation and ED, vascular aging, and arterial stiffness are all associated with elevated levels of VWF (Horvath *et al.,* 2004; Gragnano *et al.,* 2017). The role of

inflammation, endothelial activation, and ED in the development of atherosclerosis has been extensively studied in the general HIV negative population, and several biomarkers such as VCAM-1, ICAM-1, and VWF are reliable indicators of ED in atherosclerosis (D' Abramo *et al.,* 2014; Castellon and Bogdanova, 2015; Gimbrone and García-Cardeña, 2016). Results from *in vivo* animal models and clinical studies showed that inhibiting VWF with VWF antagonist drugs enhances antithrombotic and anti-inflammatory processes (De Meyer *et al.,* 2012; Gragnano *et al.,* 2017). Therefore, VWF can be considered as an attractive therapeutic target in evaluating thrombosis and inflammation. A marked elevation in VWF was observed among HIV-infected individuals, which further indicated an association between VWF and first and recurrent thrombotic events. Suggesting HIV-infection as an independent risk factor for thrombotic abnormalities (Van den Dries *et al.,* 2015). Stroke was also found to be associated with pro-thrombotic activity among HIV-infected individuals which was indicated by elevated levels in VWF (Allie *et al.,* 2015). A complex interaction exists between coagulation activity and fibrinolysis to ensure thrombo-haemorrhagic balance.

2.8 ROLE OF THE ENDOTHELIUM IN THE FIBRINOLYTIC SYSTEM

Fibrinolysis is the enzymatic breakdown of fibrin in blood clots and it specifically serves to prevent clots from becoming problematic. It promotes the smooth flow of blood in the circulatory system (Chapin and Hajjar, 2015); and is initiated through the release of tPA-1 or urokinase plasminogen-1 (uPA-1) from the endothelial cells at the site of vascular damage. It is activated simultaneously with the coagulation cascade (thrombosis). During clot formation tPA converts plasminogen into plasmin, the latter degrades fibrin into fibrinogen and other fibrin degradation products (FDPs), which include D-dimer (Urano *et al.,* 2018). A South African study pointed out that although HIV-infection is an independent cause for ED, no signs of prothrombotic activity (no PAI-1 and fibrinogen activity levels were increased) was observed but vascular aging and early atherosclerosis was evident (Fourie *et al.,* 2011). Fourie *et al*. (2011) further implicated that ethnic effects of fibrinogen concentration as a potential cause for this

finding. Fibrinogen levels had been said to differ among European Americans, African Americans, and Africans, where the highest was observed among Africans (Folsom *et al.,* 1992; James *et al.,* 2000; Cronjé *et al.,* 2017). The inherited higher fibrinogen levels among the African ancestry may put the Africans at risk for CAD.

Plasminogen activator inhibitor-1 (PAI-1), the primary inhibitor of tPA-1 and uPA-1, is considered a critical regulator of the fibrinolytic system pathway. Under normal conditions, tPA-1 or uPA-1 and PAI-1 are synthesised and released simultaneously by the endothelial cells in equimolar amounts to regulate fibrinolysis (Gong *et al.,* 2016). However, when CRP levels are elevated, dysregulation of the equilibrium between coagulation and fibrinolysis occurs, favouring coagulation and reducing the inhibiting fibrinolytic activity, where a reduction in tPA-1 levels and the increase in PAI-1 are often present (Chen *et al.,* 2008). Similarly, during ED or endothelial activation, this equilibrium is usually altered and may result in elevated tPA-1, thus generating plasmin and lysing of soluble blood clots which lead to the production of fibrinogen and FPDs, such as D-dimer (Poggesi *et al.,* 2015; Storch *et al.,* 2017). Increasing evidence exists in the general population to support the role of endothelial activation and ED in the development of plaque formation which can lead to atherosclerosis (Castellon and Bogdanova, 2015; Park and Park, 2015). Borges *et al.* (2014), found that D-dimer levels increased in HIV-positive men as they age over time, while among HIV-positive females D-dimer levels were elevated at an early age. Furthermore, it was discovered that inflammation, renal function, and TC levels were independently associated with elevated levels of D-dimer (Borges *et al.,* 2014). In addition, it was also indicated that D-dimer levels were significantly higher among PLWH compared to the HIV-negative population; which is indicative of elevated thrombotic activity (Borges *et al.,* 2014; Funderburg, 2014).

2.9 IMPACT OF HIV INFECTION AND HAART ON THE IMMUNE SYSTEM

The human immunodeficiency virus initiates inflammation in the vascular endothelial cell membranes which trigger a cascade of biochemical intracellular reactions (Chen *et al.,* 2017). HIV-induced immune cell activation and HAART-induced OS play a

crucial role in initiating these events which ultimately result in elevated levels of inflammatory molecules (Hileman and Funderburg, 2017). Although traditional cardiovascular risk factors play causative roles in the progression of CVD, current reports suggest that HIV-associated inflammation and immune cell activation are important mediators of cardiovascular risk (Virginia and Triant, 2013).

Immune cell activation reported in HIV-positive patients is said to occur when the HIV replicates (D'Abramo *et al.,* 2014). Early HAART treatment reduces immune cell activation, although in some cases the activation persists, regardless of ARV therapy (Van den Dries *et al.,* 2017). Chronic inflammation and immune cell activation contribute to the ED and subsequently lead to atherosclerosis among the HIV-positive population (Siedner *et al.,* 2015). It is argued that the immune cell activation is responsible for many complications of HIV infection, manifested as CVDs (Ahmed *et al.,* 2018). It has been reported that HIV-infection causes the atypical activation of the immune system, which is the leading cause of various disease pathologies such as CVD. Most CVDs are due to EC inflammation which causes EC hyper-activation and may lead to ED (Virginia and Triant, 2013; Beltrán *et al.,* 2015).

Immune cell activation is detrimental in that it leads to $CD4^+$ cell depletion and causes immune dysfunction (Vijayan *et al.,* 2017). It consequently activates a cascade of inflammatory and immunological reactions with the secretion of pro-inflammatory cytokines, such as tumour necrosis factor (TNF-α), IL-6, and CRP. They regulate the immune cells involved in innate and adaptive immune responses and have an enormous impact on the regulation of the immune system in general (Virginia and Triant, 2013; Sokoya *et al.,* 2017).

2.10 HIV INFECTION AND ENDOTHELIAL BIOMARKERS

Inflammation is a commonly considered key component within the pathophysiology of CHD (Shrivastava *et al.,* 2015). Identifying biomarkers of inflammation and improving treatment strategies may help to reduce the risk of CVD. Patients with normal LDL-C levels and high CRP levels still showed an equivalent risk for CVD (Shrivastava *et al.,* 2015). Cytokines, especially IL-6, stimulate the liver to synthesise and produce CRP.

Although CRP is synthesised primarily in hepatocytes, smooth muscle cells, macrophages, endothelial cells, lymphocytes, and adipocytes also produce it (Sproston and Ashworth, 2018).

C-reactive protein plays an important role in binding LDL-C, promoting LDL-C uptake by the macrophage, which further enhances the reduction of eNOS expression and subsequent reduction in NO-bioavailability. All these pathologic factors are key elements in atherogenesis, promoting the development of atherosclerotic lesions (Shrivastava *et al.,* 2015). C-reactive protein levels is even higher among the HIV-positive individuals and it was independently associated with increased risk for CVD (Triant *et al.,* 2009; Shivakoti *et al.,* 2015). The increased levels of CRP were independent of HAART (Shivakoti *et al.,* 2015; Vishwanath *et al.,* 2016).

Chronic HIV inflammation is known to induce endothelial activation and ED and, has been proposed as a potential mechanism for HIV and HAART-induced atherosclerosis (Beltrán *et al.,* 2015). Upon inflammation, pro-inflammatory cytokines, such as IL-6, CRP, and TNF-α, are released and these cause increased expression of CAMs and selectins on the surface of the endothelium (Arango and Descteaux, 2014; Mosevoll *et al.,* 2018). The expression of these CAMs and selectin genes recruit neutrophils and other leukocytes into the sub-endothelium, referred to as endothelial activation which results in leukocyte rolling to the site of inflammation (Gimbrone and García-Cardeña*,* 2016).

Selectins are a family of three related transmembrane glycoproteins which are expressed on leukocytes, activated endothelium, and platelets (McEver*,* 2015). Transient rolling of leukocytes along the endothelium is mediated by the selectins (L-selectin, E-selectin, and P-selectin) through the process of diapedesis (Leick *et al.,* 2014). L-selectin is constitutively expressed on the majority of leukocytes, while E-selectin is often expressed by activated endothelium. P-selectin is momentarily expressed on the cell surface of the platelets and the endothelium upon endothelial activation (McEver *et al.,* 2015). Endothelial activation has been related to the upregulation of VCAM-1, ICAM-1, and selectins which are further associated with the elevation in inflammation via recruitment, adhesion, and migration of activated

leukocytes. These conditions aggravate vascular permeability and thrombosis (Pober, 2002; Castro-Ferreira *et al.,* 2018).

Cell adhesion molecules are glycoproteins expressed on the cell surface of many cell types including leukocytes, platelets, and endothelium. They are key to various cellular processes such as the development and maintenance of tissue, stimulation of cell differentiation, and cell communication (Galkina and Ley, 2007; Khalili and Ahmad, 2015). These CAMs facilitate the firm adhesion and recruitment of circulating leukocytes to sites of inflammation in the endothelium. It has been shown that elevated levels of CAMs are suggestive of inflammation, ED, and atherosclerosis (Gimbrone and García-Cardeña*.,* 2016; Wu *et al.,* 2017). Cell-adhesion molecules also mediate the adhesive interaction between platelets and endothelium which results in thrombosis (Koh and Park, 2018).

The role of CAMs in leukocyte activity in both healthy and disease stages has been extensively investigated in both HIV-negative *in vivo* and *in vitro* models (Khalili and Ahmad, 2015; Koh and Park, 2018). The attachment of leukocytes to the endothelium is mediated by sICAM-1 and sVCAM-1. The transmembrane glycoproteins sICAM-1 and sVCAM-1 play a pivotal role in transmigrating leukocytes into the vascular intima (Koh and Park, 2018). The four major steps for the transmigration of leukocytes by the CAMs involves capturing, rolling, adhesion, and transmigrating (Figure 2.3).

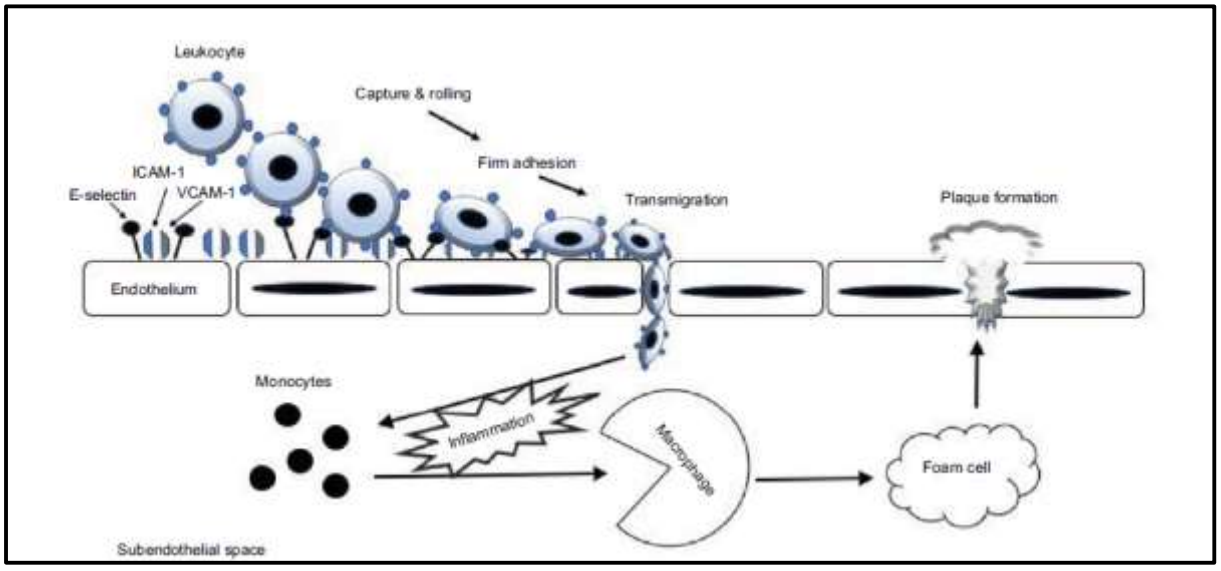

Figure 2.3: The role of CAMs in the leukocyte adhesion cascade during endothelium inflammation (Koh and Park, 2018).

Selectins only play a role in the first two steps of leukocyte transmigration (Koh and Park, 2018). Leukocytes do not adhere to the normal endothelium but only to the activated endothelium when CAMs and selectins are expressed (Galkina and Ley, 2007; Čejková *et al.,* 2016). Elevated CAMs and selectins are associated with vascular inflammation and accumulation of macrophages within the intima, which subsequently accelerates atherosclerotic progression (Koh and Park, 2018). The soluble CAMs indirectly reflect the rate of endothelial activation by CAMs expression on the endothelial cell surface (Koh and Park**,** 2018). Cell adhesion molecules and selectins are therefore considered useful biomarkers of inflammation, ED, and atherosclerosis (Hocaoglu-Emre *et al.,* 2017; Koh and Park, 2018).

Endothelial targeting of drugs via CAMs has been achieved in animal models of human pathologies (Muzykantov, 2013). The CAMs are observed more frequently among human atherosclerotic lesions as compared to healthy arterial tissue (Malik *et al.,* 2001). Knock-out mice with deficient E-selectin, P-selectin, and sICAM developed fewer atherosclerotic lesions compared to their controls (Collins *et al.,* 2000). Therefore, a reduction in the expression of P-selectin, ICAM-1, or E-selectin prevents the development of atherosclerotic lesion in a mouse model. These biomarkers may play a pivotal role in the pathogenesis of atherosclerosis.

Interleukin-6 and CRP are associated with atherosclerosis and are independent predictors of future cardiovascular complications (Lubrano and Balzan, 2015; Shrivastava *et al.,* 2015). Soluble Vascular cell adhesion molecule-1 and sICAM-1 together with VWF were highly expressed in endothelial activation and may lead to atherosclerosis (Gragnano *et al.,* 2017; Wu *et al.,* 2017). These findings support the notion that inflammation, endothelial activation, and dysfunction might play an important role in the progression of plaque formation and atherosclerosis in the HIV-positive population on HAART.

2.11 ASSOCIATION OF HAART WITH ENDOTHELIAL ACTIVATION AND DYSFUNCTION

Atherosclerosis-associated CVD is the number one cause of mortality in the HIV infected population on HAART (Laurence *et al.,* 2018; WHO, 2018). It was reported that HAART is associated with many adverse effects, which include dyslipidaemia, IR, and central obesity (Dimala *et al.,* 2018; Njuguna *et al.,* 2018). Despite all the extensive research done thus far, the mechanism of HIV-induced atherogenesis remains unclear. HIV-induced atherogenesis is further complicated by HAART, drug abuse, and co-morbidities which include amongst others metabolic abnormalities, opportunistic infections, renal disorders, and lifestyle factors (Freiberg and So-Armah, 2016; Kearns *et al.,* 2017). Evidence implicates the elevation of inflammatory processes (immune cell activation) in which HIV accelerates atherogenesis.

In vitro studies found that HIV proteins such as Tat and gp120 may activate the endothelium and cause ED before the HIV can invade, infect and replicate itself in the endothelium (Jiang *et al.,* 2010; Wang *et al.,* 2015; Hijman *et al.,* 2018). Another *in vitro* study reported that the HIV protein, Nef, also increased ROS generation; which is a mechanism specifically associated with ED (Duffy *et al.,* 2009). Human immunodeficiency virus treatment naïve patients also exhibited consistently elevated levels of ROS with a subsequent reduction in antioxidant activity (Couret and Chang, 2016). It is well documented that ROS plays a crucial role in various physiological processes in the human body (Sandström *et al.,* 2006; Cubero and Nieto, 2012; Forman *et al.,* 2015). In pathological conditions, the ROS levels are abnormally elevated to such an extent that it cannot be neutralised by its defence mechanisms. The abnormal levels of ROS can damage biological molecules which lead to the altering of physiological functions and may initiate various pathologies in the cells. Endothelial dysfunction is an example of how ROS alters the normal activity of eNOS enzyme which leads to eNOS-uncoupling where abnormal levels of superoxide are produced instead of NO (Zhao *et al.,* 2015). This may thus cause ED which is an early event in the pathogenesis of atherosclerosis.

It has been shown that plasma levels of the ED markers, VWF, tPA-1, and tPAI-1 were significantly elevated among HIV positive HAART-naïve patients (De Larrañaga *et al.,* 2004). Human immunodeficiency virus-positive HAART-naïve patients also showed higher levels of sVCAM-1, sICAM-1, and E-selectin which is indicative of endothelial

activation, ED, and suggest inflammation (Kristoffersen *et al.,* 2009; Dagenais-Lussier *et al.,* 2015). Although the implementation of HAART is beneficial in reducing the VL and various infections among HIV-infected subjects, it did not completely reverse the effects of inflammation and immune cell activation.

The beneficial effects after initiation of HAART were mostly attributed to the reduction in CRP and IL-6 (Non *et al.,* 2017). As previously mentioned, CRP and IL-6 play a pivotal role in the initiation of both inflammation, endothelial activation, and the development of ED. Chronic inflammation has been proposed as a possible mechanism for HIV and HAART-induced atherosclerosis (Beltran *et al.,* 2015). Endothelial dysfunction together with CIMT are surrogate markers of atherosclerosis that are clinically useful and are predictors of CVD. These surrogate biomarkers showed that the risk for CHD increased significantly among HIV positive patients who are on PI-based regimens (Ross *et al.,* 2009; Cerrato *et al.,* 2015). Chronic exposure to these regimens accelerates the atherosclerotic process in HIV-positive individuals and increases the risk of developing MI (Hyle *et al.,* 2017; Vachiat *et al.,* 2017).

HIV-positive patients exposed to HAART for approximately 48 weeks, showed elevated levels of high sensitivity C-reactive protein (hsCRP) and asymmetric dimethyl arginine (ADMA), thus reflected constant inflammation and ED (Luetkemeyer *et al.,* 2012). HIV-positive populations in SSA have also demonstrated endothelial activation and this persisted even after the initiation of HAART (Fourie *et al.,* 2015). However, evidence exists that HAART may be beneficial in reducing ED in HIV-positive patients, but this was only observed during a 12-month treatment period. This beneficial effect of HAART on the endothelium was specifically dependent on the duration of HIV infection prior to HAART initiation (Kristoffersen *et al.,* 2009). The duration of HIV infection before HAART initiation has more detrimental effects on the endothelium; therefore, it will take longer to observe the beneficial effect of HAART on the endothelium. *In vitro* studies have shown that HAART, especially PI-based regimens can directly cause ED in porcine coronary arteries through OS, where eNOS was down-regulated (Wang *et al.,* 2007; Chen and Mark, 2014). HIV positive patients who were on PI-based regimens and NNRTIs-based regimens for more than four years exhibited elevated levels of endothelial activation (sVCAM-1), inflammation (hsCRP,

TNF-α, IL-6), and an increased CIMT compared to the control (Ross *et al.,* 2009). Contrary, HAART initiation with either fosamprenavir/RTV or EFZ in combination with Abacavir (ABC) /3TC for 96 weeks revealed a decrease in endothelial activation (sVCAM-1) and thrombotic activity (D-dimer) except for inflammation (hsCRP) levels. It was only fibrinogen which showed a significant decline in the EFZ group (Kumar *et al.,* 2013).

While some ED biomarkers improved after HAART initiation, not all demonstrated equal improvements (Beltran *et al.,* 2014; Cerrato *et al.,* 2015). It was found that control subjects who were on a PI-based regimen Indinavir for four weeks developed ED; however, this was not accompanied by dyslipidaemia (Andrade and Cotter, 2006). The observed outcome difference(s) could be explained by the baseline differences, e.g., lower HDL-C, smoking status, and body fatness between study groups and duration on HAART treatment. It is anticipated that HAART should reduce immune activation, inflammation, and consequently maintain normal endothelial function via the reduction in viral replication. Though, this was only observed during short-term HAART treatment (Arildsen *et al.,* 2013).

Since ED is an early event in the initiation of atherosclerotic disease, it is considered a powerful diagnostic tool to predict CVD (Modena *et al.,* 2002; Mudau *et al.,* 2012; Park and Park, 2015). Therefore, the measurement of ED biomarkers in HIV-positive patients could provide important information on the effects of long-term HIV HAART therapy. Although contradictory findings exist regarding the effects of HAART on the endothelium, the present study intents to elucidate whether HAART effects early markers of CVD in HIV-positive individuals.

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

This section provides the approaches that were used in the study as it was conducted. These include the study design, study population and setting, eligibility criteria, sampling technique and sample size, organisational procedures, data collection procedure and instruments used, ethical considerations, and the data analysis.

3.2 STUDY DESIGN

The study was a cross-sectional design employing quantitative methods. It allowed the investigators to observe the effect of HAART on endothelial biomarkers. This research technique involves the testing of a theory based on measured variables and statistical analysis. The design allowed sample collection and analysis to be completed within 12 months. In this study, data were expressed in numbers from age, gender, height, weight, glucose, TC, TG, fibrinogen levels, and endothelial biomarkers.

3.3 STUDY POPULATION AND SETTING

Male and female participants were 18 years or older. The study was conducted at Mankweng Hospital and surrounding clinics, where patients were either transferred to or from the Mankweng Hospital which is situated in the Polokwane Local Municipality, in the Capricorn District, Limpopo Province, South Africa. The referral clinics included: Ga-Mothapo, Ga-Thoka, Megoring, Badimong, Mankweng, Nobody, Evelyn Lekganyane and Makanye.

3.4 ELIGIBILITY CRITERIA

The eligible groups consisted of participants who were HIV positive individuals who are on HAART (HAART-exposed group), HIV-positive treatment naïve, individuals who were diagnosed positive for HIV prior to the commencement of HAART (HAART-naïve group) and HIV negative; therefore, individuals who are free of HIV (control group). The latter group should further not have been diagnosed with any of the following conditions prior to the initiation of the study: coronary artery disease, acute myocardial ischaemia, history of DM, high triglyceride levels, high LDL-C levels, hypertension, participants taking medication for cardiovascular-related conditions, and healthy (non-HIV) who were pregnant and breastfeeding. Any co-infection such as tuberculosis (TB) was also excluded.

3.5 SAMPLING TECHNIQUES AND SAMPLE SIZE

3.5.1 Sampling techniques

The participants were selected disproportionally from the above-mentioned clinics. Consecutive sampling was applied to include accessible participants. The proportionate stratified random sampling was employed to accommodate all the subgroups (control, HAART-naïve, HAART-exposed) in the target population.

3.5.2 Sample size

The original predetermined sample size was 155; however, a total number of 158 participants were recruited in this study. The sample size (n = 155) was determined based on the prevalence of people living with HIV in the Limpopo Province, which is 11.4% with a confidence interval (CI) of 95% and margin error of 5% (Day and Gray, 2012).

The formula used to determine the sample size is as follow:

$$n=\frac{t^2\ x\ p(1-p)}{m^2}$$

Description:

n = required sample size

t = confidence level at 95% (standard value of 1.96)

p = estimated prevalence of HIV in Limpopo Province in the project at 11.4% (0.114)

m = margin of error at 5% (standard value of 0.05)

Calculation: $= \frac{1.96^2 \text{x} 0.114(1-0.114)}{0.05^2}$

$= 155.21 \sim 155$

3.5.3 Organisational procedure

The researcher(s) visited the clinics based on the clinic's established routine schedules with their patients, who were coming for regular check-ups and the collection of medication. Before the collection of the data, visits to the clinics were conducted by the researcher(s) to establish the patient's routine schedules and to identify potential participants. The study was then explained to the prospective participants (Addendum A: Information to participants); informed consent was obtained and the contact information was collected (Addendum B: Consent form). All participants were reminded a day before their normal routine visits by SMS, e-mails, or a call to fast overnight for at least 10 hours.

On the day of data collection, for confidentiality purposes, all participants were assigned a number and it was only the researcher(s) who had access to all participant's details. A questionnaire was administered to all participants to confirm eligibility; patients' medical files were screened and blood was collected for

biochemical analysis (Appendix C: Data collection form). Consecutive sampling was applied to include accessible participants.

3.6 DATA COLLECTION PROCEDURE AND INSTRUMENTS USED

3.6.1 Questionnaires and medical files

Socio-demographic questionnaires in the form of semi-constructed interviews and patient's medical files were used to obtain information regarding age, gender, ethnicity. Information on the duration of HAART, year of HIV diagnosis, and the specific regimen the patients were done at the time of sample/data collection (Appendix C). After analysis of the blood samples, a feedback letter (Addendum C1) was completed for each participant and forwarded to them.

3.6.2 Blood sample collection and preparation

Fasting blood samples were collected by a phlebotomist (qualified nurse) and transported on dry ice to the Haematology Laboratory in the Department of Medical Sciences (UL) and Lancet Laboratories (Polokwane Branch) for blood sample analysis. The blood was collected in ethylenediaminetetra acetic acid (EDTA) for plasma preparation, with sodium fluoride blood tubes for glucose measurement and Vacutainer SST II tubes for serum preparation. All blood samples were centrifuged at 2000 rpm for 15 minutes.

3.6.3 Biochemical analysis

3.6.3.1 HIV testing

On the day of blood collection, the processed serum samples were used to determine the HIV status of all participants. These tests were performed at the Medical Sciences Laboratory (UL) (Alere Determine HIV-1/2, Alere to Abbott Medical Co Ltd., Japan).

3.6.3.2 Fasting blood glucose determination

Glucose was determined on the Cobas® Integra 400 plus auto-analyser with the enzymatic (hexokinase) colorimetric method (Roche Diagnostics). Hexokinase (HK) catalysed the phosphorylation of glucose which was present in the sample by ATP in the presence of magnesium (Mg^{2+}) in the reagent to produce glucose-6-phosphate (G6P) and adenosine diphosphate (ADP). Glucose-6-phosphate becomes oxidized by glucose-6-phosphate dehydrogenase (G6PD) in the presence of nicotinamide adenine dinucleotide phosphate ($NADP^{+}$) to produce 6-phosphogluconate and the production nicotinamide adenine dinucleotide phosphate (NADPH). The concentration of the NADPH formed at the subsequent increase in absorbance at 340nm is directly proportional to the glucose concentration. A list of reference ranges was used for this parameter (Appendix I).

3.6.3.3 Lipid profile determination

Serum samples were sent to the Lancet Laboratories in Polokwane to determine the lipid profiles (TG, TC, and HDL) for all participants. At the Lancet Laboratory, all samples were also registered on the information system. The lipid profile was determined with the use of Roche reagents (Roche Diagnostics) which are based on the enzymatic colorimetric assays on the Cobas® Integra 400 plus auto-analyser.

3.6.3.3.1 Triglyceride measurement

The serum TG was determined on the Cobas® Integra 400 plus auto-analyser by the enzymatic colorimetric method (GPO/PAP) (Systemic Liquicolour Reagent test kit, Germany). The method for this assay is based on a modified Trinder colour reaction to produce a linear endpoint reaction. Triglyceride was completely hydrolysed by lipases to glycerol and fatty acids.

Glycerol is converted to glycerol-3-phosphate by glycerol kinases. Glycerol-3-phosphate is then converted into dihydroxyacetone phosphate (DHAP) and hydrogen peroxide (H_2O_2) by glycerophosphate oxidase. The H_2O_2 reacts with 4-aminoantipyrine and 3, 5 dichloro-2-hydroxybenzene (Chlorophenol) in a reaction catalysed by peroxidase to yield a red coloured quinoneimine fluorescent dye. The fluorescent intensity of the colour produced is directly proportional to the concentration of TG in the sample at an absorbance 510nm is directly related to the concentration of TG in the sample (Friedewald *et al*., 1972).

3.6.3.3.2 Total cholesterol measurement

The TC was determined by an enzymatic colorimetric method (CHOD/PAP) on the Cobas® Integra 400 plus auto-analyser (Systemic Liquicolour Reagent test kit, Germany). The TC method is based on the determination of 4-cholestenone after enzymatic cleavage of the TC ester by the enzyme cholesterol esterase, conversion of TC by cholesterol oxidase and subsequent measurement by the Trinder reaction of H_2O_2 formed. The absorbance readings at a wavelength of 500nm are proportional to the concentration of TC in the sample (Friedewald *et al*., 1972).

3.6.3.3.3 High-Density Lipoprotein cholesterol quantification

The HDL-C was also done on Cobas® Integra 400 plus auto-analyser employing the enzymatical method by cholesterol esterase and cholesterol oxidase coupled with polyethylene glycol to the amino groups (Systemic Liquicolour Reagent test kit, Germany). Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase. Cholesterol is oxidized by cholesterol oxidase to 4-cholestenone and H_2O_2 in the presence of Oxygen. Hydrogen peroxide further forms a dye, where the intensity of dye measured concentrations of the HDL-C at the wavelength of 500nm determines the HDL-C concentration.

3.6.3.3.4 Calculating Low-Density Lipoprotein Cholesterol (LDL-Cc_{cal})

The LDL-C levels were automatically calculated on the Laboratory information system. The calculation used to determine LDL-C are as follow:

$LDL\text{-}Cc_{cal} = TC - (\frac{TG}{2.2}) + HDL\text{-}C$

3.6.3.4 $CD4^+$ cell count determination

Whole blood samples which were collected in the EDTA vacutainer tubes were sent to the Lancet Laboratories on the day of collection to determine the $CD4^+$ counts of only the HAART-exposed participants and HAART-naïve participants who were treatment naïve. The $CD4^+$ count for the control participants were not determined because we were more concerned of the probable situation of a low $CD4^+$ count due to HIV itself. Since we were unable to determine the VL, $CD4^+$ count will also provide information on the severity HIV-infection. The $CD4^+$ count was determined using the Cytomics FC500 Flow Cytometer Multi-Platform loader (MPL) which is an automated tube-based acquisition device for clinical assays (Beckman Coulter). The following reagents were used to determine the $CD4^+$ counts, CD45 FITC and CD4 PE. The enumeration of the $CD4^+$ T-cells by Panleucogating (PLG) involves two measurements. Identifying the white cell count (WCC) and positive $CD4^+$ T cells within the total white cell population. The samples reacted with Fluorochrome labelled CD4 and CD45 monoclonal antibodies (Flow Care PLG CD4 Reagent) that are specific for the cell surface antigens CD4 and CD45. During Flow cytometric analysis, cells bind to the labelled antibody and were identified based on their specific fluorescence emission related to the specific Fluorochrome attached to either the CD45 or the CD4 antibody. All white blood cells were CD45 positive, but the brightest fluorescence is emitted by the lymphocytes. The CD4 positive T-lymphocytes were further defined from the rest of the white blood cells based on their complexity (side scatter) and their specific CD45 and CD4 expression. The additional serum and plasma samples were stored at -80°C for later analysis of the biomarkers of interest.

3.6.3.5 Multiplex bead-based immunoassay

In this study two commercially available bead-based multiplex kits (were used to analyse the 8 serum biomarkers on a Luminex 200™ device. The multiplex system can quantify a large array (>100) of protein and peptide targets simultaneously in a single 5µl blood sample. The principle of Luminex is similar to flow cytometry. However, the Luminex sorts pre-coated analyte-specific beads (same size) which are ligated with specific mean fluorescence intensities (MFI) (different concentrations of the red dye). Luminex 200 has two lasers to examine the pre-coated analyte-specific beads. The lasers examine the spectral property (MFI, the concentration of the red dye content) of the beads and the reaction around the analyte (antibody) of the specific bead.

Fibrinogen, CRP, L-selectin, and D-dimer concentrations were simultaneously determined in serum samples, using human CVD magnetic bead panel 2 (EMD Millipore Corporation, Billerica, USA, 2017). The second Luminex assay (human CVD magnetic bead panel 3) consisted of the following biomarkers, P-selectin, VWF, ICAM-1, and sVCAM-1 concentrations were also simultaneously determined in serum samples. Assay sensitivity for the analytes are in the pg/mL range, and intra-assay and inter-assay coefficients of variation are <10% and <20%, respectively.

Briefly, distinct sets of fluorescently dyed beads (150µl) specific for each biomarker of interest were mixed with capture monoclonal antibodies specific for each biomarker. A serum sample serial dilution (1:40 000) for human CVD magnetic bead panel 3 and serum sample serial dilution (1:100) for human CVD magnetic bead panel 2 was prepared. Assay buffer (25µl) was added to the entire 96-well plate. The diluted serum which was combined with premixed beads solution and monoclonal antibodies (25µl) were placed in the EMD Millipore's MILLIPLEX® 96-well plate together with the quality controls (25µl) and standards (25µl) according to the predetermined well map. The 96-well micro-plates were incubated (12 hours) in the plate shaker overnight (2 – 8°C, 600rpm).

The following day, the 96-well plate was placed in a magnetic plate and incubated for 90 seconds, where after it was washed thrice in the washing buffer (200µl).

Fluorescent detection antibody mixture was added to the 96-well plate and incubated for 1-hour in the plate shaker (22°C, 600rpm). Streptavidin-PE conjugate (50µl) was added to the 96-well plate, incubated for 30 minutes.

The 96 well-plate were re-suspended in Luminex sheath fluid and analysed in the Luminex 200 device. Before the running of the samples, calibration and performance verification test was run to ensure that the system operates correctly and maintaining data accuracy. The xPONENT® software package 5.1 was used for data acquisition. xPONENT® software package from the Milliplex company was used in this study to acquire the biomarkers data. Milliplex bead based anylytes data are retrieved with the aid of the software.

3.7 ETHICAL CONSIDERATIONS

The relevant documentation was submitted to the Faculty Higher Degrees Committee (Faculty of Science and Agriculture) for approval, and to the UL Turfloop Campus Research Ethical Committee (TREC) for ethical clearance (Appendix E and F). Permission was granted by the senior managers at the Department of Health and at Primary Health Care and Social Development to conduct the study at the selected clinics (Appendix G and H). The study was explained and informed consent was obtained from all participants (Appendix B). The participants were informed that the study is voluntary and that they have the right to withdraw at any point from the study. Very strict confidentiality principles were adhered to in the handling of personal information and the obtained research results. The participants were also informed that blood will be collected by a qualified nurse and that they might experience some pain and discomfort during the blood collection procedure. They were also informed that all samples will be used for study purposes only. A list of all standard operating procedures which include general laboratory procedures, how to handle blood inside and outside the laboratory, and personal protective procedures were used in this study (Appendix D).

3.8 RELIABILITY, VALIDITY AND BIAS

3.8.1 Reliability and validity

Reliability is the extent to which an assessment or experiment produces stable and consistent results (Iwata *et al.,* 2013). Validity refers to how well a testing instrument measures what it is supposed to measure (Gable *et al.,* 2012). The reliability and validity of all tests were tested with the use of coefficient of variation, quality assurance and interference. All instruments were calibrated, quality control and standard samples were used to monitor quality. Blood lipid levels were determined with standard laboratory procedures. To ensure accuracy, the analyser was calibrated prior to analysis where Serodos and Serodos plus were respectively used to establish normal and abnormal human sera reference ranges. Autocal is a multiparametric calibration serum. The concentration and activities have been collected to optimum calibration of the analyser (Tamang *et al*., 2013)

3.8.2 Bias

Bias is defined as any tendency which prevents unprejudiced consideration of a question and leads to the distortion of the measurement process (Pannucci *et al.,* 2010). The questionnaire was self-reported assessment and was therefore subjective to bias; however, the researcher ensured that all participants understood the questions. Standardised procedures were used for the analysis of all the biomarkers in order to minimise bias. In the study random sampling was applied in order to give all subjects equal chances to be included in the study.

3.9 DATA ANALYSIS

All the data were statistically analysed with the Statistical Package for Social Sciences (SPSS) software program version 25. Descriptive statistics were performed on all variables. All normally distributed variables were expressed as mean ± standard

deviation (SD) and all non-parametric data were expressed as median and Interquartile ranges (IQRs). Categorical variables were expressed as percentages. For Gaussian distributions, parametric statistical test, one-way analysis of variance (ANOVA) was used to compare means across the study groups. The p-values of the variables which showed significant differences were further adjusted by the Bonferroni correction for multiple tests to reduce the chances of obtaining type 1 error. The Bonferroni Post-hoc test was consequently applied for normally distributed continuous variables. Kruskal-Wallis test was used for variables with non-Gaussian distributions.

Significance values were adjusted with the use of the Independent Samples Kruskal-Wallis Post-hoc test for multiple tests where appropriate. All categorical variables were analysed with the Chi-square test and the Chi-square Bonferroni Post Hoc-test were used where appropriate. One-way analysis of covariance (ANCOVA) was used to compare means of the main parameters of interest (BMI, fasting glucose concentration, lipid profile which include HDL-C, LDL-C, TC, TRIG, L-selectin, P-selectin, sICAM-1, sVCAM-1, VWF, CRP, D-dimer, and Fibrinogen) across the study groups while adjusting for age and gender. Bonferroni tests were performed to obtain differences between the study groups.

Pearson and partial correlations were used to assess the associations of the main parameters of interest with HAART. All partial correlation analysis was adjusted for age and BMI. Multiple regression analyses were used to test for associations between HAART regimens and CVD risk factors, endothelial, inflammatory, and coagulating biomarkers. We performed multivariate adjusted regression analyses with endothelial biomarkers (L-selectin, P-selectin, sICAM-1, sVCAM-1, VWF), inflammatory biomarker (CRP), and coagulating biomarkers (fibrinogen, D-dimer) as dependent variables. This was done to determine the contributions of HAART in CVD risk factors**.** The covariates that were selected and entered into the model included age, gender, TG, duration on HAART, $CD4^{+}$ cell count. The covariates were selected based on appropriate selection criteria which are mainly based on the strength of association between the covariates and main variables of interest. Age, gender, TG and duration on HAART were included because they are patient variables. CD4+ cell count represents the immune status of the participants as a confounder and it is related

to both the exposure and the outcome. A p-value of < 0.05 was considered statistically significant.

CHAPTER 4

SOCIO-DEMOGRAPHIC CHARACTERISTICS

4.1 RESULTS AND DISCUSSION

Normally distributed data were presented as means and SD, non-normally distributed data were expressed as means and IQRs, and categorical data were expressed in frequencies and percentages. This section focuses specifically on analysing and interpreting the socio-demographic characteristics which included age, age categories, and gender of all study groups (HAART-exposed, HAART-naïve, and control) which are followed by the discussion.

4.1.1 Results: Description of the participants and groups

In this cross-sectional study 158 (n = 158) participants from both genders were recruited. The study population consisted of three groups, where 51 (31.9%) were the control, 36 (22.5%) were HAART-naïve and 73 (45.6%) were HAART-exposed groups. The age of the participants ranged from 18 – 81 years (Table 4.1). The mean ± standard deviation (STD) (M ± SD) of the total population was 40.25 ± 12.67 while the HAART-exposed group was 43.19 ± 10.15 years, for the HAART-naïve group it was 39.06 ± 11.70 years and for the control group was 36.88 ± 15.53 years (Table 4.1)**.** In terms of the three groups, the control group had younger participants compared to HAART-naïve and HAART-exposed groups.

There was a significant difference in the general age and age categories across the groups (p = 0.001). The Bonferroni Post Hoc-test further revealed a significant difference in terms of age between the control group versus HAART-naïve group (p = 0.018) and HAART-exposed group versus HAART-naïve group (p = 0.018). The majority of participants in the overall population were in the age category of 30 – 39 years (36.3%). For both the control and HAART-naïve groups the majority of

participants were in the age categories of 18 – 29 years and 30 – 39 years, respectively, while most participants in the HAART-exposed group were in the age categories of 30 – 39 years and 40 – 49 years (Table 4.1).

No significant difference in terms of gender across the three groups was observed (Table 4.1, p = 0.536). The overall study sample consisted of more females (63.8%) than males (36.3%) which were similar in all three groups (Table 4.1). Due to the uneven gender distribution it was anticipated that gender might be a confounding factor and the results would be adjusted accordingly.

Table 4.1: Age, age categories, and gender of the study population.

Parameter	**Total** (N = 158)	**HAART-exposed** (n = 71)	**HAART-naïve** (n = 36)	**Control** (n = 51)	**p-value**
Age, years (mean ± SD)	40.23 ± 12.72	43.24 ± 10.19	39.06 ± 11.70	36.88 ± 15.53	**0.019**
Age, years Categories					
18 – 29, n (%)	35 (22.2)	5 (7.0)	10 (27.8)	20 (39.2)	**0.000**
30 – 39, n (%)	57 (36.1)	23 (32.4)	14 (38.9)	20 (39.2)	
40 – 49, n (%)	36 (22.8)	26 (36.6)	6 (16.7)	4 (7.8)	
≥50, n (%)	30 (19.0)	17 (23.9)	6 (16.7)	7 (13.7)	
Gender					
Female, n (%)	100 (63.3)	46 (64.8)	20 (55.6)	34 (66.7)	0.536
Male, n (%)	58 (36.7)	25 (35.2)	16 (44.4)	17 (33.3)	

p-values of ≤ 0.05 were considered statistically significant. The largest participation was in the treated group and also in terms of gender.

4.1.2 Discussion

In the study HIV infection was predominantly in the age group 30 – 39 years (64.91%). This finding was supported by Stats SA (2017) which also indicated that the age group 30 – 39 has the highest HIV transmission rate. They were also more likely to engage in multiple sexual partnerships which may explain the high rate of HIV infection transmission in this particular group. This could be aggravated by the high levels of

unemployment affecting the HIV-positive population, where they may be looking for financial security. The prevalence of HIV-infection in urban settings are higher compared to the rural settings. The migration of men to the urban areas may also create a mode of HIV transmission, especially those men who frequently return to their rural homes from urban settings. Females may be more vulnerable as they may be seeking financial security, accepting any sexual proposal in exchange for money. Gender inequality and male's sexual and economic dominance over females have been identified as major cause of HIV transmission (Ayuttacorn *et al.,* 2019), and it is highly likely that it is at play in the current study population.

Some individuals in this age group (30 – 39 years) fall in the most economically active sector of the population. HIV-infection is associated with an increase in various other co-morbidities resulting in days away from work, a decrease in productivity, early pension and premature death (Gallant *et al.,* 2017; Ghiasvand *et al.,* 2019). This results in a loss of expertise. However, it also affects the family income which has a dire socio-economic impact. The findings suggest that there may still be a lack in education on the importance of understanding the cultural norms which are associated with poor sexual behaviours among rural South African population.

4.2 RESULTS AND DISCUSSION: SOCIO-DEMOGRAPHICS IN TERMS OF ETHNICITY, $CD4^+$ T-CELL COUNT, AND HAART REGIMENS

This section focuses on analysing and interpreting the socio-demographic characteristics which included ethnicity and the type of HAART regimen of all study groups (HAART-exposed, HAART-naïve and control).

4.2.1 Results

The three groups did not show any significant differences with regard to ethnicity and the type of HAART regimen (Table 4.2). The majority of participants were Northern Sotho speaking across all three groups (Table 4.2).

Table 4.2: Ethnic distribution of the study population.

Parameter	**Total** (N=158)	**HAART-exposed** (n=71)	**HAART-naïve** (n=36)	**Control** (n=51)	**p-value**
Ethnicity					
Zulu, n (%)	1 (0.6)	0	0	1 (2.0)	0.441
Ndebele, n (%)	2 (1.3)	1 (1.4)	1 (2.8)	0	
Southern Sotho, n (%)	1 (0.6)	0	0	1 (2.0)	
Venda, n (%)	3 (1.9)	2 (2.8)	0	1 (2.0)	
Tsonga, n (%)	4 (2.5)	2 (2.8)	0	2 (3.9)	
Tswana, n (%)	2 (1.3)	1 (1.4)	1 (2.8)	0	
Northern Sotho, n (%)	141 (89.2)	64 (90.2)	31 (86.0)	46 (90.2)	
Zimbabwean, n (%)	3 (1.9)	1 (1.4)	2 (5.6)	0	
Mozambican, n (%)	1 (0.6)	0	1 (2.8)	0	

The HAART-exposed groups were mainly on the first-line regimen (83.8%) compared to the second-line regimen (16.2%) (Table 4.2). There were none of the participants that were on the third-line regimen. In the study, we observed a huge disparity between the first-line (n = 57) and second-line regimens (n = 11) in terms of the number of participants. Subsequently it was decided to group them as one; thus, the HAART-exposed group. This was to prevent the detection of differences between the two different lines of regimen which were not clinically relevant. The majority of participants who were on first-line (82.2%) regimen specifically used the following three ARV drug combinations, (i) EVF+FTC+TDF (77.64%), (ii) 3TC+AZT+NVP (2.98%) and (iii) 3TC+ABC+EVF (1.49%). The participants on the second line regimen (17.8) were either on 3TC+AZT+LPV/r (16.4%) or FTC+TDF+LPV/r (1.49%) ARV drug combination (Figure 4.1).

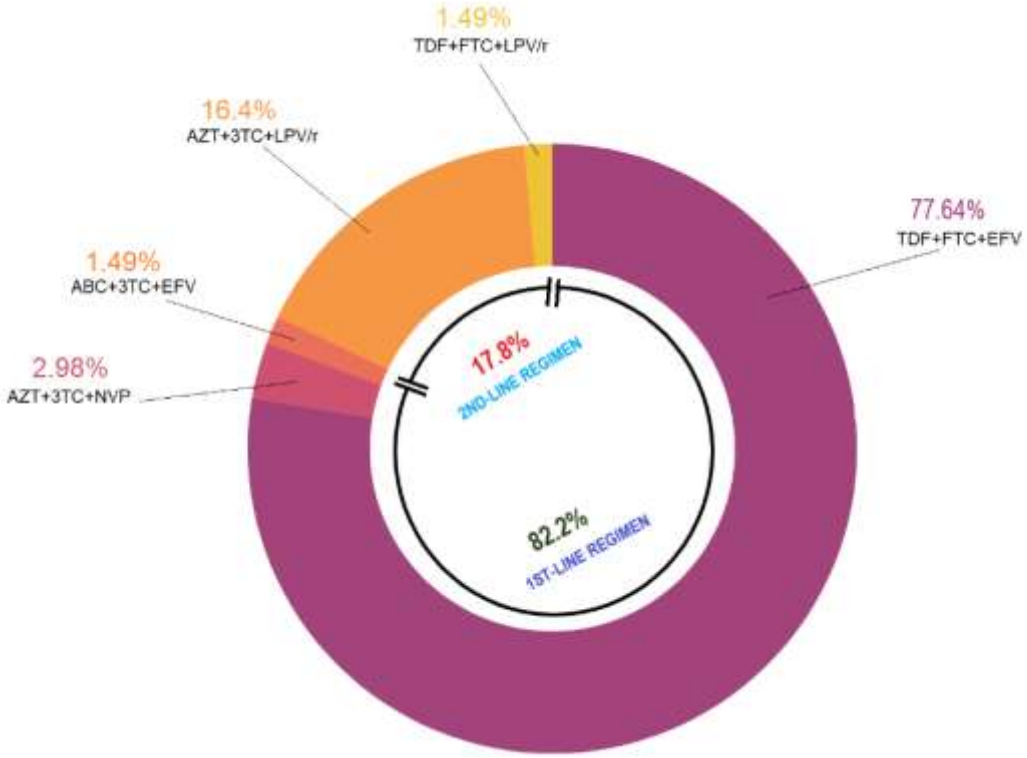

Figure 4.1: The distribution (%) of the different types of ARV drugs in first-line compared to the second-line regimens. [EVF: Efavirenz, FTC: Emtricitabine, TDF: Tenofovir, 3TC: Lamivudine, ABC: Abacavir, AZT: Zidovudine, NVP: Nevirapine, LPV/r: Lopinavir/ritonavir].

The HAART-exposed group were further divided into specific categories of duration on HAART. Most participants were on HAART for more than 5 years (39.7%) followed by those who were on HAART for less than 1 year (23.5%), between 1 and 2.9 years (19.2%), and between 3 and 4.9 years (Table 4.3). There was no significant difference observed in the HAART-exposed group compared to HAART-naïve group in terms of the average $CD4^+$ T-cell count ($p = 0.053$, Table 4.3).

When evaluating HAART-exposed in terms of WHO clinical disease stages there were 29.6% in stage I, 26.8% in stage II, 22.5% in stage III, and 11.3% in stage IV. In terms of WHO clinical disease stages for the HAART-naïve group, most participants

were in stage IV (38.9%) followed by stage I (25%), stage III (13.9%) then stage II (11.1%) (Table 4.3)**.** The missing values observed can be attributed to the information that could not be retrieved from the patient's files or the laboratory was unable to analyse the $CD4^+$ T-cell count of some participants.

Table 4.3: HIV related and immunological variables of the HAART-exposed and HAART-naïve groups.

Parameter	**HAART-exposed n (%)**	**HAART-naïve n (%)**	**p-value**
Gender			
Females	46 (64.8)	20 (55.6)	–
Males	25 (35.2)	16 (44.4)	–
line of Regimen,			
First-line regimen	57 (83.8)	–	–
Second-line regimen	11 (16.2)	–	–
Missing values	3	–	
HAART Duration			
< 1 year	16 (23.5%)	–	–
1-2.9 years	13 (19.2%)	–	–
3-4.9 years	12 (17.6%)	–	–
≥ 5 years	27 (39.7%)	–	–
$CD4^+$ Cell Count (cells/µl) (mean ±SD)			
	433.02 (± 214.04)	341 (± 281.44)	0.053
WHO Clinical Stage			
I (>500 cells/µl)	21 (29.6)	9 (25.0)	–
II (350–499 cell/µl)	19 (26.8)	4 (11.1)	–
III (200–349 cells/µl)	16 (22.5)	5 (13.90)	–
IV (<200 cells/µl)	8 (11.3)	14 (38.9)	–
Missing values	7	1	

p-values of ≤ 0.05 were considered statistically significant.

4.2.2 Discussion

4.2.2.1 Ethnicity and $CD4^+$ T-cell count

The $CD4^+$ T-cell count in both the HAART-exposed and HAART-naïve groups were below the reference range. Ethnicity is a major determinant of $CD4^+$ T-cell count. Klein *et al*. (2014), reported that individuals of African descent in developing countries have a lower $CD4^+$ T-cell count. This may translate to a longer asymptomatic phase where it is difficult to detect HIV infection, which increases the opportunity for HIV transmission.

Smaller participation numbers made it challenging to sensibly comment on other ethnic groups. Higher HIV infection rates were expected among the Northern Sotho speaking groups, based on the fact that the study was conducted mainly among the Northern Sotho speaking people. Madiba *et al*. (2017), indicated that black women from the SSA continue to be highly affected by HIV infection and little attention is given on the sociocultural practices regarding safe sex, especially in the rural regions. Labour migration promotes multiple sexual partnerships that favours HIV transmission (Crush *et al.,* 2007; Smith *et al.,* 2015). It would mostly be females who were vulnerable to HIV infection following this migration labour practice (Mswela, 2009). This trend was consistent in all three groups. This can explain the larger percentage of females who participated in the study.

As previously mentioned, Klein *et al*. (2014) reported that individuals of African descent's $CD4^+$ T-cell count is naturally low; thereby, advocating the need for the development of African reference points. The low $CD4^+$ T-cell count observed in the HAART-exposed and HAART-naïve groups is a cause of concern in the present study population. This may create a vehicle for easy HIV transmission since a low $CD4^+$ T-cell count is often associated with an increase in VL.

4.2.2.2 Percentage ARV drug distribution and immunological characteristics

The findings of the study indicated that the majority of participants were on first-line regimens specifically the ARV drug combination EVF+FTC+TDF. Most were in stages I and II of WHO clinical disease stages and were on HAART for more than 5 years. It was further observed that the HAART-naïve group were more at the disease progression stages III and IV compared to HAART-exposed group. As previously mentioned, these findings provide evidence of the importance of HAART initiation. The advent of HAART changed both HIV progression rate (VL) and immunological responses ($CD4^+$ T-cell count) among the HIV-positive population globally and also in SSA, which resulted in an increased life expectancy. It is important to determine the immune status of HIV-positive patients. An inverse relationship exists between $CD4^+$ T-cell count and the degree of immunosuppression (Nyiramana *et al*., 2017). The $CD4^+$ T-cell count is considered to be the strongest predictor of disease progression and survival (Ford *et al.,* 2017). The $CD4^+$ T-cell count is a common indicator used to determine the immune status which gives ARV therapy guidance, monitoring disease progression, deciding when to commence therapy, and determining treatment failure (Meintjies *et al.,* 2017).

The implementation of the immediate UTT policy may be attributed to the latter findings (Koenig *et al.,* 2017). The motivation behind the implementation of the UTT policy comes from the findings of two studies that were consistent in producing evidence of clinical benefits when HAART is initiated immediately in patients with $CD4^+$ T-cell counts >500 cells/μL (NSIGHT START Study Group, 2015; Koenig *et al.*, 2017). Other factors that could have contributed to the improved $CD4^+$ T-cell count in the treatment group included good adherence to treatment due to proper counselling, professional practices (confidentiality), and lesser side effects of the drugs (Harberer *et al.,* 2017).

More females than males participated in the study. The majority of the HIV-infected participants (HAART-exposed and HAART-naïve groups combined) were in the age group 30 – 39 years, an age group that contains most of the sexually active participants (Stats SA, 2017). In this study, the age groups 18 – 29 and 30 – 39

contained higher numbers of participants that are HAART-naïve, either deliberately or because they did not know their HIV status, both favouring HIV transmission. Some HIV-infected patients might be in denial or afraid to disclose their HIV status to their families and/or partners. Others may also move from clinic to clinic to confirm their HIV status while they are still sexually active. Perhaps out of fear to disclose their status to their partners, they continue to have unprotected sex which increases HIV transmission. Lack of a morally enlightened society based on low education and poor socioeconomic backgrounds are considered as predisposing factors to the spread of HIV infection (Ogunmola *et al.,* 2014). It is important to keep these HAART-exposed individuals on first-line regimen because it will increase the chances of long-term viral suppression. A great need exists to strengthen the link between HIV status and treatment to prevent HIV progression among young females. This can play an important role in reducing the increase HIV transmission in this study population.

CHAPTER 5

THE IMPACT OF HIGHLY ACTIVE ANTIRETROVIRAL THERAPY ON BODY MASS INDEX AND METABOLIC INDICATORS

5.1 THE IMPACT OF HAART ON BODY MASS INDEX

In this chapter, the impact of HAART on BMI, glucose, and the lipid profile (lipogram) was analysed and discussed.

5.1.1 Results

The BMI as an indicator of body fatness was compared between HAART-exposed, HAART-naïve, and control groups. The mean BMI across all three groups of the study population showed no significant difference (Table 5.1).

A significant difference was observed across the three groups in terms of BMI categories ($p = 0.045$, Table 5.1). The Chi-square Post Hoc test was performed where the p-value was adjusted to 0.003 to prevent a type 1 error. After the p-value adjustment, the prevalence of obesity was significantly higher among the HAART-exposed group compared to the HAART-naïve group (22.5% vs 2.8%, $p \leq 0.003$, Table 5.1). The prevalence of obesity further was also significantly higher in the control group compared to HAART-naïve group (33.3% vs 2.8%, $p \leq 0.003$, Table 5.1). The H_0 was accepted with respect to control group compared to HAART-naïve group. However, the H_0 was rejected with respect to HAART-exposed group compared to the HAART-naïve group. Although the obesity levels were the highest among the control group, no significant difference was observed between control group and HAART-exposed group. In this study, the HAART regimen increased the likelihood of obesity and overweight in the HAART-exposed group.

Table 5.1: Body fatness, lifestyle factors, and metabolic indicators of the study population.

Parameter	Total (N = 158)	HAART-exposed (n = 71)	HAART-naïve (n = 36)	Control (n = 51)	p-value
BMI (kg/m²)	25.75 ± 5.82	25.71 ± 5.97	24.10 ± 5.21	27.00 ± 5.82	0.071
Categories of BMI (n, %)					
Underweight	10 (6.3)	7 (9.9)	2 (5.6)	1 (2.0)	**0.045***
Healthy	68 (43.0)	28 (39.4)	19 (52.8)	21 (41.2)	
Overweight	44 (27.8)	19 (26.8)	13 (36.1)	12 (23.5)	
Obese	34 (21.5)	16 (22.5)	1 (2.8)	17 (33.3)	
Morbidly obese	2 (1.3)	1 (1.4)	1 (2.8)	0	
Parameter	**Total (N = 158)**	**HAART-exposed (n = 71)**	**HAART-naïve (n = 36)**	**Control (n = 51)**	**p-value**
Lifestyle factors (n, %)					
Alcohol consumption	33 (21)	18 (25.3)	10 (27.8)	5 (10)	
Tobacco usage	36 (22.7)	18 (25.3)	11 (30.6)	7 (19.4)	
Parameter	**Total (N = 158)**	**HAART-exposed (n = 71)**	**HAART-naïve (n = 36)**	**Control (n = 51)**	**p-value**
Metabolic indicators (mmol/L)					
Glucose	5.21 ± 1.56	5.44 ± 1.95	5.09 ± 1.45	4.95 ± 0.91	0.214
HDL-C	1.35 ± 0.42	1.45 ± 0.35	1.22 ± 0.50	1.31 ± 0.44	**0.024***
LDL-C	2.36 ± 0.92	2.47 ± 0.87	1.87 ± 0.80	2.55 ± 0.98	**0.001****
TC	4.28 ± 1.05	4.49 ± 0.98	3.66 ± 0.87	4.43 ± 1.13	**0.000****
TG	1.26 ± 0.69	1.26 ± 0.64	1.26 ± 0.71	1.25 ± 0.77	0.997

All categorical variables were expressed as frequencies and percentages and continuous variables as mean ± SD, *: $p < 0.05$. Bonferroni post hoc test: Post Hoc test chi-square Obese: adjusted p value is less 0,003 for Obese versus control (33.3%, p = 0,002). HIV-treatment versus HAART-naïve for LDL-C (p = 0.002) and TC (p = 0.002); Control versus HIV-treatment for HDL-C (p = 0.029), LDL-C (p = 0.002) and TC (p = 0.002); Control versus HAART-naïve p-value is less than 0.05 for HDL-C (p = 0.029), LDL-C (p = 0.003) and TC (p = 0.000) *: $p < 0.05$ or **: $p < 0.01$ were considered significant. Note. Overweight and obesity was Adapted from (WHO, 2004). Diabetes mellitus and dysglycaemia was adopted from (WHO, 2006). Dyslipidaemia was adopted from the South African Dyslipidaemia Guideline Consensus Statement (2014).

Table 5.2: Analysis of covariance for BMI and metabolic indicators of the study population after adjusting for age and gender.

Parameters	SS	Df	F	p-value
BMI (kg/m²)	133.938	2	2.165	0.118
Metabolic indicators (mmol/L)				
Glucose	5.677	2	1.171	0.313
HDL-C	1.371610	2	3.896	0.22
LDL-C	18.383	2	7.501	**0.001****
TC	18.279	2	9.174	**0.0002****
TG	0.075	2	0.081	0.922

SS: Sum of Squares, df: Degrees of freedom, p<0.05 were considered significant. Bonferroni post hoc test: For LDL-C, control vs HAART-naïve, HIV-treatment vs HIV-naïve, the p-value was less than 0.05. For TC, control vs HAART-naïve, HIV-treatment vs HAART-naïve p-value was less than 0.05. *: p<0.05 or **: p<0.01 were considered significant.

Table 5.3: Pearson versus partial correlation coefficients between HAART regimen and BMI and metabolic indicators of the study population after adjusting for age and gender.

	HAART (Pearson)	HAART (Partial)
BMI (kg/m²)	r = -0.107, p = 0.278	r = -0.136, p = 0.172
Glucose	r = -0.118, p = 0.235	r = -0.111, p = 0.264
HDL-C	**r = -0.267, p = 0.006****	**r = -0.268, p = 0.006****
LDL-C	**r = 0.314, p = 0.001****	**r = -0.312, p = 0.001****
TC	**r = 0.380, p = 0.0001****	**r = -0.376, p = 0.0001**
TG	r = 0.006, p = 0.951	r = 0.028, p = 0.779

Pearson correlation (unadjusted) vs Partial correlation (adjusted for age and gender combined),
*: p < 0.05 or **: p < 0.01 were considered significant.

The ANCOVA was thereafter calculated to compare the different groups (HAART-exposed vs HAART-naïve vs control) based on BMI while adjusting for age and gender. BMI continued to show no statistical difference across all three groups after eliminating the effect of age and gender (F (2, 154) = 2.165, p = 0.118, Table 5.2). We

further performed both Pearson and Partial correlations (Adjusted for age and gender combined) between HAART and BMI. The correlation continued to show no significant association between HAART and BMI (Table 5.3).

5.1.2 Discussion

In the present study, one of the objectives was to determine the effect of HIV and HAART on body fat among the HIV-positive population in a rural district in the Limpopo Province. Even when age and gender were taken into consideration, BMI did not show any significant difference between the three groups, suggesting that HAART and HIV did not have any effect on BMI. Although HAART is commonly associated with an increase in BMI, in the present study it was not observed. This may be explained by other factors such as smoking status, alcohol consumption, poor diet, $CD4^+$ T-cell count, duration of HIV infection and duration on HAART (> 60% were on HAART for < 5 years) in the study population which could have weakened the association. Also, the BMI levels of the study population were high, which made it difficult to observe potential differences. This can be confirmed by the control group which had the highest BMI compared to both HAART-exposed and HAART-naïve groups who also exhibited high BMI's (not significant). The finding of the present study was in agreement with that of the study conducted by Nell *et al*. (2015), where they also observed high levels in BMI among HAART-exposed group. The finding of this study may be attributed to HIV-positive patients who already have a high BMI before the commencement of HAART.

The huge disparity in the present study in terms of the number of participants per group may perhaps also explain why we did not observe an increase in BMI among the HAART-exposed group. This study population consisted of 63.3% black African females. Women of African descent is known to have a high BMI which is driven by cultural values which promote women with large body sizes as an indication of fertility and wealth (Miecklesfield *et al*., 2013; Okop *et al.,* 2016). Since this study was conducted in a rural setting, these cultural norms may still be practiced in this population. A large body of evidence exists which considers central fat distribution (measured by waist

circumference) as a better indicator of body fatness compared to relative weight (measured by BMI) (Janssen *et al.*, 2004; Yang *et al*., 2017). Unfortunately, we did not measure the central fat distribution, which could have provided a different outcome. The high BMI levels observed in the present study are worrying because high levels of BMI are associated with CVDs.

Even though the levels of obesity were significantly higher among the control group compared to HAART-naïve group, no significant difference was observed compared to the HAART-exposed group. The higher female numbers in the present study population may explain the increased manifestation of obesity. The reasons for this it may be attributed to cultural or lifestyle factors, for example factors such as energy dense diets and lack of physical activity (Padmapriyadarsini *et al.,* 2017). However, additional studies are needed to confirm these aspects within rural populations. Although HAART may have improved the $CD4^+$ T-cell count, it could have had side effects where it also increased body fatness among the HAART-exposed group.

The high levels of obesity observed in the HAART-exposed group may also be ascribed to an overweight state prior to the initiation of HAART. Besides obesity, other well-known risk factors of CVD, which include high levels of alcohol consumption and tobacco use, were quite prominent among both HAART-exposed and HAART-naïve groups. The majority of the HAART-exposed group were at WHO clinical stage I and stage II indicative of higher $CD4^+$ T-cell count while the HAART-naïve group were in WHO clinical stage III and IV, indicative of a lower $CD4^+$ T-cell count. The HAART-naïve population are more likely to present with reduced body fat, while evidence indicate that exposure to HAART may further increase body fat (Nduka *et al.,* 2015). However, without proper assessment of the effects of HAART on body fat, it would be challenging to accurately estimate the impact of the burden in different population groups.

Some studies have reported that HIV infection is associated with both fat and muscle-wasting disease (decrease in BMI) where malnutrition, elevated cytokine production, and endocrine dysfunction were implicated (Erlandson *et al.,* 2016; Koethe *et al*., 2016). However, there is also evidence in support of HAART-naïve groups being more obese than their HAART-exposed counterparts (Crum-Cianflone *et al.,* 2010; Anyabolu, 2016). These findings were attributed to the fact that the HAART-naïve

group may have had a normal $CD4^+$ T-cell count and were therefore classified according to the WHO HIV clinical criteria at stage 1 (Anyabolu, 2016). HAART itself has been associated with changes in body fat among HIV-positive individuals and may signify a considerable global health burden (Nduka *et al.,* 2015). Therefore, a great need exists to distinguish weight gain as part of the "return to health" phenomenon, from clinically undesirable and excessive weight gain that places an individual at high risk to become overweight and obese.

In the present study, an increase in CVD among the HIV-positive patients due to elevated levels of BMI among HAART-exposed group was anticipated. With their increased survival rate, the HIV-infected population is growing older and is facing new challenges in the form of the comorbidities such as CVDs that are well known in the aging general population. In patients living with HIV, CVD has emerged as an important cause of morbidity. We have observed high levels of overweight incidences among the HAART-naïve group which may explain the development of obesity as observed in HAART-exposed group when HAART is initiated in the HAART-naïve group which are already overweight. In the present study, we also observed that the prevalence of obesity was 12 times higher among the HAART-exposed compared to the HAART-naïve group, which is quite disturbing. Obesity is associated with increased mortality and several health complications which include DM and various heart diseases. The use of HAART by HIV-positive individuals, who are already overweight as observed in the present study, may enhance the increase in obesity and put these individuals at increased risk of future cardio-metabolic complications such as dyslipidaemia and dysglycaemia which are CVD risk factors. At this stage, it is fair to argue that both HIV and HAART are potential risk factors for the development of CVD.

The HAART-exposed patients may be at higher risk of becoming overweight or obese compared to the HAART-naïve patients. One general misconception is that many believe that PI-based regimens are the major cause of lipodystrophy in particular lipohypertrophy, among the HIV-positive population. Furthermore, a study performed by McComsey *et al*. (2016), showed that all HAART-naïve patients who were put on three different HAART regimens, two different PI-boosted regimens versus an

integrase inhibitor Raltegravir-based regimen for two years, had a 25% increase in visceral abdominal fat. Raltegravir, an integrase inhibitor the novel ARV drug is considered more tolerable towards lipid disturbances and insulin resistance, was also found to induce weight gain similar to that observed for PI-boosted regimens. In the former era before the implementation of the UTT program, HAART was initiated during the late phases of the HIV disease progression when $CD4^+$ T-cell count and BMI levels were very low which consequently improved virological control and increased BMI back to normal levels ("return-to-health"). In the current era as observed in the present study, it is most likely when HAART is immediately administered (UTT program) in the HIV-positive patients who already have a high BMI (overweight) that these HIV-positive patients may further develop into obesity. Approximately 84% of HIV-positive patients were on first-line regimen where approximately 95% consisted of TDF+FTC+EFV combination. Our study suggests that first-line regimens may increase the chances of overweight and obesity among the HIV-positive population.

Dolutegravir is an Integrase inhibitor which was introduced in South Africa early in December 2018. Although DTG holds multiple advantages, it raises concerns whether the Department of Health and other health care providers are aware that the prevalence of overweight and obesity is already high among our general population in the present study. In addition, individuals who may become infected by HIV being already overweight and commence on HAART, are at an increased risk for developing CVD risk factors such as obesity, DM, and IR. Moreover, alcohol and tobacco consumption was quite high among both HAART-exposed and HAART-naïve groups which may potentially exacerbate the development of CVD risk factors.

The increased CVD risk factors may put a huge financial burden on the Department of Health to further treat other co-morbidities which include various NCDs among the HIV-positive population and cardiovascular complications among the general population unless preventative measures are implemented. Therefore, this study supports the importance of periodic screening for overweight and obesity, especially amongst the sexually active portion of the population, to mitigate future cardiovascular complications. Furthermore, it is also imperative to screen the HAART-naïve individuals before the initiation of HAART so that other co-morbidities such as

CVDs can be treated in conjunction with HAART. More longitudinal studies are required to elucidate on the change in BMI from the commencement of HAART.

5.2 THE IMPACT OF HAART ON FASTING GLUCOSE METABOLISM

5.2.1 Results

The mean fasting blood glucose concentrations were determined to draw a comparison between HAART-exposed, HAART-naïve, and control groups. The prevalence of dysglycaemia was further determined among the latter groups (Refer to the cut-off point in Appendix I).

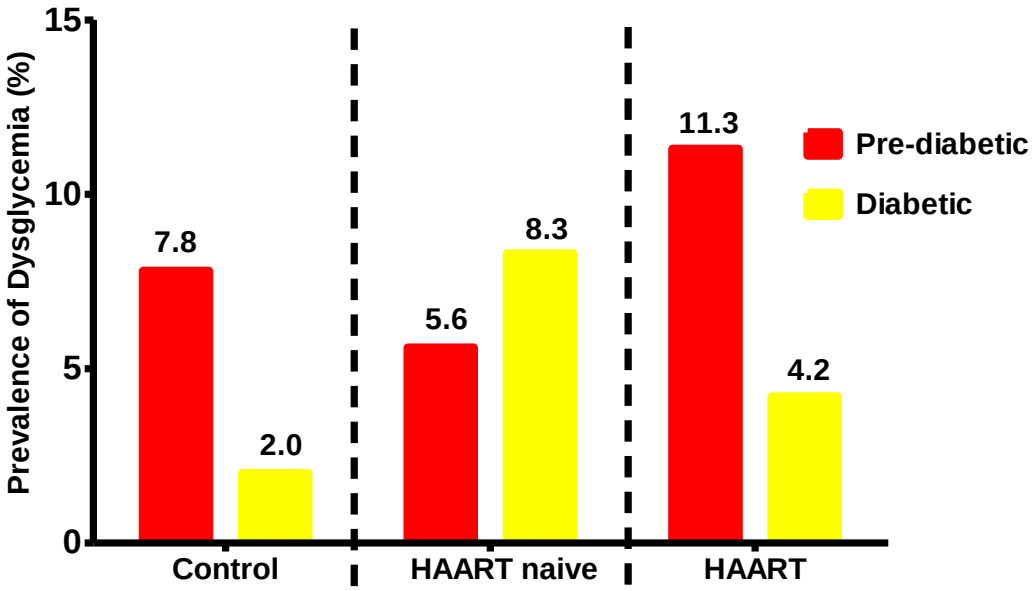

Figure 5.1: The prevalence of dysglycaemia in the overall population between control, HAART-naïve, and HAART-exposed groups. [Note. Diabetes mellitus and dysglycaemia was adopted from (WHO, 2006)].

In the overall population, no difference was observed in terms of the prevalence of both pre-diabetes and DM (Figure 5.2). Although a trend where HAART-exposed group (11.3%) indicated a higher prevalence of pre-diabetes compared to HAART-naïve (8.3%) and control groups (7.8%) (Table 5.2). We also observed a trend where

the prevalence of DM was higher in the HAART-naïve group (8.3%) compared to the HAART-exposed (4.2%) and control (2%) groups (Table 5.2).

In this study, no significant differences were observed across the three groups ($p < 0.497$, Table 5.1). The ANCOVA was thereafter performed to compare the different groups (HAART-exposed vs HAART-naïve vs control) based on fasting blood glucose while adjusting for age and gender. Fasting blood glucose continued to show no statistical difference across the three groups after eliminating the effect of age and gender ($F (2, 154) = 1.171$, $p = 0.313$, Table 5.2). The correlation analysis showed no significant association between HAART and fasting blood glucose (Table 5.3).

5.2.2 Discussion

The management of glucose levels among the HIV-positive populations are important to decrease the incidence of co-morbidities, such as DM. Although contrasting reports exist regarding the impact of both HIV and HAART on glucose metabolism, it is known that chronic exposure to high circulating blood glucose levels will eventually result in IR, glucose intolerance, and DM (Kramer *et al.,* 2009; Husain *et al.,* 2017; Njuguna *et al.,* 2018).

The prevalence of both pre-diabetes and DM differed among the three groups. This may suggest that the contribution of the risk factors for both pre-diabetes and DM in all the three groups were equal at this point. In the control group obesity could have contributed to the increase in the prevalence of both pre-diabetes and DM. In the HAART-naïve group the HIV could have contributed to the increase in the prevalence of both pre-diabetes and DM. Among the HAART-naïve group we also observed a trend whereby its prevalence of DM was the highest among the three group. This may be explained by the high prevalence of males, alcohol consumption, and tobacco use in this group. Alcohol consumption can reduce insulin sensitivity and it can also cause destruction of the beta (β)-cells of the pancreas (Dembele *et al*., 2009; Lee *et al*., 2015). The nicotine in tobacco has been shown to directly disturb glucose metabolism by causing β-cell dysfunction and increased β-cell apoptosis (Chen *et al*., 2018). This collectively can increase the prevalence of DM among the HAART-naïve male

participants as observed in the present study. Moreover, the HAART-naïve group had significantly lower HDL-C, LDL-C, and TC levels. This lipid profile pattern is a common feature among HAART-naïve patients which are associated with premature development of cardiovascular risk factors such as DM (Mashinya *et al.,* 2014; Njuguna *et al.,* 2018).

The findings of this study further did not find any significant changes in mean glucose levels across the three groups. Some studies did identify that the risk of glucose abnormalities can be increased by specific HAART regimens which include PIs and some ARV drugs such as Stavudine and Indinavir (Hruz, 2008; Husain *et al.,* 2017). Higher VL (HIV RNA copies) and BMI have also been associated with increased risk for dysglycaemia (Erlandson *et al.,* 2014). Several other factors which include duration on HAART, duration of HIV infection, large abdominal circumference, overweight/obesity, advanced age, gender, and low income were all contributing factors for developing dysglycaemia (Erlandson *et al.,* 2014; Levitt *et al.,* 2016; Njuguna *et al.,* 2018). In the present study, approximately 83% of the HAART-exposed group were on first-line regimens, the average age for the entire group was middle-age adults (40.23 ± 12.72) and the $CD4^+$ T-cell count was at normal levels which may explain why no changes in the fasting glucose levels were observed in the HAART-exposed group.

A Zambian study reported a high incidence of DM among HAART-exposed patients compared to the general population which suggests poor metabolic imbalance management (Shankalala *et al.,* 2017). The majority of participants in this study were relatively older compared to our study with a median age of 46 years, which may explain why the difference in glucose metabolism was not observed in the current study. Both HIV and HAART are risk factors for dysglycaemia. However, we also observed that the prevalence of obesity which is also a risk factor for dysglycaemia was also the highest in the control group. The similar findings observed in the present study in terms of dysglycaemia may suggest that the general population, HAART-exposed population and HAART-naïve population are at equal risk to developed dysglycaemia which is, therefore, a call for concern. Screening for dysglycaemia

should become routine and more awareness campaigns regarding glucose abnormalities are needed in this rural population.

5.3 THE IMPACT OF HAART ON THE LIPID PROFILES

5.3.1 Results

The mean serum lipid concentrations were measured and compared between HAART-exposed, HAART-naïve, and control groups. Further, the prevalence of dyslipidaemia was determined among the latter groups (Refer to the cut-off points in Appendix I).

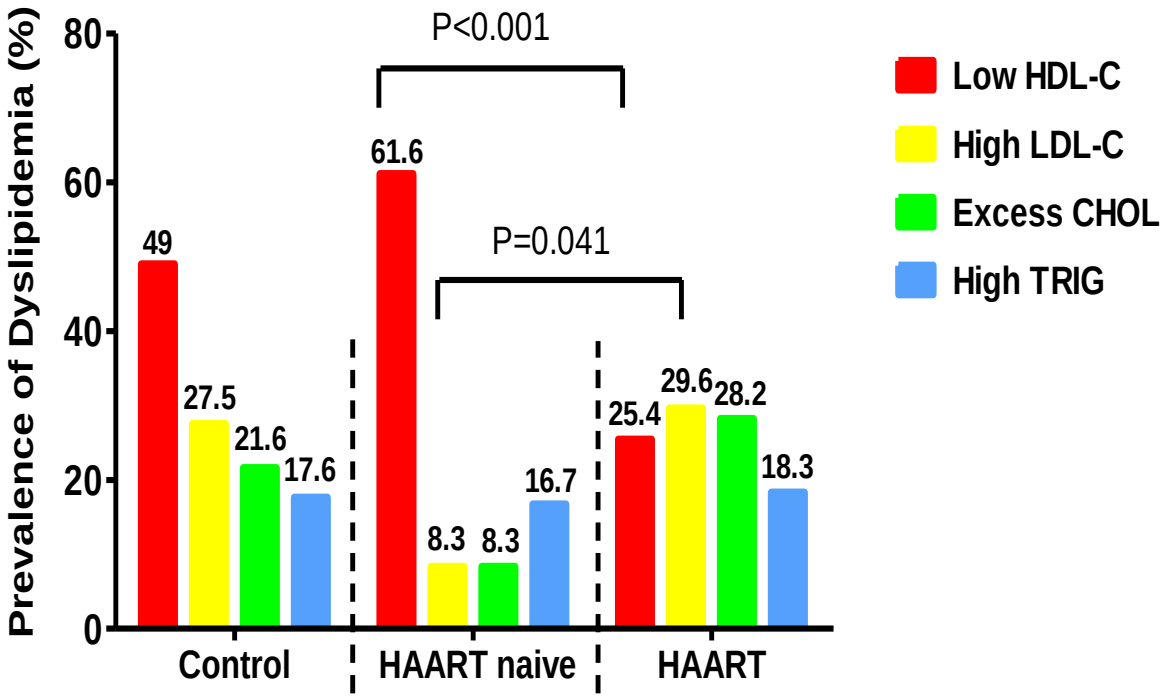

Figure 5.2: The prevalence of dyslipidaemia in the overall population between the control, HAART-naïve, and HAART-exposed groups. [Note. Dyslipidaemia was aadopted from the South African Dyslipidaemia Guideline Consensus Statement (2014)]. (Refer to Appendix I for cut-off points)

In the overall study population, the prevalence of low HDL-C levels was significantly higher in the HAART-naïve group (61.6%) compared to the HAART-exposed group

(25.4%) (Table 5.3, $p < 0.001$). Also, the prevalence of high LDL-C levels was significantly higher in the HAART-exposed group (29.6%) compared to the HAART-naïve group (Table 5.3, $p = 0.041$).

A significant difference was observed in TC, LDL-C, HDL-C and TG lipid levels across the HAART-exposed HAART-naïve, and control groups (Table 5.1). When the Chi-square Bonferroni post-hoc test was performed, it specifically indicated that the HAART-exposed group had a significantly higher LDL-C and TC concentration compared to the HAART-naïve group (Table 5.1, $p = 0.002$). The HDL-C ($p = 0.041$) and TC ($p = 0.002$) concentrations were also significantly higher among HAART-exposed group compared to control group (Table 5.1). The HAART-naïve group also showed a significantly lower HDL-C ($p = 0.041$) and TC ($p = 0.0001$) compared to the control group (Table 5.1). The control group showed significantly higher LDL-C levels compared to both HAART-exposed group ($p = 0.002$) and HAART-naïve group ($p = 0.003$) (Table 5.1).

The ANCOVA compared the different groups based on their lipid profiles (HDL-C, LDL-C, TC, and TG) while adjusting for age and gender. It revealed a significant difference across all groups for LDL-C ($F (2, 154) = 7.501$, $p = 0.001$) and TC ($F (2, 154) = 9.174$, $p = 0.0002$) (Table 5.2), but not for HDL-C ($F (2, 154) = 3.896$, $p = 0.22$) and TG (($F (2, 154) =18.780$, $p = 0.671$) (Table 5.2). One-way analysis of covariance Bonferroni post-hoc test revealed that the control group had a significantly higher LDL-C level compared to HAART-naïve ($p = 0.001$). Further, LDL-C was also significantly higher among the HAART-exposed compared to the HAART-naïve group ($p = 0.004$). The age and gender-adjusted ANCOVA Bonferroni post-hoc test specifically indicated that the control group had significantly higher TC levels compared to HAART-naïve group ($p = 0.001$). HAART-exposed group also showed significantly higher TC levels compared to the HAART-naïve group ($p = 0.0003$).

Initially, prior to any adjustments, HAART showed a significant negative association with HDL-C ($r = -0.267$, $p = 0.006$) and a significant positive association with LDL-C ($r = 0.314$, $p = 0.001$) and TC ($r = 0.380$, $p = 0.0001$) respectively (Table 5.3). After adjustment for age and gender, the Partial correlation analysis showed a

negative correlation between HAART and HDL-C (r = -0.268, p = 0.006), LDL-C (r = -0.312, p = 0.001) and TC (r = -0.376, p = 0.0001) (Table 5.3) respectively.

Table 5.4: Independent associations of markers of CVD risk factors with HAART regimens, first-line regimen, and second-line regimen.

	Adjusted R^2	Adj. P_1	Std β (95% CI)	P_2
HAART Regimens				
Variables:				
CVD Risk factors				
BMI (kg/m²)	0.056	0.149	0.060 (-4.118; 4.238)	0.635
Glucose	0.039	0.149	-0.149 (-1.495;1.197)	0.248
HDL-C	0.105	0.208	-0.117 (-0.350; 0.116)	0.346
LDL-C	-0.014	0.102	0.056 (-0.553; 0.665)	0.670
TC	0.117	0.218	0.008 (-0.638; 0.654)	0.951
TG	0.084	0.174	0.075 (-0.360; 0.510)	0.549
First-line Regimen				
BMI (kg/m²)	0.125	0,232	0.141 (-1.505; 1.787)	0.486
Glucose	0.02	0,160	-0.130 (-0.731; 0.471)	0.385
HDL-C	0.077	0,209	0.049 (-0.045; 0.143)	0.732
LDL-C	0.122	0,247	-0.253 (-0.486; -0.019)	0.077
TC	0.187	0,303	-0.208 (-0.460; 0.044)	0.131
TG	0.162	0,264	-0.089 (-0.212; 0.034)	0.517
Second-line Regimen				
BMI (kg/m²)	0.711	0,884	0.409 (-3.481;4.299)	0.119
Glucose	0.735	0,920	-0.655 (-1.370;0.060)	0.101
HDL-C	0.015	0,705	-0.434 (-1.053;0.185)	0.478
LDL-C	-0.182	0,645	0.194 (0.188;0.199)	0.712
TC	0.267	0,780	0.004 (-1.403;1.411)	0.994
TG	0.315	0,726	0.194 (0.188;0.199)	0.384

Standardized β (Std. β) represents the change in the dependent variable for every 1 SD change in the independent variable: partial regression coefficient; Adj. R^2: adjusted R^2; 95% CI: 95% confidence interval of β; Adj. P_1: p-value for Adjusted R^2; P_2: p-value of Std. β; HAART: Highly active antiretroviral therapy, TDF: Tenofovir; FTC: Emtricitabine, EFV: Efavirenz; 3TC: Lamivudine; AZT: Zidovudine; LPV/R: Lopinavir/Ritonavir. All models included the following covariates: age, gender, BMI: Body mass index; TG, CD4+ T-cell counts, duration on HAART. *: $p < 0.05$ or **: $p < 0.01$ were considered significant.

5.3.2 Multi-variable adjusted regression analyses based on CVD risk factors

Multiple regression analysis was specifically used to determine the strength of association between HAART (combined HAART regimens, first-line regimens, second-line regimens, specific ARV drug combination of the first-line and second-line regimens) and CVD risk factors (BMI, glucose, HDL-C, LDL-C, TC, and TRIG) as the dependent variables in HAART-exposed group. The covariates were selected based on appropriate selection criteria which are mainly based on the strength of association between the covariates and main variables of interest. The covariates that were selected and entered into the model included age, gender, TG, duration on HAART, $CD4^+$ T-cell count.

We found no significant association between the combined HAART regimens (first and second-line regimen combined) and all CVD risk factors. The multiple analysis with the first-line and second-line regimens respectively also did not provide any significant association with CVD risk factors (Table 5.4). When further multiple analysis was performed in terms of specific ARV three-drug combination, TDF_FTC_EFV (first-line regimen) and 3TC_AZT_LPV/R (second-line regimen) continued not to show any significant association with CVD risk factors (Table 5.5).

Table 5.5: Independent associations of markers of CVD risk factors with specific ARV three-drug combinations of first-line regimen TDF_FTC_EFV (first-line regimen) and 3TC_AZT_LPV/R (second-line regimen).

	Adj. R^2	Adj. P_1	Std β (95% CI)	P_2
TDF_FTC_EFV				
Variables:				
CVD risk factors				
BMI	0.069	0.16	-0.073 (-0.080; -0.065)	0.326
Glucose	0.05	0.159	0.185 (-0.996; 1.366)	0.162
HDL-C	0.091	0.196	0.036 (-0.171; 0.243)	0.78
LDL-C	-0.004	0.111	0.113 (-0.422; 0.648)	0.404
TC	0.129	0.229	0.112 (-0.454; 0.647)	0.374
TG	0.105	0.193	-0.160 (-0.534; 0.214)	0.204
3TC_AZT_LPV/R				
BMI	0.053	0.146	-0.024 (-4.230; 4.182)	0.852
Glucose	0.029	0.14	-0.115 (-1.473; 1.243)	0.374
HDL-C	0.091	0.196	-0.034 (-0.269; 0.201)	0.784
LDL-C	0.006	0.12	0.149 (-0.456; 0.754)	0.258
TC	0.132	0.231	0.118 (-0.526; 0.762)	0.335
TG	0.093	0.182	0.117 (-0.314; 0.548)	0.346

Standardized β (Std. β) represents the change in the dependent variable for every 1 SD change in the independent variable: partial regression coefficient; Adj. R^2: adjusted R^2; 95% CI: 95% confidence interval of β; Adj. P_1: p-value for Adjusted R^2; P_2: p-value of Std. β; HAART: Highly active antiretroviral therapy, TDF: Tenofovir; FTC: Emtricitabine, EFV: Efavirenz; 3TC: Lamivudine; AZT: Zidovudine; LPV/R: Lopinavir/Ritonavir. All models included the following covariates: age, gender, BMI: Body mass index; TG, CD4+ T-cell counts, duration on HAART. *: p<0.05 or **: p<0.01 were considered significant.

5.3.3 Discussion

The risk for CVD is substantially increased by HIV infection and HAART (Feinstein *et al.,* 2016; Hsue and Waters, 2019). Exposure of HIV-infected individuals to ARV therapy predisposes them to dyslipidaemia and result in various cardiovascular complications (Hejazi *et al.,* 2013; Ahmed *et al.,* 2016; Toth, 2016). Therefore, it was imperative to establish the lipid profile in the current population to determine the prevalence of dyslipidaemia and the risk factors associated with dyslipidaemia in the present study population. This insight may also create a platform on which effective

prevention and intervention strategies can be established to mitigate cardiovascular complications.

5.3.3.1 Lipid metabolism of the HAART-naïve group

The findings of the study indicated that the low HDL-C levels are highly prevalent among the HAART-naïve group which suggests that the burden of CAD may rapidly increase among HAART-naïve patients. Our findings agree with previous studies who reported that low HDL-C levels during HIV infection are strongly associated with increased inflammatory markers and disease progression (Marin-Palma *et al.,* 2018). The high prevalence of low HDL-C among the HAART-naïve group may be a result of altered lipid metabolism which is known to which is often displayed in this group. The different forms of dyslipidaemia among the HAART-naïve group were similar to the control group, which further suggest that the risk of CAD may also be rapidly increased among the control group. Therefore, the data suggests that dyslipidaemia is present in both the HAART-naïve and control groups. Urgent measures which include effective public health education, routine screening, and initiating appropriate treatment should be implemented for both HAART-naïve and control groups.

The mean HDL-C, LDL-C, and TC levels in this study declined following HIV infection among HAART-naïve group (non-significant). This finding is consistent with previous studies which were performed before the implementation of HAART which observed that the lipid levels of HIV patients differed (decreased TC, LDL-C, and HDL-C) from those of the general population (Rose *et al.,* 2006; Shen *et al.,* 2015). However, a study performed in Nigeria observed a significant elevation in LDL-C, reduction in HDL-C, and TC among HAART-naïve group compared with control group (Adewole *et al.,* 2010). In the latter study, they noted that it was only the HAART-naïve patients with co-infections of TB who presented with elevated LDL-C levels. In our study, no TB co-infections were observed among the HAART-naïve patients which may explain the different findings.

Reverse cholesterol transport (RCT) is the mechanism the body uses to remove high TC from the peripheral tissue. The high TC is either redistributed or removed

where HDL-C plays a vital role in the entire process (Marques *et al.,* 2018). Some studies have pointed out that HIV specifically targets and impairs key proteins such as the ATP-binding cassette A1 (ABC1) which plays a critical role in RCT, resulting in the reduction in TC efflux from the peripheral tissues (Mujawar *et al.,* 2006; Khoury *et al.,* 2015). The functional impairment of ABC1 is associated with severe HDL-C deficiency (Mujawar *et al.,* 2006). This may therefore explain the low levels of HDL-C observed in this study.

The TG levels among the HAART-naïve group in this study remained unchanged which may be due to early stages of HIV infection. The different outcomes in TG levels were attributed to HIV disease progression (Grunfeld *et al.,* 1992; Fourie *et al.,* 2010). Several studies are in agreement with our findings that HIV-induced dyslipidaemia is associated with lower LDL-C and HDL-C levels among the HAART-naïve population and that the decline in $CD4^+$ T-cell count is an independent predictor of dyslipidaemia (Rose *et al.,* 2008; Teto *et al.*, 2013; Shen *et al.,* 2015; Njoroge *et al.,* 2017). Chronic inflammation and immune cell activation may lead to $CD4^+$ T-cell count depletion and may result in alteration in lipid metabolism. Virginia and Triant (2013) and Sokoya *et al*. (2017), reported that chronic inflammation and immune cell activation are important mediators that may increase the risk for CVD among the HIV-positive population.

Grunfeld (2010), reported that hypertriglyceridemia together with low levels of HDL-C and TC are considered as advanced stages of HIV disease progression. In the present study, we did not observe elevated levels of TG, which may suggest that the HAART-naïve group were at the early stages of HIV disease progression. The high prevalence of low HDL-C levels observed further may suggest that these patients may have less protection against atherosclerosis and if no intervention such as the introduction of HAART is implemented at an early stage of HIV disease progression that these patients may be at increased risk for CAD. However, HIV itself may be the primary cause of elevated risk for CAD prior to the initiation of HAART. The HAART-naïve group was at the early stages of HIV disease progression and its different forms of dyslipidaemia was similar to the general population. This finding may also suggest that the prevalence of dyslipidaemia observed in the HAART-naïve group may be attributed more to the conventional risk factors which were already present prior to

HIV infection, and not to HIV per se. This may also imply that the general population is at an equal risk for CAD. The entire study population must be routinely screened for viral replication, but also appropriate measures should be implemented routinely to screen and treat all lipid abnormalities in this rural population.

5.3.3.2 Lipid metabolism of HAART-exposed group

The mean LDL-C and TC levels of the HAART-exposed group were significantly higher compared to the HAART-naïve group in the present study. The study also found that high LDL-C levels were highly prevalent among the HAART-exposed group. Female gender, high BMI, and advanced age are among the risk factors which can rapidly increase the risk for atherosclerosis (Spence and Pilote, 2015; Head *et al.,* 2017). The HAART-exposed group in the present study consist of group where the majority were predominantly females, advanced in age, over 50% had a high BMI (BMI indicative of overweight or obesity), and either used alcohol and or tobacco. The latter risk factors recorded together with the HIV itself and HAART respectively may explain the elevated LDL-C and TC levels observed among the HAART-exposed group. The higher TC levels in the HAART-exposed group may also be accredited to higher HDL-C and LDL-C levels. Dyslipidaemia in the form of elevated LDL-C and TC levels among the HAART-exposed group can increase the risk for CVDs (Ogundahunsi *et al.,* 2008; Hejazi *et al.,* 2013; Nduka *et al.,* 2015; Ahmed *et al.,* 2016; Toth, 2016). These findings are consistent throughout different study populations across the globe (Mills *et al.,* 2011; Deeks *et al.,* 2013). HAART-exposed groups are therefore at increased risk for dyslipidaemia and consequently CHD. High levels of LDL-C and TC particles in the blood vessels may contribute to atherosclerotic plaque formation and can increase the incidences of MI and stroke among the HAART-exposed group.

A significant negative association was reported between HAART and LDL-C and TC in the current study. The finding suggests that HAART may serve a beneficial role in improving lipid metabolism by decreasing the LDL-C and TC back to normal levels; whilst participants improved clinically through the increase in $CD4^+$ T-cell count and the reduction in VL. We reported in the present study that the prevalence of high LDL-

C levels was significantly lower among the HAART-exposed compared to the HAART-naïve group. Which is similar to that reported in a Kenya study (Njoroge *et al.,* 2017). From the 71 participants in our study that were on treatment, 57 participants were on first-line regimens where 94% were on ARV three-drug combination TDF+FTC+EFV which is an NNRTI-based regimen. The beneficial effect observed in the present study may be due to the greater use of NNRTI-based regimens compared to PI-based and Stavudine-based regimens which are well-known to have more detrimental effects on the lipid metabolism. Our findings are in agreement with previous studies that observed a positive effect of the NNTRI-based regimen on lipid metabolism (Dave *et al.,* 2016; Yang *et al.,* 2019).

Besides the normal $CD4^+$ T-cell count levels of the HAART-exposed group, the elevated levels in HDL-C observed in this HAART-exposed group may also be attributed to lifestyle factors, such as increased physical activity levels. It has been reported that physical activity is associated with increased HDL-C levels (Kokkinos and Fernhall, 1999; Wang and Xu, 2017). It is well known that rural populations are more physically active compare to urban populations (Assah *et al.,* 2015). The elevated HDL-C levels in the current study may be attributed to high levels of physical activity which are associated with people living in rural populations. The elevated HDL-C levels in the HAART-exposed group is indicative of a cardio-protective role. However, the negative association observed between the HAART-exposed group and HDL-C may suggest detrimental effects of the NNRTI-based regimen on lipid metabolism. In the present study, we observed that the NNRTI-based regimen may play a beneficial role in reducing the LDL-C and TC levels. However, the continuous decrease in HDL-C levels over time may result in dyslipidaemia.

Triglyceride levels among the HAART-exposed group remained unchanged in the present study even after adjusting for the effect of age and gender. The unchanged TG levels observed in the present study may be because NNRTI-based regimens are more cardio-protective. European and American based studies reported on elevated levels of TG (De Wit *et al.,* 2008). The latter specifically observed an association between Stavudine and TG, an ARV drug that was phased-out and is currently replaced with TDF or EFV due to its detrimental effects on both glucose and lipid

metabolism. The difference observed in terms of TG levels may be attributed to ethnicity, $CD4^+$ T-cell count, duration on HAART, stage of HIV disease progression, and the difference in regimens used by the study populations. Although the TG levels remained unchanged in the present study, routine screening of lipid profiles after the initiation of HAART should become a standard health care practice among the HIV-positive population.

In the present study, HAART treatment seems to play both a protective and harmful role in lipid metabolism. This study highlights the importance of ARV therapy immediately after HIV infection to reduce dyslipidaemia among HIV-positive patients in this rural population. The data further suggests that these patients are at increased risk of developing dyslipidaemia in the near future; however, we could not establish over how many years after the commencement of HAART. Routine screening of the HAART-exposed group is important to monitor lipid abnormalities. Urgent measures should also be implemented which include effective public health education and initiating appropriate treatment for lipid abnormalities among the HAART-exposed individuals.

Despite that, the correlation analysis reported significant associations of HAART with HDL-C, LDL-C, and TC, multivariate analysis ruled out any association after controlling for age, gender, BMI, $CD4^+$ T-cell count, and duration on HAART. Fasting glucose was the strongest negatively associated predictor variable associated with second-line regimen. These participants were non-responsive to the first-line regimen which are associated with elevated levels of VL, and very low $CD4^+$ count. The HIV-positive participants may have been at early phases of the second-line regimens administration. At the initial stage of second-line regimen commencement, the VL was probably still elevated and $CD4^+$ T-cell count was very low. It was reported that $CD4^+$ T-cell count is negatively associated with glucose levels (Khosa *et al.,* 2018). As the HIV-positive participant advances in his/her second-line regimen the VL load will decrease and $CD4^+$ T-cell count will increase. The negative correlation observed between second-line regimen and glucose levels may be explained by the fact that at this point in the present study the second-line regimen might have effectively improved immune functionality which was mediated by the increase in $CD4^+$ T-cell count and

weakening of the VL and consequent reduction in glucose levels. The different findings observed in the present study may be attributed to the sample size which was too small to detect any significant associations. Although no significant associations were observed in the multivariate analysis between HAART and the CVD risk factors. This study still reiterates the importance of immediate initiation of ARV treatment and the routine screening for CVD risk factors among the HIV-positive population who are on ARV treatment.

CHAPTER 6

IMPACT OF HAART BIOMARKERS ON ENDOTHELIAL DYSFUNCTION

6.1 RESULTS

In this chapter, the impact of HIV infection and HAART on endothelial function was analysed and discussed extensively through a critical review of the literature to gain insight into the manifestations of ED in the study population. Several CVDs induced by both HIV and HAART are associated with atherosclerosis and ED. Measuring ED may serve as a preventative strategy that could represent a promising approach to detect CVD at an early stage of development.

The serum endothelial biomarker levels were measured and compared between HAART-exposed, HAART-naïve, and control groups. All endothelial biomarkers were expressed as median and IQRs. The median serum concentrations of the endothelial biomarkers included L-selectin, P-selectin, sICAM-1, sVCAM-1, and VWF. Table 6.1 indicates that there was a significant difference observed across the three groups for L-selectin ($p = 0.010$), P-selectin ($p = 0.001$), sICAM-1 ($p = 0.017$) and VWF ($p = 0.013$). There was no significant difference observed for sVCAM-1 ($p = 0.123$) (Table 6.1) across all three groups.

Further statistical analysis was performed on endothelial biomarkers which showed significant differences for L-selectin, VWF, P-selectin, and sICAM-1. Kruskal-Wallis Bonferroni Post hoc test revealed that the median L-selectin concentration ($p = 0.008$) (Figure 6.1, A) of the HAART-naïve group were significantly higher than the control group. The median P-selectin concentrations of the HAART-naïve group were also significantly higher compared to both the control group ($p = 0.001$) (Figure 6.1, B) and the HAART-exposed group ($p = 0.02$) (Figure 6.1, B). The median sICAM-1 concentrations ($p = 0.013$) (Figure 6.1, C) of the HAART-naïve group were significantly higher than the control group. The median VWF concentrations ($p = 0.018$) (Figure

6.1, E) of the HAART-exposed group were significantly higher compared to the control group.

Table 6.1: The Kruskal-Wallis statistical test analysis of the measured endothelial function across all three groups.

Biomarkers (ng/mL)	**Total** (N = 158)	**HAART-exposed** (n = 71)	**HAART-naïve** (n = 36)	**Control** (n = 51)	**p-value**
L-selectin	0.06 (0.11 – 0.03)	0.06 (0.13 – 0.04)	0.09 (0.17 – 0.05)	0.05 (0.08 – 0.03)	**0.010***
P-selectin	0.87 (1.23 – 0.56)	0.86 (1.07 – 0.54)	1.11 (18.78 – 0.74)	0.75 (1.02 – 0.34)	**0.0018***
sICAM-1	0.71 (1.47 – 0.28)	0.68 (2.11 – 0.26)	1.00 (1.56 – 0.65)	0.49 (0.98 – 0.19)	**0.017***
sVCAM-1	6.83 (11.75 – 4.16)	7.46 (11.12 – 5.19)	6.15 (9.65 – 0.92)	6.68 (13.47 – 3.98)	0.123
VWF	1.43 (2.47 – 0.59)	1.67 (2.91 – 0.94)	1.05 (2.33 – 0.34)	1.03(2.01 – 0.41)	**0.013***

All endothelial biomarkers were expressed as median and interquartile ranges (IQR), *: $p < 0.05$, Adj.: Adjusted

Table 6.2: Analysis of covariance for the endothelial biomarkers between all participants after adjusting for age and gender.

Parameters	**Sum of Squares**	**df**	**F**	**p-value**
L-selectin	44.411	2	0.622	0.517
P-selectin	9151.054	2	7.253	**0.001***
sICAM-1	3548.076	2	0.710	0.493
sVCAM-1	14736.725	2	1.070	0.346
VWF	30.566	2	4.556	**0.012***

SS: Sum of Squares, df: Degrees of freedom, $p<0.05$ were considered significant, Bonferroni post hoc test: P-selectin, HAART-naïve vs control, HIV-treatment vs HAART-naïve p-value was less than 0.05. For VWF, HAART- naïve vs control, HIV-treatment vs HAART-naïve p-value was less than 0.05.

After adjusting for age and gender with the use of the ANCOVA statistically test, P-selectin ($F(2, 154 = 7.253$, $p = 0.001$) and VWF ($F(2, 154 = 4.556$, $p = 0.011$) showed significant differences across all three groups (Table 6.2). There was no significant

difference observed for L-selectin, sICAM-1, sVCAM-1 after the ANCOVA Bonferroni post-hoc test.

The ANCOVA Bonferroni post-hoc test further revealed that the P-selectin concentration for HAART-naïve group was significantly higher compared to both control (p = 0.001) and HAART-exposed (p = 0.009) groups (Table 6.2). The ANCOVA Bonferroni post-hoc test also showed that the HAART-exposed group had significantly higher VWF levels compared to the control group (p = 0.01) (Table 6.2). The Partial correlation further also showed a positive significant correlation between HAART-exposed group and P-selectin (r = 0.227, p = 0.021). However, between the HAART-exposed group and VWF (r = -0.249, p = 0.011), a negative significant association was observed (Table 6.3).

Table 6.3: Pearson and partial correlation coefficients between the HAART regimen and biomarkers of endothelial after adjusting for age and gender.

	HAART (Pearson)	**HAART (Partial)**
L-selectin	r=-0.089, p=0.369	r=0.083, p=0.405
P-selectin	**r=0.226, p=0.021***	**r=0.227, p=0.021***
sICAM-1	r=-0.125, p=0.208	r=-0.125, p=0.208
sVCAM-1	r=0.120, p=0.224	r=-0.114, p=0.250
VWF	r=0.137, p=0.167	**r=-0.249, p=0.011***

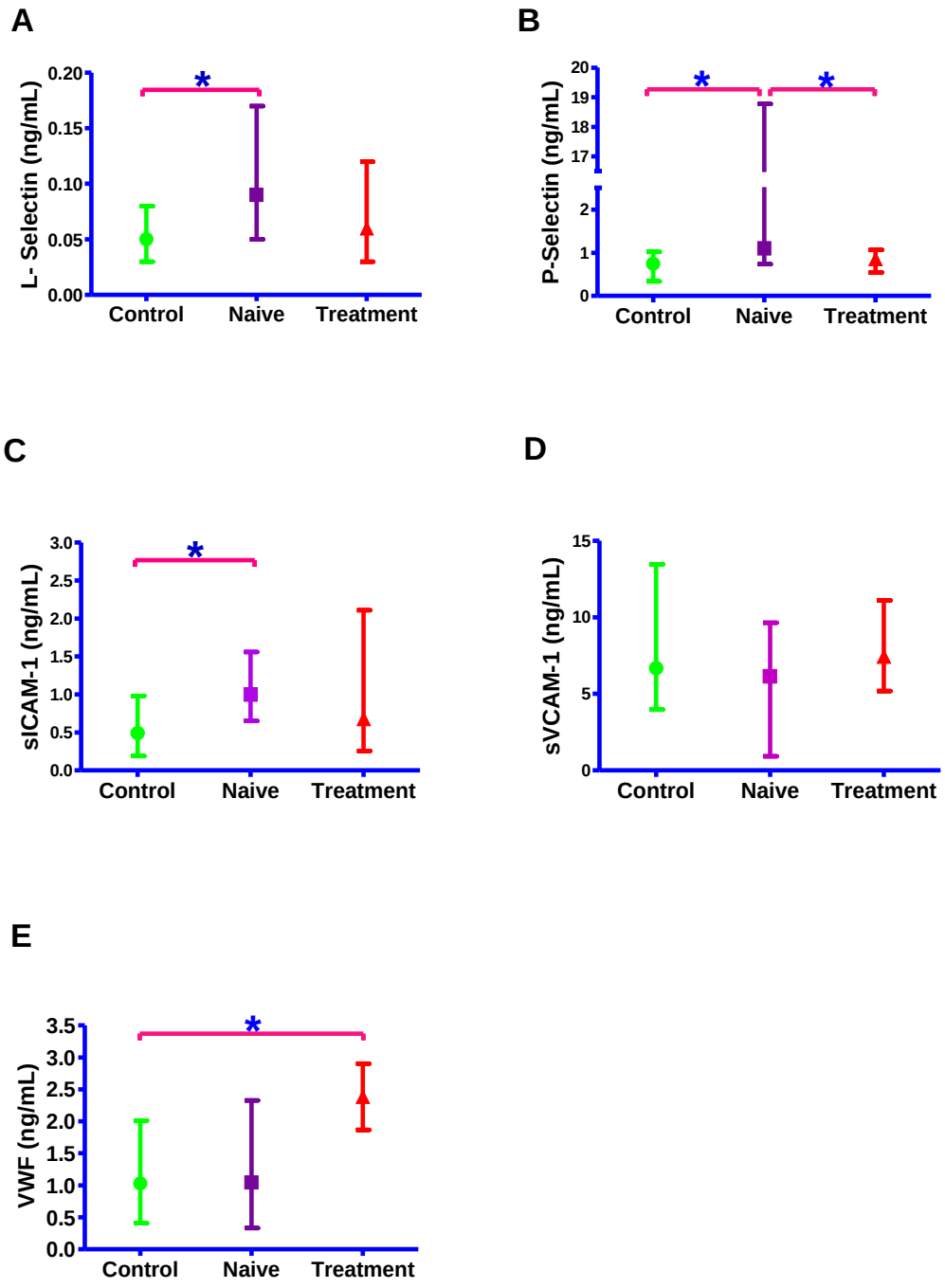

Figure 6.1: Evaluating endothelial function with A: L-selectin, B: P-selectin (ng/ml), C: soluble vascular cell adhesion molecule (sVCAM-1), D: intercellular adhesion molecule (sICAM-1), E: von Willebrand factor (VWF) between the control, HAART-naïve and HAART-exposed groups. Values are expressed as median and Interquartile ranges (IQR), ng/mL: nanogram per milliliter, *: $P < 0.005$ (ANOVA analysis).

6.1.1 Multi-variable adjusted regression analyses result based on endothelial biomarkers

Multiple regression analysis was specifically used to determine the strength of association between HAART (combined HAART regimens, first-line regimens, second-line regimens, specific ARV drug combination of the first-line and second-line regimens) and biomarkers of endothelial function (L-selectin, P-selectin, sICAM-1, sVCAM-1 and VWF) as dependent variables in the HAART-exposed group. A significant independent association was observed between the HAART-exposed group (first and second-line regimens combined) and P-selectin (Std β = 0.219, p = 0.032) (Table 6.4). The multiple analysis revealed a significant independent association between first-line regimen and both P-selectin (Std β = 0.434, p = 0.004) and sVCAM-1 Std β = 0.328, p = 0.031) (Table 6.4). We further also observed a significant independent association between the second-line regimens and L-selectin (Std β = 1.032, p = 0.005) (Table 6.4). When further multiple analysis was performed in terms of specific ARV three-drug combination, TDF_FTC_EFV (first-line regimen) and 3TC_AZT_LPV/R (second-line regimen) did not show any significant association with all the endothelial biomarkers (Table 6.5).

6.2 DISCUSSION

6.2.1 L- and P-selectin

HIV infection and HAART are both associated with vascular dysfunction where ED is often implicated as a common precursor event prior to the manifestation of CVD. Under normal circumstances, the endothelium provides an anti-inflammatory and non-adhesive surface to enhance normal blood perfusion. Thus, any alterations in the vascular endothelium homeostasis due to diverse stimuli, such as HIV infection or adverse effects of ARV drugs may convert the healthy endothelium from resting to an activated state. This study investigated the impact of HIV infection and HAART on specific biomarkers of endothelial function.

Table 6.4: Independent associations of markers of endothelial function with HAART regimens, first-line regimen, and second-line regimen.

Model				
	Adj. R^2	**Adj. P_1**	**Std β (95% CI)**	**P_2**
Endothelial Biomarkers				
Variables:				
HAART Regimens				
L-selectin	0.029	0.067	-0.115 (-1.116; 0.886)	0.252
P-selectin	0.015	0.054	0.219 (-4.257; 4.695)	**0.032***
sCAM-1	0.07	0.106	-0.129 (-9.850; 9.592)	0.191
sVCAM-1	0.001	0.04	-0.100 (-14.617; 14.417)	0.325
VWF	-0.01	0.029	-0.145 (-0.448; 0.158)	0.157
first-line Regimen				
L-selectin	0.24	0.349	0.000 (-2.479; 2.479)	0.998
P-selectin	0.104	0.232	0.434 (-4.907; 5.775)	**0.004***
sCAM-1	-0.099	0.058	0.002 (-20.770; 20.774)	0.992
sVCAM-1	0.037	174	0.328 (-39.299; 39.955)	**0.031***
VWF	0.017	0.158	-0.067 (-0.696; 0.562)	0.655
Second-line Regimen				
L-selectin	0.932	0.980	1.032 (0.988; 1.075)	**0.005**
P-selectin	0.563	0.869	-0.422 (-10.331; 9.487)	0.324
sCAM-1	0.402	0.821	-0.094 (-208.165; 207.977)	0.836
sVCAM-1	0.006	0.702	0.233 (-9.731; 10.197)	0.696
VWF	0.757	0.927	1.081 (-0.747; 2.909)	0.027

Standardized β (Std. β) represents the change in the dependent variable for every 1 SD change in the independent variable: partial regression coefficient; Adj. R^2: adjusted R^2; 95% CI: 95% confidence interval of β; Adj. P_1: p-value for Adjusted R^2; P_2: p-value of Std. β; HAART: Highly active antiretroviral therapy, TDF: Tenofovir; FTC: Emtricitabine, EFV: Efavirenz; 3TC: Lamivudine; AZT: Zidovudine; LPV/R: Lopinavir/Ritonavir. All models included the following covariates: age, gender, BMI: Body mass index; TG, $CD4^+$ T-cell counts, duration on HAART. *: $p<0.05$ or **: $p<0.01$ were considered significant.

Table 6.5: Independent associations of biomarkers of endothelial function with specific ARV three-drug combinations of first-line regimen TDF_FTC_EFV (first-line regimen) and 3TC_AZT_LPV/R (second-line regimen).

Model				
	Adj. R^2	**Adj. P_1**	**Std. β (95% CI)**	**P_2**
Endothelial Biomarkers				
Variables:				
TDF_FTC_EFV				
L-selectin	0.095	0.199	0.165 (-5.154; 5.484)	0.200
P-selectin	-0.057	0.064	-0.086 (-11.675; 11.503)	0.536
sCAM-1	0.101	0.204	-0.095 (-52.329; 52.139)	0.454
sVCAM-1	-0.063	0.059	0.060 (-81.995; 81.875)	0.667
VWF	-0.066	0.056	0.062 (-1.335; 1.459)	0.655
3TC_AZT_LPV/R				
L-selectin	0.075	0.181	-0.089 (-6.200; 6.022)	0.482
P-selectin	-0.063	0.059	-0.044 (-13.248; 13.160)	0.744
sCAM-1	0.114	0.216	0.144 (-58.755; 59.063)	0.246
sVCAM-1	-0.062	0.06	-0.066 (-93.120; 92.988)	0.624
VWF	-0.06	0.061	-0.094 (-1.677; 1.489)	0.486

Standardized β (Std. β) represents the change in the dependent variable for every 1 SD change in the independent variable: partial regression coefficient; Adj. R^2: adjusted R^2; 95% CI: 95% confidence interval of β; Adj. P_1: p-value for Adjusted R^2; P_2: p-value of Std. β; TDF: Tenofovir; FTC: Emtricitabine, EFV: Efavirenz; 3TC: Lamivudine; AZT: Zidovudine; LPV/R: Lopinavir/Ritonavir. All models included the following covariates: age, gender, BMI: Body mass index; TG, CD4+ T-cell counts, duration on HAART. *: p<0.05 or **: p<0.01 were considered significant.

Selectins play a central role in initiating the adhesion of leukocytes and platelets during inflammatory processes in mediating the tethering and rolling interaction of both the circulating leukocytes and endothelial cells (Leick *et al.,* 2014; Gimbrone and García-Cardeña*.,* 2016). P-selectin and L-selectin are promising biomarkers of ED, and predictive measures of future cardiovascular complications (Mulvihill *et al.,* 2002; Kaur *et al.,* 2019).

Our results found that both L-selectin and P-selectin levels were significantly elevated in HAART-naïve group compared to the control group. After controlling for age and gender, the study found that only P-selectin levels were significantly higher in the HAART-naïve group compared to the control group while no significant difference

was observed for L-selectin. Previous studies showed the effect of HIV infection on L-selectin was controversial (Hayes *et al.,* 1999; Koh and Park, 2018; Kononchik *et al.,* 2018). Several studies, in support of our findings, reported that L-selectin is most likely elevated in the acute inflammatory phases, rather than the chronic HIV infection stages (Mastroianni *et al.,* 2000; Kononchik *et al.,* 2018). L-selectin plays a critical role during the initial stages of inflammation. It enables the rolling and adhesion of leukocytes on the surface of endothelial cells for migration to the site of inflammation (Sipsas and Sfikakis, 2004). Kononchik *et al*. (2018), showed that during the acute phase of HIV infection, the HIV targets L-selectin on the surface of the $CD4^+$ T cells. The viral proteins such as Nef and Vpu can sequester and redirect L-selectin into subcellular compartments and over time cause the downregulation of L-selectin and subsequent reduction of L-selectin levels (Guiliani *et al.,* 2015).

In the present study HAART-naïve participants were in the chronic phase of HIV infection, indicated by the $CD4^+$ T-cell count which was below normal (<500 cells/m^3) (Hoffman *et al.,* 2018; Tinarwo *et al.,* 2020). Therefore, this may explain the low levels of L-selectin observed in the present study. L-selectin is probably elevated during the acute phase of HIV infection and not the chronic phase. On the other hand, the control group is already overweight and obese to such an extent that even during HIV infection where L-selectin should be elevated no differences were observed between the control and HAART-naïve groups. Perhaps the HAART-naïve group are still at the early stages of HIV disease progression and the combined effect of the co-morbidities such as dyslipidaemia observed among the HAART-naïve group on L-selectin is not yet visible. We did observe that the prevalence of obesity in the general population (33%) was higher compared to the HAART-naïve (2.8%) group which may also explain why no difference in terms of L-selectin between the HAART-naïve and control groups were observed. The findings of the study suggest that L-selectin may not be a valuable marker for HAART-naïve individuals who are in the chronic phase of HIV infection or individuals who are at the early stages of HIV infection with comorbidities such as overweight, obesity, and dyslipidaemia. Since L-selectin is one of the markers used to indicate endothelial activation and ED, alternative markers should be used when the

HAART-naïve individuals are in the chronic phase of HIV infection or predispose to any risk factors of CVD.

P-selectin levels continued to be elevated among the HAART-naïve group even when age and gender were taken into consideration. This is in agreement with the earlier studies by Calza *et al.* (2009) and Arildsen *et al.* (2012). Although virion particles Vpu and Nef also target P-selectin to gain entry into the host cell, P-selectin expression may be protected by P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is a dimeric, mucin-like, 120kDa glycoprotein that is primarily expressed on the surface of lymphoid and myeloid cells (Fu *et al.,* 2020; Murakami *et al.,* 2020). PSGL-1 specifically binds to P-selectin, E-selectin and L-selectins which are elevated during inflammation to assist leukocyte tethering and rolling on the surface of the endothelium to migrate to the site of inflammation (de Gaetano Donati *et al.,* 2004; Fu *et al.,* 2020). PSGL-1 has a higher affinity for P-selectin compared to L-selectin and was found that the binding of PSGL-1 to P-selectin blocks the infectivity of HIV-1 particles by preventing the binding of the virion particles to the target cells (Fu *et al.,* 2020). PSGL-1; therefore, prevents sequestration of P-selectin and subsequent up-regulation of P-selectin expression (Kappelmayer and Nagy, 2017).

From the general population, evidence exist which suggest that elevated levels of P-selectin were associated with hypertension, dyslipidaemia, and atherosclerosis (de Gaetano Donati *et al.,* 2004; Eikendal *et al.,* 2018). In a large-scale prospective study among women, elevated P-selectin levels were associated with greater risk for future MI, stroke, and cardiovascular death even after controlling for lipid and non-lipid risk factors (Frijns and Kappelle, 2002). From our findings, P-selectin may be a valuable biomarker for ED among HAART-naïve group. The HAART-naïve group may be at increased risk of future cardiovascular complications such as MI, stroke, and cardiovascular death. This study suggests that P-selectin needs to be monitored on a routine basis among the HAART-naïve population which may reduce future cardiovascular morbidity and mortality.

In the present study it was only P-selectin in the HAART-exposed group that was significantly reduced to nearly the levels of the control group even after controlling for age and gender. Endothelial dysfunction measured by L-selectin between HAART-

naïve and HAART-exposed groups was similar. A positive change in the P-selectin levels among the HAART-exposed group was observed in the present study. HAART can reduce endothelial activation in HIV subjects, restoring the normal release of P-selectin by the endothelial cells (Siedner *et al.,* 2015). There could be conflicting findings in endothelial biomarkers (Beltran *et al.,* 2014; Cerrato *et al.,* 2015), depending on several factors such as the composition of HAART and the duration of the treatment (Luetkemeyer *et al.,* 2012). In the present study, we observed an improvement in the endothelial function where the majority of the HAART-exposed group were on the NNRTI-based regimen and treatment for < 5 years. Since HIV infection causes the activation of endothelial cells with subsequent release of more P-selectin, the change in the P-selectin levels following treatment reflects repairing activity in the endothelial cells, especially following low VL before HAART initiation. It is known that endothelial damage may predispose one to CVD (Collins *et al.,* 2000). The NNRT-based regimen may therefore play an important role in reducing the risk for future cardiovascular complications such as MI, stroke, and cardiovascular death in the present study population. P-selectin may therefore be a useful biomarker of ED among HIV-positive patients.

6.2.2 sICAM-1 and sVCAM-1

The findings of the study indicated that the median sICAM-1 levels of HAART-naïve group were significantly higher than the control group. However, after adjusting for both age and gender, no differences were observed. This may suggest that both the HAART-naïve and the control groups have equal risk for the development of ED at this point.

It has been previously reported that it is important to understand the role of inflammatory proteins in the development of atherosclerosis which may provide critical information on effective strategies to lower the risk for CVDs (Fatkhullina *et al*., 2016). Cell adhesion molecules are some of the biomarkers of vascular inflammation that are expressed on the surface of the endothelium or released in response to various stimuli such as cytokines, chronic inflammation, and tumour necrosis factor-alpha (TNF-α)

(Kacimi *et al.,* 1998). These endothelial CAMs (sICAM-1 and sVCAM-1) play a crucial role in the process of leukocyte rolling, firm adhesion, and trans-endothelial migration which are very early inflammatory events (de Gaetano Donati *et al.,* 2004). Evidence exists that elevated levels of both sICAM-1 and sVCAM-1 are indicative of inflammation, ED and may predict atherosclerosis and various cardiovascular complications (Koh and Park, 2018). Two prospective cohort trials performed by Ridker *et al*. (2000) and Porsch-Oezçueruemez *et al*. (1999) respectively, indicated that elevated levels of particularly sICAM-1 were associated with CHD among healthy males and females in the general populations. These studies specifically showed that elevated levels of sICAM-1 were detected many years before incidences of MI. Elevated levels of both sICAM-1 and sVCAM-1 have been reported in HIV-positive individuals (Fourie *et al.,* 2011; Graham *et al.,* 2013; Mosepele *et al.,* 2018). In the present study, ICAM-1 and sVCAM-1 levels were equally elevated in both HAART-naïve and control groups, indicative of equal risk for ED. Obesity, dyslipidaemia, pre-diabetes, and DM were present among the control group in the present study. All these risk factors of CVD have been previously reported to be associated with biomarkers of ED which include ICAM-1 and sVCAM-1 in the general population (Bošanská *et al.,* 2010, Aburawi *et al.,* 2016; Derosi and Maffioli, 2016). In the HAART-naïve group the HIV together with a high prevalence of alcohol consumption and tobacco use could have potentially contributed to the elevated ICAM-1 and sVCAM-1 levels observed in the present study. Several other factors that include the duration of HIV infection and the $CD4^+$ T-cell count which was at normal levels may explain why no changes in the ICAM-1 and sVCAM-1 levels were observed between the HAART-naïve and the control groups. The findings of the present study suggest that vascular inflammation and ED is present among the HAART-naïve group. Based on our findings both the control and HAART-naïve groups may therefore be at increased risk for atherosclerosis and cardiovascular complications.

The HAART-exposed group showed no significant difference in either sICAM-1 or sVCAM-1 levels even after adjusting for age and gender. This may suggest that ED continued irrespective of viral suppression. Our findings that sICAM-1 and sVCAM-1 remained elevated despite the intervention of HAART is similar to that of Rönsholt *et*

al. (2013) and Mosepele *et al.* (2018). In the present study dyslipidaemia was the highest among the HAART-exposed group. The HAART exposed individuals in this study population were predominantly treated with an NNRTI-based regimen. Friss-Moller *et al*. (2003), showed that both NNRTI-based and PI-based regimens were associated with dyslipidaemia. Dyslipidaemia is also associated with an increase in circulating CAMs (de Gaetano Donati *et al.,* 2003). Therefore, this may explain why both sICAM-1 and sVCAM-1 levels remained elevated regardless of viral suppression. Our findings, together with Rönsholt *et al*. (2013) and Mosepele *et al*. (2018), further emphasises the concept that HIV itself has a strong effect on ED. Other factors that could have further contributed to the persisted elevated sICAM-1 and sVCAM-1 levels among the HAART-exposed group includes lifestyle factors such as tobacco use and alcohol consumption.

The disparity observed in literature based on the effect of HAART on sICAM-1 and sVCAM-1, may be explained by the variation between different study populations in terms of their $CD4^+$ T-cell count, VL, sample size, and duration on HAART. HAART may have a protective role in preventing ED which is subject to the type of HAART because some studies have previously implicated NNRTs and PIs in inducing ED. The elevated levels in sICAM-1 and sVCAM-1 which indicate ED suggest that the HAART-exposed group may be at increased risk for future cardiovascular complications. Both sICAM-1 and sVCAM-1 biomarkers may serve a meaningful role in the identification of HIV-positive patients who are at risk for developing CVD. This study reiterates the importance of routine screening for ED with the use of sICAM-1 and sVCAM-1 biomarkers among the HIV-positive population.

6.2.3 Von Willebrand factor levels

Von Willebrand factor is a multimeric glycoprotein that plays a key role in the coagulation cascade. Von Willebrand factor has received a considerable amount of attention over the past decade (Agostini and Lionetti, 2017), and is well-established as a biomarker of ED (Horvath *et al.,* 2004; Graham *et al.,* 2013). It has been reported that VWF has a better predictive value in high-risk patients with either pre-existing

cardiovascular complications such as atherosclerosis or DM (Spiel *et al.,* 2008; Gragnano *et al.,* 2017). Interest has escalated on the potential effect of HIV and HAART on endothelial function. Therefore, the effects of HIV and HAART on VWF were investigated in this rural cohort**.**

In the present study even after adjusting for age and gender, VWF levels continued to show no significant difference between HAART-naïve and control groups. Despite the findings of the current study where no elevated VWF levels were observed among the HAART-naïve group, several investigators reported a marked elevation in VWF among HAART-naïve individuals (De Larrañaga *et al.,* 2004; Van den Dries *et al.,* 2015; Huson *et al.,* 2016). Van den Dries *et al*. (2015), further observed a correlation between VWF, first, and recurrent thrombotic events which suggest HIV infection as an independent risk factor for thrombotic abnormalities. Von Willebrand factor has been negatively associated with $CD4^+$ T-cell count and positively associated with VL (Lafeuillade *et al.,* 1992; Graham *et al.,* 2019). In agreement with our findings, a study performed by Allie *et al*. (2015) in Cape Town (Groote Schuur Hospital) investigated the role of VWF in the pathogenesis of HIV-related stroke among HIV-positive patients. They also found no significant difference in the median VWF levels between the HAART-naïve group compared to control group. They favour the notion that VWF is slowly released following HIV infection. The release of VWF is driven by chronic inflammation which is often demonstrated by elevated CRP levels. Aukrust *et al*. (2000), showed that elevated VWF levels correlated with HIV disease progression which may explain our findings that the HAART-naïve group were still in the early stages of HIV disease progression, indicative of VWF levels similar to basal control levels. In the present study, no significant difference was observed between HAART-naïve group and the control group in terms of CRP levels. We expected that VWF levels to be significantly elevated among the HAART-naïve compared to the control groups. This may be attributed to the fact that HAART-naïve group was at the early stages of HIV disease progression, indicative of TG levels that remained unchanged. Evidence exists that HIV infection promotes arterial inflammation which in turn gives rise to ED, atherosclerosis, and thrombosis (Horvath *et al.,* 2004; Graham *et al.,* 2013; Zanetti *et al.,* 2018). This may suggest early stages of chronic inflammation among the

HAART-naïve group and may also explain the non-significant results observed in VWF levels between HAART-naïve group vs control group.

The prevalence of obesity among the control group was the highest in the present study. We further also observed among the control group that the prevalence of pre-diabetes, DM, dyslipidaemia was similar compared to the HAART-naïve group, suggesting an equal risk for CAD. A study performed by Schaefer *et al*. (2019) reported that obesity is associated with elevated VWF levels. Elevated levels of VWF is associated with CAD (Graham *et al.,* 2013; Beltran *et al.,* 2015). Von Willebrand factor which serves as a prognostic marker for CAD is associated with cardiovascular risk factors such as DM and dyslipidaemia (Spiel *et al.,* 2008; Gragnano *et al.,* 2017). These cardiovascular risk factors were present and elevated to similar levels among both the HAART-naïve and control groups which may explain why no difference in terms of VWF levels was observed. The present study showed that both the HAART-naïve and control groups have equal levels of VWF, indicative of ED. Therefore, both HAART-naïve and control groups are at increased risk for developing CAD such as atherosclerosis. Routine screening for ED among both HIV-positive and the general population with the use of the biomarker VWF may serve a meaningful role in identifying individuals at risk for CAD.

The median VWF levels of the HAART-exposed group were significantly higher compared to the control group after adjusting for both age and gender. In agreement with our findings others also found that HAART significantly elevated VWF levels (Van den Dries *et al.,* 2015; Huson *et al.,* 2016; Graham *et al.,* 2019). The ARV induced toxicity can damage the endothelial cells in HIV-positive subjects which may lead to the pathological release of VWF by the damaged endothelium (Kearns *et al.,* 2017). Graham *et al*. (2019), reported that HDL-C and ADAMTS13 (proteolytic enzyme) play a decisive role in regulating self-association of VWF, a process where VWF is clustered into larger molecular structures with a high affinity for platelet binding. The HDL-C levels in the present study were significantly reduced in the HAART-exposed group which may explain the elevated levels in VWF in the HAART-exposed group. Wu *et al*. (2017), reported that VWF is considered an emerging mediator of vascular

inflammation, indicating that pathological elevation of VWF among the HAART-exposed group may be a product of inflammatory endothelial damage.

In the present study, the prolonged HAART therapy (±40% were on HAART for > 5 years) could have contributed to the endothelium damage which was demonstrated by the increase in VWF into the circulation. We already indicated that HAART reduced P-selectin which is suggestive of ED repair. In addition, HAART also exacerbates ED which was observed by the increase in VWF. In the present study, the prevalence of dysglycaemia was similar among the groups; however, high LDL-C levels were more prevalent among the HAART-exposed group. A common occurrence among the HAART-exposed population (Ahmed *et al.,* 2016; Lee *et al.,* 2017). Alcohol consumption and tobacco usage were also elevated in the HAART-exposed group compared to the control group. All these risk factors which include elevated high LDL-C, alcohol consumption, and tobacco usage may also explain the elevated levels of VWF observed in the HAART-exposed group. The persistent release of VWF, a multimeric adhesive protein that plays a key role in plaque formation and over time can promote atherosclerosis.

Our findings propose that HAART may have a dual effect on the endothelium. It may potentially repair ED by reducing HIV replication, as a result reducing the VL. The $CD4^+$ count (433.02 cells/uL) of the HAART-exposed group were also higher compared to the HAART-naïve group (341 cells/uL) which may explain the positive effect HAART had on these patients and may be linked to the repair in ED observed. Even though HAART may contain HIV replication, it cannot completely remove or destroy the virus, subsequently chronic inflammation persists which will enhance the pathology of ED and will put these HIV-positive patients at increased risk for future cardiac complications such as atherosclerosis. The VWF may be a reliable indicator of ED and a predictive measure for atherosclerosis, the forerunner of CVD (D' Abramo *et al.,* 2014; Bryckaert *et al.,* 2015). This could predict the development of CVD in the present HAART-naïve and HAART-exposed groups. Measurement of VWF can thus be used to detect CVD development in HIV infection, whether before or following HAART initiation.

6.2.4 Multi-variable analysis of HAART and endothelial biomarkers.

The multi-variate analysis conducted based on the established model showed a positive association between HAART and P-selectin. Sub-analysis further also showed a positive association between the first-line regimen and both P-selectin and sVCAM-1. The second-line regimen was also strongly associated with L-selectin. Several studies have demonstrated associations between biomarkers of endothelial activation and clinically important outcomes in HIV-1 infection (Calza *et al.,* 2009; Ronsholt *et al.,* 2013; Mosepele *et al.,* 2018). Ronsholt *et al*. (2013), reported a persistent activation of the endothelium and inflammation in HIV-positive patients on HAART for at least 12 years. In addition, Mosepele *et al*. (2018) confirmed that even in immunosuppressive HIV-positive patients, endothelial activation persists. Raised levels of endothelial biomarkers in the HAART-exposed group may reflect high ARV drug toxicity towards the endothelium or reduced effectiveness of the ARV drug which may prompt an alternative treatment regimen to be considered.

Although HAART did not show any association with sICAM-1, VWF levels, the findings of this study suggest a modifying effect of HAART on L-selectin, P-selectin, and sVCAM-1 if replicated in future studies, these biomarkers of ED may be used to predict cardiovascular risk which may act as an effective intervention to reverse atherosclerosis while it is still at its early stages of development in the HIV-positive population. Additional large-scale prospective investigations to determine the utility of endothelial activation and biomarkers for risk stratification and prediction of adverse outcomes are warranted.

CHAPTER 7

IMPACT OF HAART ON BIOMARKERS OF INFLAMMATION AND COAGULATION

7.1 RESULTS

In this chapter, the impact of HIV infection and HAART on inflammation and coagulation was assessed and discussed. Polovina and Potpara (2014), reported that endothelial damage is associated with various phenotypic changes which include pro-inflammatory and coagulating activity. Immune activation and inflammation in HIV patients may serve as a predictive measure for cardiovascular risk. These HIV-positive patients may also experience high coagulating activity which may predispose them to atherosclerosis. Measuring both the inflammatory and coagulating activity may provide important information that could serve as preventative strategies to combat CVD at an early stage of development among the HIV-positive population.

In the present study the serum CRP, fibrinogen, and D-dimer levels were measured in the HAART-exposed, HAART-naïve, and control groups. All endothelial biomarkers were expressed as median and interquartile ranges (IQR). Kruskal-Wallis statistical analysis revealed a significant difference in the CRP ($p = 0.007$), fibrinogen ($p = 0.0001$), and D-dimer (0.008) levels across the three groups (Table 7.1).

In an attempt to identify where the significant differences were among the three groups, the Kruskal-Wallis Bonferroni Post-Hoc test was performed on all three biomarkers. The HAART-exposed group had a significantly higher CRP concentration compared to the control group ($p = 0.008$, Figure 7.1, A). The fibrinogen concentration of the HAART-naïve group was significantly higher compared to the control group ($p = 0.001$, Figure 7.1, B). Moreover, the fibrinogen concentration was significantly reduced in the HAART-exposed group compared to the; HAART-naïve group ($p = 0.001$, Figure 7.1, B). The D-dimer concentration was significantly elevated in the HAART-exposed group compared to the HAART-naïve group ($p = 0.008$, Figure 7.1, C). Further, the

Kruskal-Wallis Bonferroni Post-Hoc analysis specifically showed that the HAART-exposed group had a significantly higher D-dimer concentration compared to HAART-naïve group (p = 0.014).

Table 7.1: Biomarkers of inflammation and atherosclerosis.

Biomarkers (ng/mL)	**Total** (N = 158)	**HAART-exposed** (n = 71)	**HAART-naïve** (n = 36)	**Control** (n = 51)	**p-value**
CRP	0.71 (2.05 – 0.20)	0.87 (3.90 – 0.27)	0.90 (2.71 – 0.26)	0.36 (1.05 – 0.06)	**0.007**
Fibrinogen	0.18 (0.55 – 0.07)	0.17 (0.40 – 0.07)	0.64 (1.19 – 0.17)	0.12 (0.39 – 0.05)	**0.0001**
D-dimer	26.07 (105.54 – 10.30)	41.27 (191.27 – 14.78)	17.55 (54.69 – 1.95)	15.60 (94.42 – 9.12)	**0.008**

All endothelial biomarkers were expressed as median and interquartile ranges (IQR)

Table 7.2: Analysis of covariance for inflammatory and coagulating biomarkers between all participants after adjusting for age and gender.

Parameters	**Sum of Squares**	**df**	**F**	**p-value**
CRP	639.305	2	0.765	0.467
Fibrinogen	224.269	2	1.354	0.261
D-dimer	172462.519	2	2.541	0.082

SS: Sum of Squares, df: Degrees of freedom, *: p<0.05 were considered significant

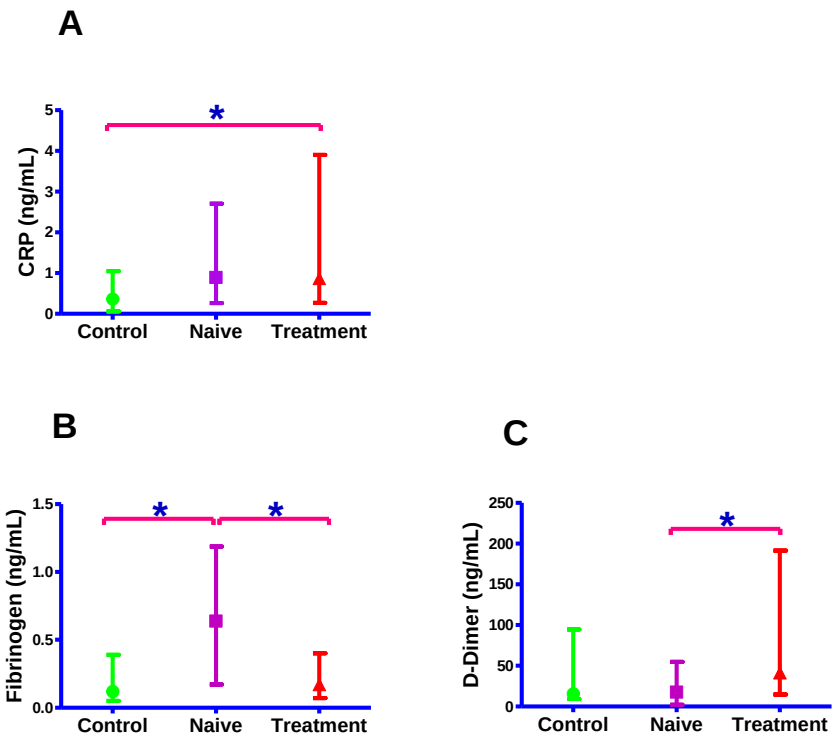

Figure 7.1: Evaluating inflammatory and coagulating activity in the study population with A: C-Reactive protein (CRP), B: Fibrinogen, C: D-dimer between the control, HAART-naïve and HAART-exposed groups. Values are expressed as median and interquartile ranges, ng/mL: nanogram per millilitre, *: P<0.005 (ANOVA analysis).

Table 7.3: Pearson and partial correlation coefficients between the HAART regimen and inflammatory and coagulating biomarkers after adjusting for age and gender.

	HAART (Pearson)	**HAART (Partial)**
CRP	r = -0.073, p = 0.462	r = -.073, p = 0.462
Fibrinogen	r = 0.009, p = 0.928	r = 0.013, p = 0.896
D-dimer	**r = -0.247, p = 0.011***	**r = -0.249, p = 0.011***

Pearson correlation (unadjusted) vs Partial correlation (adjusted for age and gender combined), *: p<0.05 or **: p<0.01 were considered significant.

Table 7.4: Independent associations of biomarkers of inflammation and coagulation with HAART regimens, first-line regimen, second-line regimen, TDF_FTC_EFV, and 3TC_AZT_LPV/R respectively.

Model				
	Adj. R^2	**Adj. P_1**	**Std β (95% CI)**	**P_2**
HAART Regimens				
Variables:				
CRP	0.058	0,166	-0.078 (-20.401; 20,245)	0,540
Fibrinogen	-0.016	0,101	-0.097 (-4.154; 3.960)	0,461
D-dimer	0.011	0,124	-0.153 (-158.36; 158.05)	0,243
First-line Regimen				
CRP	0.280	0,383	-0.013 (-7.931; 7.905)	0,921
Fibrinogen	-0.053	0,098	-0.003 (-0.592; 0.586)	0,985
D-dimer	0.591	0,649	0.741(-44.097; 45.579)	**0,0001****
Second-line Regimen				
CRP	0.540	0,862	0.584 (-11.530; 12.698)	0,210
Fibrinogen	0.450	0,835	0.529 (0.156; 0.901)	0,279
D-dimer	-0.351	0,595	-0.290 (-206.999; 206.419)	0,677
TDF_FTC_EFV				
CRP	0.077	0,183	0.158 (-65.192;65.508)	0,224
Fibrinogen	0.006	0,120	0.177 (-3.366;3.72)	0,190
D-dimer	0.001	0,115	-0.122 (-140.475;140.231)	0,364
3TC_AZT_LPV/R				
CRP	0.058	0,166	-0.079 (-20.461;20.303)	0,535
Fibrinogen	-0.021	0,096	-0.065 (-4.147;4.017)	0,624
D-dimer	0,002	0,116	-0.122 (-159.540;159.296)	0,352

Standardized β (Std. β) represents the change in the dependent variable for every 1 SD change in the independent variable: partial regression coefficient; Adj. R^2: adjusted R^2; 95% CI: 95% confidence interval of β; Adj. P_1: p-value for Adjusted R^2; P_2: p-value of Std. β; HAART: Highly active antiretroviral therapy, TDF: Tenofovir; FTC: Emtricitabine, EFV: Efavirenz; 3TC: Lamivudine; AZT: Zidovudine; LPV/R: Lopinavir/Ritonavir. All models included the following covariates: age, gender, BMI: Body mass index; TG, CD4+ T-cell counts, duration on HAART. *: p<0.05 or **: p<0.01 were considered significant.

After controlling for both age and gender combined with the use of ANCOVAs, the statistical significance initially observed in terms of CRP, fibrinogen, and D-dimer disappeared (Table 7.2). Both the Pearson (r = -0.247, p = 0.011) and partial (r = -

0.249, p = 0.011) correlation showed a significant association between HAART-exposed group and D-dimer (Table 7.3).

We further performed a correlation analysis to evaluate the strength of association between HAART and CRP, fibrinogen, and D-dimer. Both Pearson and partial correlation (adjusted for age and gender combined) did not show any significant associations between HAART-exposed group and both CRP and fibrinogen (Table 7.3). A significant negative association was reported by both Pearson (r = -0.247, p = 0.011) and partial (r = -0.249, p = 0.011) correlation test between D-dimer and HAART-exposed group (Table 7.3).

7.1.1 Multi-variable analysis of HAART, inflammatory and coagulating biomarkers.

The multi-variate analysis conducted based on the established model only showed a positive independent association between the first-line regimen and D-dimer (β = 0.741, p = 0.0001, Table 7.4).

7.2 DISCUSSION

7.2.1 C-Reactive Protein

HIV infection and HAART have been implicated in arterial inflammation which may lead to vascular dysfunction where ED is one of the early phenotypic changes observed. Inflammatory biomarkers such as CRP are often elevated among HIV-positive populations which may reflect vascular inflammation and ED, precursor events of atherosclerosis, and various cardiac complications (Fourie *et al.,*2011; Shrivastava *et al.,* 2015; Kearns *et al.,* 2017). This study investigated the impact of HIV infection and HAART on CRP, a commonly used biomarker of inflammation. Our results suggested that HIV infection did not have any effect on CRP levels although a trend of higher CRP levels (not-significant) was observed among the HAART-naïve group compared to the control group. Following HAART, no significant effect on CRP levels was observed although an inclination towards lower CRP levels was evident.

Furthermore, the CRP levels of the HAART-exposed group were higher compared to the control group.

Our study agrees with a similar study performed in South Africa which also found no difference in CRP among the HAART-naïve compared to the control group (Fourie *et al.,* 2015). Although no explanation was provided by Fourie *et al*. (2015), the high levels of obesity observed among the control group in the present study may explain why no difference between HAART-naïve and control groups was observed. Fourie et al. (2015) reported that the BMI levels of their entire study population was at normal while for the present study was high (overweight). In the general population, obesity is considered a major determinant of the increase in CRP (Aronson *et al.,* 2004). Our expectation to observe elevated levels of CRP in the HAART-naïve group could have been masked by the high prevalence of obesity in the control group.

Contrary to our findings, Boulware *et al*. (2011) reported elevated levels of CRP prior to HAART initiation. The HAART-naïve group of the latter study had a very low $CD4^+$ T-cell count (163 cells/µL), indicative of advanced stages of HIV disease progression. In addition, 38% of HAART-naïve group presented with AIDS-defining illness. In the present study the HAART-naïve group had higher $CD4^+$ T-cell count (341 cells/mm^3), indicative of early HIV disease progression. A more recent study performed in both South Africa and Uganda reported that immune activation, systemic inflammation, and coagulopathy are highly elevated before HAART initiation (Siedner *et al.,* 2019). It is important to note that in all of these studies where CRP levels were elevated, the median or mean $CD4^+$ T-cell counts were very low, which are indicative of advanced stages of HIV disease progression. In the present study we observed no difference in CRP levels between HAART-naïve and control groups, and this may be attributed to the early stages of the HIV disease progression.

A well-recorded observation in HAART-naïve populations is the reduction in HDL-C levels together with increased CRP levels (Fourie *et al.,* 2010; Souza *et al.,* 2013; Marín-Palma *et al.,* 2018). Marín-Palma *et al*. (2018), emphasised that the regularly observed low levels of HDL-C in the HAART-naïve population is associated with increased inflammation and HIV disease progression. High-density lipoprotein cholesterol (HDL-C) plays an important role in modulating inflammatory responses;

therefore, reducing inflammation (Fourie *et al.,* 2015). The elevation of CRP levels among the HAART-naïve group may be attributed to the reduction in HDL-C activity. HIV proteins which include Tat, gp120, and Nef have been implicated as one of the causes for the elevation in CRP levels observed among HAART-naïve individuals (Lui *et al.,* 2005; Anand *et al.,* 2018).

In the present study, we did observe a trend of elevated CRP levels in the HAART-naïve group compared to the control group. Although the trend was not significant, it may still indicate arterial inflammation and injury which may induce endothelial activation and dysfunction. This was supported by the elevation in P-selectin observed in the HAART-naïve group. D'Abramo *et al*. (2014), indicated that immune cell activation starts at the onset of HIV infection. This is associated with the depletion of $CD4^+$ cells and consequently activates a cascade of inflammatory and immunological reactions with the secretion of pro-inflammatory biomarkers such as CRP. Chronic inflammation has been proposed as a possible mechanism for HIV and HAART-induced atherosclerosis (Beltran *et al.,* 2015). Although early HAART cannot eradicate HIV infection, it has been reported that it can reduce chronic immune activation and rapid CVD progression. The present study suggests that HAART-naïve participants may be at increased risk for future cardiovascular complications such as atherosclerosis. The findings of this study reiterate the importance of immediate testing and initiation of treatment of HIV-infected individuals to reduce the rate of chronic inflammation and to subsequently reduce the rate of cardiovascular disease progression.

When HAART-exposed and HAART-naïve groups were compared in terms of CRP levels no difference was observed, indicative of consistent chronic inflammation. Several studies observed similar findings of persisted chronic inflammation regardless of HAART initiation (Erlandson and Campbell, 2015; Fourie *et al.,* 2015; Shivakoti *et al.,* 2015; Mosepele *et al.,* 2018). Contrary to our findings, some also reported that HAART is strongly associated with a reduction in CRP levels (Arildsen *et al.,* 2013). The different outcomes observed may be attributed to the time of HAART initiation, for example, the study performed by Arildsen *et al*. (2013) HAART was initiated when the HIV-infected participants were at very low $CD4^+$ T-cell count of 160 cell/mm^3 when the

CRP levels were highly elevated. It is well known that CRP is negatively associated with $CD4^+$ T-cell count, where CRP levels were highly elevated in groups with $CD4^+$ T-cell counts below 200 cells/µL (Chaudhary *et al.,* 2008).

The present study is in agreement that HAART can reduce endothelial activation and ED; however, we also observed that HAART did not completely reverse ED since VWF was still elevated. Shivakoti *et al*. (2015), also reported on similar median CRP levels before and after HAART, they advised that looking at only median CRP values might not always be sufficient because the change in inflammatory status differs from one patient to another after the initiation of HAART. The conflicting findings among the various studies can be attributed to several factors which include the time of HAART initiation, the stage of HIV disease progression, and the type of HAART administered. The initial VL status prior to HAART initiation will also influence the CRP levels (Luetkemeyer *et al.,* 2012; Vishwanath *et al*., 2016). The increase in cardiovascular risk caused by chronic inflammation observed among the HIV population may be alleviated in part by HAART associated viral suppression and by careful selection of ARVs which will mitigate the increase in inflammation and various other adverse changes in the lipid profile, and platelet and endothelial functions (Baker *et al.,* 2017). Therefore, it is recommended that CRP levels in HIV infection be monitored, because it can be a risk associated with CVD among the HIV-positive population.

7.2.2 Fibrinogen and D-dimer

Since the introduction of HAART, mortality due to opportunistic infections has drastically reduced. However, the evidence is showing that more HIV-positive individuals are at increased risk of CVD. Chronic inflammation which is associated with HIV infection may activate the coagulation cascade and increase the risk of atherosclerosis among the HIV-positive population (Baker *et al.,* 2017). This study investigated the impact of HIV infection and HAART on the coagulating factors, fibrinogen, and D-dimer.

The findings of the study indicated that the median fibrinogen and D-dimer levels of the HAART-naïve group were significantly higher than the control group. However, after adjusting for both age and gender, no significant differences were observed. A

trend of elevated fibrinogen levels in the HAART-naïve group compared to the control group was observed. Our results suggest that HIV infection itself can elevate the median fibrinogen levels, while in the HAART-exposed group the fibrinogen levels were reduced. Although HIV infection did not have any effect on D-dimer levels, the HAART-exposed group showed elevated levels of D-dimer levels.

Several studies have reported elevated levels of fibrinogen in HAART-naïve patients (Funderberg *et al.,* 2014; Pogessi *et al.,* 2015; Huson *et al.,* 2016). Fibrinogen is an acute-phase reactant (Hoffman *et al.,* 2018), and during acute inflammation as observed during HIV infection, fibrinogen is usually elevated (Subramanya *et al.,* 2019) This may explain the elevation in fibrinogen levels observed among the HAART-naïve group in the present study. Therefore, acutely increased fibrinogen levels may indicate early stages of HIV disease progression among HAART-naïve individuals. Contrary to our findings, Fourie *et al*. (2011) reported no significant difference in the levels of fibrinogen between the HAART-naïve group compared to control group. They attributed their findings based on what James *et al*. (2000) previously reported that black South Africans, in general, have elevated levels of fibrinogen and that this may explain the masking effect of HIV on the fibrinogen concentration.

Under normal conditions, the healthy endothelium regulates fibrin formation. It forms a barrier between thrombogenic sub-endothelial matrix constituents and coagulation proteins (Yau *et al.,* 2015). During inflammatory conditions, such as with HIV infection, endothelial cell function becomes compromised (Chien, 2007; Camare *et al.,* 2017). Endothelial cells may trigger increased fibrinogen and fibrin formation during endothelial damage (Pogessi *et al.,* 2015). This was demonstrated in the present study through observed elevated P-selectin levels as well as elevated levels of fibrinogen (activation of coagulation) in the HAART-naïve group. Hypercoagulability is common in HIV infection (Arildsen *et al.,* 2013; Vachiat *et al.,* 2017). In the present study a trend of increased fibrinogen levels was observed in the HAART-naïve group. Although HIV infection was associated with ED, no difference was observed in D-dimer levels. These findings may be explained by early HIV disease progression. It has been previously reported that elevated D-dimer predicts cardiovascular risk and long-term cause-specific mortality (Choi *et al.,* 2016; Simes *et al.,* 2018). Dyslipidaemia, obesity,

smoking, and tobacco use were all present in the control group which may also explain why no difference in D-dimer levels was not observed between the control and HAART-naïve groups. The findings of this study suggest that the elevated levels in fibrinogen observed in the HAART-naïve individuals may indicate thrombotic events in association with CAD which may put this group at increased risk for atherosclerosis.

The HAART-exposed group showed a trend of a reduction in the median fibrinogen levels and an increase in D-dimer levels. The study also found a significant negative association between D-dimer levels and HAART. A longitudinal study performed by Freiberg *et al*. (2016) reported elevated levels of D-dimer before and after ARV therapy. Although we did not observe a significant increase in the D-dimer levels in our HAART-naïve group, many studies are in agreement with our study that D-dimer is elevated in the HAART-exposed group (Kuller *et al.,* 2008; Tenorio *et al.,* 2014; Freiberg *et al.,* 2016; Grund *et al.,* 2016). However, some reported either a reduction in the levels of D-dimer (Arildsen *et al.,* 2013) or no difference at all after ARV therapy (Hsue *et al.,* 2012). It is important to note that Hsue *et al*. (2012) also found no difference in the immune ($CD4^+$ T-cell count) and inflammatory (hsCRP) activity between HAART-naïve vs HAART-exposed groups which may explain why no significant difference in D-dimer levels was observed. The longitudinal study performed by Arildsen *et al*. (2013) showed a reduction in D-dimer levels after treatment among patients who had an initial median $CD4^+$ T-cell count of 160 cells/µl (WHO stage IV of HIV disease progression). The initial median $CD4^+$ T-cell count of 160 cells/µl which are indicative of the immune-compromised stage suggests that the high VL may have induced the elevation in D-dimer levels. Immediately after the initiation of HAART, the $CD4^+$ T-cell counts improved due to viral suppression, which in turn reduced chronic inflammation. These events prompted the reduction in D-dimer in HAART-exposed individuals.

Hoffmann *et al*. (2018), confirmed that D-dimer has an inverse relationship with $CD4^+$ T-cell count and a positive relationship with hsCRP. The data may suggest that irrespective of ARV therapy, the immune and inflammatory status of HIV-positive patients may determine D-dimer levels (coagulation activity). Furthermore, it was found that D-dimer predicts incidents of CVD in chronic HIV infection (Hemkens and

Bucher, 2014; Tenorio *et al.,* 2014; Vos *et al.,* 2016). During the inflammatory process, activation of the vascular endothelium and the coagulation system may occur and thus contribute towards cardiovascular events currently observed among the HIV population on HAART.

As previously described, during endothelial damage the endothelial cells trigger fibrin formation (clot formation) (Poggesi *et al.,* 2015). Yau *et al*. (2015), reported that endothelial cells release pro-fibrinolytic agents that initiate fibrinolysis to degrade the clot, thus, yielding D-dimers (FDPs). In the present study, increased D-dimer levels in the HAART-exposed group were observed. This study further also found a significant negative correlation between D-dimer and HAART, suggesting HAART to have a protective role in reducing hypercoagulation (clot formation), possibly through a reduction in inflammation that results from decreased viral replication. We also observed that P-selectin was significantly reduced by HAART in the present study, which further may explain the negative association observed between D-dimer and HAART. Therefore, HAART may play a protective role among HIV-positive individuals in preventing the development of atherosclerosis.

The study found that D-dimer was the strongest predictor variable associated with the first-line regimen. HAART may activate endothelial cells and promote fibrinolysis; therefore, generating more D-dimers, indicative of hypercoagulation. Collectively, the findings of this study suggest that HIV infection may enhance coagulation activity; however, coagulation persisted regardless of the positive effect of HAART on viral suppression which subsequently resulted in the reduction in inflammation. The results; therefore, reflect ongoing immune activation in conjunction with coagulation even with successful viral suppression. Elevated levels in D-dimer have been associated with atherosclerosis which may put these HIV patients at increased risk for future cardiovascular complications. D-dimer may serve as a useful tool in measuring coagulation activity to prevent CVD complications such as atherosclerosis. Monitoring the coagulation status in HIV-infected patients who are on HAART might be an important strategy to combat the increase in CVD observed among the HIV-positive population.

CHAPTER 8

CONCLUSION

8.1 CONCLUDING REMARKS

The focus of the study centred on the effect of HAART on the cardio-metabolic profile, endothelial function, inflammation, and coagulation activity among a rural black South African population. Although HAART and HIV itself are strongly correlated with cardiometabolic changes in people living with HIV globally. In the present study, both HAART and HIV infection did not have any effect on BMI and glucose metabolism. As a result, the study accepted the H_0 that HAART regimen does not increase dysglycaemia. This may be reflective of inheriting poor lifestyles (smoking, alcohol consumption, and poor diet) and poor cardio-metabolic profiles before HIV infection and the commencement of HAART. This cohort demonstrated that HAART did have a detrimental effect on lipid metabolism which was confirmed by elevated LDL-C and TC levels. The findings of this study therefore gathered sufficient evidence to reject the H_0 that HAART regimen does not increase dyslipidaemia. Overall, dyslipidaemia was present across the entire population; however, no difference in the prevalence of dyslipidaemia was observed in the control group compared to the HAART-naïve and HAART-exposed groups. While HIV-associated inflammation and HAART are key to the alteration in the cardiometabolic profile. The established cardiovascular risk factors before HIV infection and HAART initiation may confer an increased cardiovascular risk which will warrant unique preventative strategies that will take into consideration the complex mechanistic factors that are at play.

An array of serological biomarkers for endothelial function (L-selectin, P-selectin, sICAM-1, sVCAM-1, and VWF); inflammation (CRP), and coagulation (fibrinogen and D-dimer) were measured. In this rural black population, HAART illustrated both a protective (repairing ED) and damaging effect (inducing ED) on the endothelial function. The study accepted the H_0 that HAART regimen does not increase L-selectin,

P-selectin, sICAM-1, sVCAM-1 and CRP. However, for VWF sufficient evidence was generated to reject the H_0. Endothelial dysfunction is commonly associated with increased inflammation; this was not reflected in the present study. There is a need to elucidate on the potential links between inflammation and ED in the context of HIV positive individuals on HAART, especially among African populations.

We found no difference in both fibrinogen and D-dimer levels among the HAART-exposed individuals compared to HIV-naïve and control groups, though, a negative association was observed between HAART and D-dimer levels. Therefore, this study accepted the H_0, stating that the HAART regimen does not increase D-dimer and fibrinogen levels. HAART may therefore serve a protective role in reducing coagulative activity.

Despite conflicting findings regarding the effect of HAART on the CVD markers, the study succeeded in elucidating that HAART affects endothelial activation, ED, inflammation, coagulation, and cardio-metabolic profiles of HIV positive individuals. Multiple analysis revealed that P-selectin is the strongest predictor of ED and that D-dimer is the strongest predictor variable of coagulating activity among first-line regimen users. It was further also discovered that L-selectin was the strongest predictor variable for ED among the second-line regimen users.

Most studies assessing ED make use of one or two ED biomarkers. In the present study we were able to demonstrate the complex interplay of the role of HAART on endothelial function with the use of five different biomarkers of ED in a HIV-positive rural black South African population. This allowed us to observe both the protective and damaging effect of HAART on the endothelium. Since endothelial damage and hypercoagulability are reliable predictor conditions for CVD, L-selectin, and D-dimer biomarkers can be useful in evaluating future cardiovascular complications.

8.2 A CRITERION MODEL FOR THE ASSESSMENT OF CVD DEVELOPMENT PREDISPOSITION IN HAART TREATMENT

The study reflected on a need for an effective and comprehensive management strategy for HAART in the treatment of HIV-infected patients. Managing both the HIV

infection and HAART is important in the reduction of the risk of developing CVD. From the present study perspective, biomarkers of interest and metabolic parameters can be used to monitor the effect of HAART on the development of CVD in HIV-positive patients. Cardiovascular disease risk assessment of the HIV-positive patients based on the biomarkers of interest could be incorporated in the current HIV/AIDS management program guidelines. The study further necessitated the development of a proposed criterion model which outline the diagnostic parameters based on both traditional risk factors and biomarkers of endothelial function, inflammation and coagulation activity to assess the CVD risk together with potential interventions (Figure 8.1). The purpose of the proposed criterion model is to predict CVD risk in order to inform preventative interventions. The earlier the CVD risk is identified, whether it is over the next 5–15 years or over the course of a life time, the greater the benefit of CVD risk reduction from appropriate therapeutic interventions for the HAART-exposed individuals. In addition, this can potentially reduce the high levels of CVD-related comorbidities and mortalities currently observed in our HIV-positive population.

The conceptual approach for the criterion model are as follows. Clinicians or health care workers could initiate the routine screening process by assessing firstly for traditional risk factors which include lifestyle behaviour (smoking, alcohol consumption, physical activity and dietary intake), blood pressure, anthropometric measures (BMI and waist to hip ratio) and cardio-metabolic profiles (dysglycemia and dyslipidemia screening). This is followed by biochemical analysis of endothelial, inflammation and coagulation biomarkers. After the CVD risk assessment of the HAART-exposed patient, the estimated CVD risk is determined and the patient is classified under one of the four CVD risk categories which include criterion 1 (low risk), criterion 2 (moderate risk), criterion 3 (high risk) and criterion 4 (very high risk). The clinician or health worker will engage the patient regarding health literacy where the CVD risk factors in the HAART-exposed population are discussed. This will be followed by a consultation with each individual patient where the outcome of the CVD risk assessment is disclosed and recommendations provided regarding how the CVD risk(s) can be controlled. The clinician or health worker will advise the patient on the appropriate lifestyle modifications according to the CVD risk outcome of each

individual patient. The lifestyle recommendations will include the termination or reduction of smoking and alcohol consumption, increased physical activity, weight-reduction plan and dietary modifications. Other CVD risk factors will include the importance of controlling blood pressure, glucose and lipids concentrations, endothelial, inflammation and coagulation biomarkers. It is important to advice the patients of the benefits and the adverse effects of any drug therapy. The regular monitoring of the CVD risk indicators among the HAART-exposed patients will be integral for effective management in primary care facilities.

Criterion-1 is based on the status of traditional CVD risk factors (blood pressure, cardio-metabolic parameters, BMI and waist-to-hip ratio) in conjunction with the evaluation of endothelial, inflammation and coagulation biomarkers in the HAART-exposed patients. This criterion will reflect no signs of any of the traditional CVD risk factors with normal levels of endothelial function, inflammation and coagulation activity which will be indicative of low risk for CVD development (Figure 8.1). The endothelial, inflammation and coagulation biomarkers will serve as predictor variables to inform the clinicians or health workers of potential future CVD risk among the HAART-exposed population where appropriate therapeutic interventions can be implemented to reduce the CVD risk. Although the criterion-1 represents low risk for CVD development where for example therapeutic interventions which include statins (lipid lowering drugs) or antihypertensive drugs is perhaps not needed, it is still imperative to promote the health literacy among the HAART-exposed population.

Criterion-2 is based on the status of endothelial function, coagulation activity, and inflammation activity. The criterion will still evaluate the all traditional CVD risk factors in conjunction with endothelial, inflammation and coagulation biomarkers. Criterion-2 is characterised by elevated endothelial, coagulation and inflammation activity where none of the traditional risk factors are present (Figure 8.1). Criterion-2 actually represents the essence of the proposed criterion model, because it is at this stage where potential CVD risk can be identified among the HAART-exposed population since the biomarkers of endothelial function, inflammation and coagulation activity can predict future CVD risk. It is at this stage where clinicians and health workers can advise the HAART-exposed patients on the importance of lifestyle modification

(alcohol and smoking cessation, increased physical activity, weight loss plans and dietary changes) and also provide the option of therapeutic interventions (lipid lowering drugs).

Criterion-3 is based on the status of endothelial damage, coagulation activity, and inflammation activity together with all traditional CVD risk factors (Figure 8.1). Elevation of endothelial, coagulation and inflammation biomarkers in combination with anthropometric measures (blood pressure, BMI and waist to hip ratio) and cardio-metabolic profiles (dysglycemia and dyslipidemia screening) will be characteristic of criterion-3. Any of the traditional CVD risk factors which include dysglycaemia, dyslipidaemia, overweight or obesity, abdominal obesity and hypertension in combination with any signs of ED, inflammation or hypercoagulation thus far would be characteristic of the criterion-3. This criterion would be graded as a moderate to high-risk situation for the development of CVD (Figure 8.1). High levels of CRP and VWF, for example, are associated with increased risk for atherosclerosis and independent predictors of future cardiovascular complications (Lubrano and Balzen, 2015; Shrivastava *et al.*, 2015). Soluble vascular cell adhesion molecule-1 and sICAM-1 together with VWF were highly expressed in endothelial activation and may lead to atherosclerosis (Gragnano *et al.*, 2017; Wu *et al.*, 2017). Classifying an HAART-exposed patient under criterion-3, is a major health concern. Clinicians and health workers should sensibly convey the CVD risk outcome to the HAART-exposed patient. The importance of adhering to both lifestyle modifications and therapeutic interventions are vital. At this stage it would also be important that clinicians and health workers should consider HAART regimen switch for some ARV drugs such as PIs can increase the risk for CVD.

Criterion-4 represents the combination of traditional CVD risk factors which include dysglycaemia, dyslipidaemia, overweight and obesity, abdominal obesity, and hypertension in concurrence with ED, elevated coagulation activity, and elevated inflammation in any form would be characteristic of the criterion-4 (Figure 8.1). This phase would reflect very high or severe risk for CVD development through risk factor synergism. It is imperative to evaluate the status of the HAART therapy from time to time to detect risks for CVD predisposition in good time for prompt intervention. Both

lifestyle modification and therapeutic intervention are imperative because criterion-4 is a life threatening stage. All interventions should be carefully considered in order to optimise the reduction of CVD risk. For example, change in the treatment regimen should be reviewed, the chronic exposure to specifically PI-based regimens can accelerate the atherosclerotic processes and can increase the risk for developing MI (Alvi *et al.,* 2018).

This criterion model emphasizes the need for routine screening of cardiovascular risk in HIV-infected patients on HAART, especially with the use of endothelial, coagulation, and inflammatory biomarkers as cardiovascular risk indicators in these patients. Future studies should explore the mechanisms of both HIV and HAART-induced ED and HIV and HAART-induced coagulative disorders to better understand the findings of these results. Programs should be established that target the prevention and appropriate treatment of HIV and HAART-related vascular diseases to combat the high CVD-related deaths among HAART-exposed populations.

The model can be relevant to clinics and hospitals where doctors are available or routinely visit, and they have access to advanced laboratory services. This model will not be directly applicable in the ordinary rural clinics where there are only nurses or where doctors occasionally visits. The doctors or health professionals will need to be trained on how, for example, to interpret CVD risk assessment results and to appropriately assign each patient to his/her specific risk criterion. Furthermore, the doctor or health professional should be able to recommend appropriate lifestyle and therapeutic interventions. It is important to note that biochemical analysis of this model which include the endothelial, inflammation and coagulation biomarkers are very expensive. The National Laboratory Services especially in the Limpopo Province will have to invest in buying the appropriate equipment in order to analyse these biomarkers since it is not currently available. Even though a significant amount of investment will be required to implement this model, it is envisaged that the long term health benefits for the HAART-exposed population, especially in the rural communities of the Limpopo Province, will outweigh this.

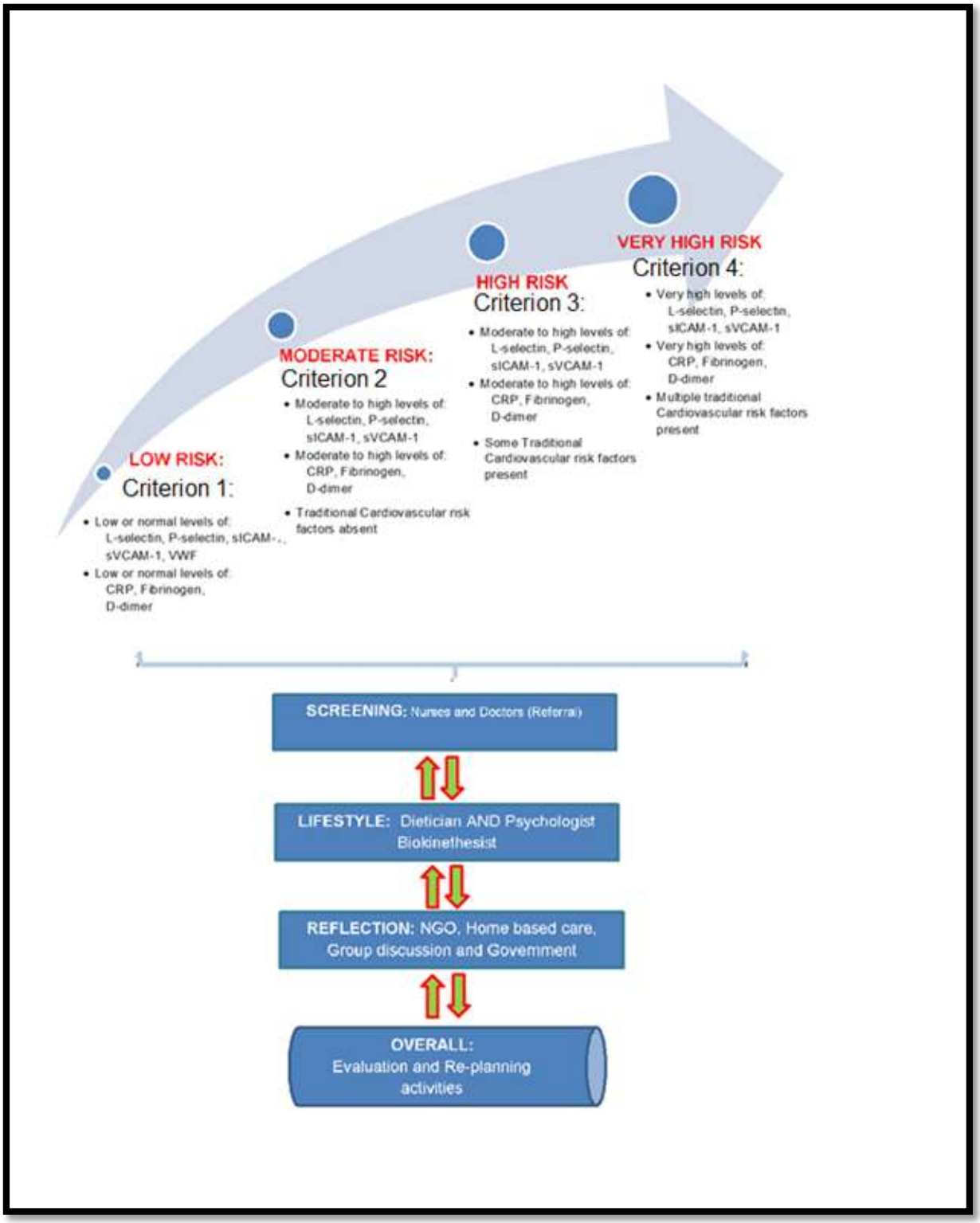

Figure 8.1: A proposed criterion model to evaluate the predisposition of a patient for CVD development on HAART treatment.

8.3 LIMITATIONS OF THE STUDY

This cross-sectional and comparative study did not allow us to infer causation. As a result of financial constraints, we could not plan for a longitudinal study to track the effects of HAART. The findings of the study were limited to only black South Africans. Other ethnic groups should also be included in the study to establish any variation in the outcome of this study. Unequal sample groups and a strong gender bias could have influenced the outcome of this study. The gender bias may be due to males being generally the sole provider in the family and hence unable to take time off from work for medical care or to participate in research projects. Also, we were unable to include VL results due to financial constraints. We did not measure the central fat distribution, which could have provided a different outcome. Future studies should recruit equal amounts of specific ethnic groupings in Limpopo to enhance the quality of conclusions reached. An element of recall bias was evident in this study since there was a level of reliance on information provided by patients and the accuracy of the documented medical files. The impact of HIV itself and HAART on central fat distribution and other measure of fat distribution needs to be elucidated.

8.4 RECOMMENDATIONS

Given the limitations of this study, a prospective longitudinal study with primary data should be conducted to infer causation. Awareness campaigns advocating the importance of physical activity and the dangers of overweight and obesity; smoking and alcohol consumption among the HIV population on HAART is recommended. The findings of this study provide population-based evidence that can be used by various stakeholders in policymaking. It may also advise the Department of Health and various stakeholders to make more informed decisions to enhance the existing strategies or interventions by empowering stakeholders to carefully scrutinise the efficacy of HAART regimens. These findings also promote awareness of the importance of designing more effective treatment regimens for CVD in the HIV population.

Furthermore, based on the findings of this study, BMI, glucose, lipid profile together with the biomarkers of endothelial function and coagulation activity should be regularly monitored on HAART-exposed individuals. Regular testing intervals will provide oversight of these parameters and assist in the early treatment and management of body weight and metabolic abnormalities such as hyperglycaemia and dyslipidaemia among HIV-positive patients on HAART. It is pertinent to note that these components (metabolic abnormalities, ED, and coagulation biomarkers) are all modifiable. Programs that focus on promoting a healthy diet, high intake of fruits and vegetables which will assist in the reduction of LDL-C and TC among the HIV-positive individuals on HAART is highly recommended.

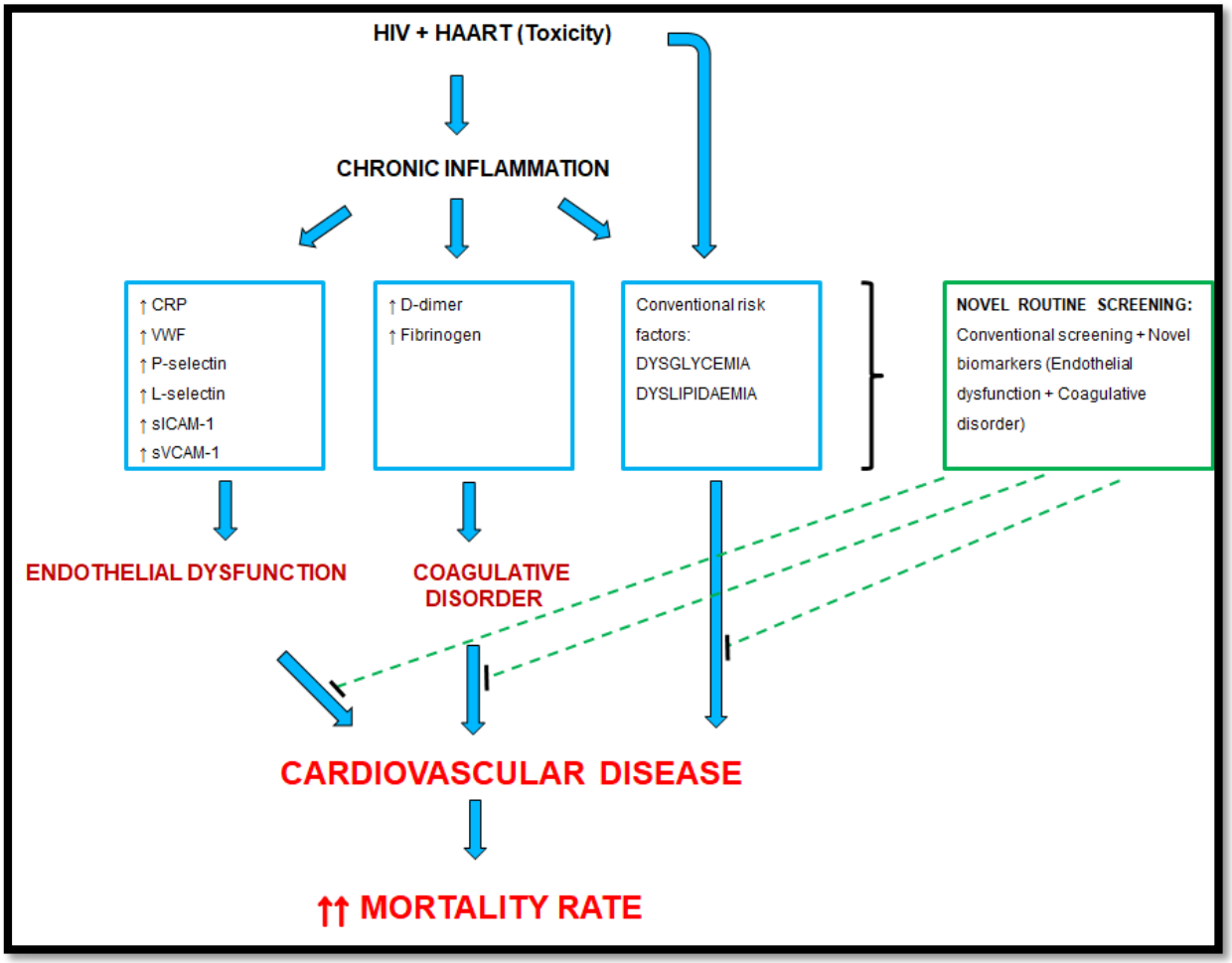

Figure 8.2: Schematic representation for routine screening for CVD with the use of conventional targets in conjunction with novel targets of ED and

coagulative disorder among HIV positive HAART exposed individuals. [HIV: Human immunodeficiency virus, CRP: C-reactive protein, VWF: von Willebrandt factor, sICAM-1: Soluble intracellular cell adhesion molecule, sVCAM-1, Soluble vascular cell adhesion molecule].

Routine testing of ED and coagulation biomarkers in resource-limited settings is not currently practiced since the equipment is expensive. The findings of this study suggest that health practitioners should consider routine screening of the conventional CVD risk factors coupled with the novel targets of ED and coagulative disorder among the HIV- positive HAART-exposed population (Figure 8.2). This may assist in reducing the high levels of CVD-related deaths currently observed among HAART-exposed populations.

This provided important information on the effects of HAART treatment on the vascular endothelium. The information gathered from this study can assist the Department of Health and various stake holders to make more informed decisions in regards to the different regimens of HAART. This information of this study may enhance the existing strategies or interventions by empowering stakeholders to design more effective treatment for CVD in the HIV population.

The findings of the study indicate that communities and HIV-positive individuals in this rural population should know that the chances of developing CVD among both HAART-naïve and HAART-exposed populations are relative high. They should therefore regularly visit their nearest clinics or health facilities to asses CVD risk so that the condition can be managed and the risk for CVD reduced. The Department of Health should incorporate CVD risk assessment programs in their primary health care services. For example, in the budget for primary health care services, allocations should be prioritized for biochemical analysis of glucose, lipids, endothelial function, inflammation status and coagulation activity. The health professionals should be trained to perform and interpret CVD risk assessment results. The scientific community should strengthen their focus on studies which can optimise CVD risk assessment strategies so that it can accommodate communities of different socio-economic backgrounds.

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APPENDICES

APPENDIX A: INFORMATION OF PARTICIPANTS

PROJECT TITLE: Investigating the effects of HAART on early markers of cardiovascular disease among HIV-positive patients in the Mankweng District, Limpopo Province.

PROJECT LEADERS/SUPERVISORS:
Prof M Van Staden
Prof LJC Erasmus
Prof MM Moraba

1. You are invited to participate in the following research project:
   This study investigates the effects of HAART on early markers of cardiovascular disease among HIV-positive patients by assessing body weight, blood pressure, metabolic profile and endothelial function.
   Name: .....

2. Participation in the project is completely voluntary and you are free to withdraw from the project (without providing any reasons) at any time.

3. It is possible that you might not personally experience any advantages during the project, although the knowledge that may be accumulated through the project might prove advantageous to others.

4. You are encouraged to ask any questions that you might have in connection with this project at any stage. The project leader and her/his staff will gladly answer your question. They will also discuss the project in detail with you.

5. The nature of the specific project, the alleged risk-factors, factors that might possibly cause discomfort, the expected advantages and the known and/or likely

side-effects should be explained under this item. (it is compulsory for the researcher to complete this field before submission to the Ethics Committee).

6. Should you at any stage feel unhappy, uncomfortable or is concerned about the research, please contact Ms Noko Shai-Ragoboya at the University of Limpopo, Private Bag X1106, Sovenga, 0727, Tel: 015 268 2401.

APPENDIX B: CONSENT FORM

PROJECT TITLE:

Investigating the effects of HAART on early markers of cardiovascular disease among HIV-positive patients in the Mankweng District, Limpopo Province.

PROJECT LEADER/SUPERVISOR:
Mr S Hanser (MSc Medical Physiology)
Prof M Van Staden (PhD)
Prof LJC Erasmus (PhD)
Prof MM Moraba (PhD)

I, hereby voluntarily consent to participate in the following project:
This study investigates the effects of HAART on early markers of cardiovascular disease among HIV-positive patients by assessing body weight, blood pressure, metabolic profile and endothelial function.

1. The study deals with effect of HAART on the human body.

2. The procedure or treatment envisaged may hold some risk for me that cannot be foreseen at this stage.

3. The Ethics Committee has approved that individuals may be approached to participate in the study.

4. The research project, i.e. the extent, aims and methods of the research, has been explained to me.

5. The project sets out the risks that can be reasonably expected as well as possible discomfort for persons participating in the research, an explanation of the anticipated advantages for myself or others that are reasonably expected from the research and alternative procedures that may be to my advantage.

6. I will be informed of any new information that may become available during the research that may influence my willingness to continue my participation.

7. Access to the records that pertain to my participation in the study will be restricted to persons directly involved in the research.

8. Any questions that I may have regarding the research, or related matters, will be answered by the researcher/s. TURFLOOP RESEARCH ETHICS COMMITTEE.

9. If I have any questions about, or problems regarding the study, or experience any undesirable effects, I may contact a member of the research team or Ms Noko Shai Ragoboya.

10. Participation in this research is voluntary and I can withdraw my participation at any stage.

11. If any medical problem is identified at any stage during the research, or when I am vetted for participation, such condition will be discussed with me in confidence by a qualified person and/or I will be referred to my doctor.

12. I indemnify the University of Limpopo and all persons involved with the above project from any liability that may arise from my participation in the above project or that may be related to it, for whatever reasons, including negligence on the part of the mentioned persons.

SIGNATURE OF RESEARCHED PERSON SIGNATURE OF WITNESS SIGNATURE OF PERSON THAT INFORMED SIGNATURE OF PARENT/GUARDIAN THE RESEARCHED PERSON Signed at_____ this ____ day of _____ 20__

APPENDIX C: DATA COLLECTION FORM

Recruitment date: …….....

DEMOGRAPHIC DATA						
Subject number/Code						
Gender	Female			Male		
Age						
HAART regimen						
Duration on HAART	12–24 Months		24–36 Months			
Specific month on HAART						
Do you take your medication every day?	Yes			No		
If no specify why						

MEDICAL HISTORY					
Any history of chronic disease of life style? Yes or No If yes specify:					
Coronary artery disease					
Type 1/2 diabetes		Hypertension		Dyslipidaemia	
Stroke		Metabolic syndrome		Hypercholesterolemia	
Other (specify)					
Breast feeding or Pregnant	Yes		No		
Have you reached menopause	Yes		No		

ANTHROPOMETRY	
Weight (kg)	
Height (m^2)	

LIPID PROFILES		
Cholesterol (mmol/l)		
Triglycerides (mmol/l)		

High-density lipoprotein cholesterol (mmol/l)		
Low-density lipoprotein cholesterol (mmol/l)		

ENDOTHELIAL BIOMARKERS		
P-selectin		
L-selectin		
Soluble Vascular cell adhesion molecule		
Soluble intracellular cell adhesion molecule		
von Willebrand factor		
INFLAMMATORY BIOMARKERS		
C-Reactive Protein		
COAGULANT AND ATHEROSCLEROTIC BIOMARKERS		
Fibrinogen		
D-dimer		

APPENDIX C1: FEEDBACK LETTER

PhD Project:

INVESTIGATING EARLY MARKERS OF CARDIOVASCULAR DISEASE AMONG HIV-POSITIVE PATIENTS IN THE MANKWENG DISTRICT, LIMPOPO PROVINCE

Name: .....

Gender: Male ☐ Female ☐ Date: ……..... Age: ……

Hereby we would like thank you for giving consent by participating in the above mentioned study. The following information regarding your health status was retrieved and the feedback thereof is as follow.

Fasting Plasma Glucose measurement:

High levels of glucose in the blood pose increased risk of complications, such as eye disease, kidney disease, myocardial infarction and strokes and many more. If your glucose levels are above 5.5mmol/L please seek medical advice. The table below serves as an indicator for your fasting blood glucose level.

	Fasting Plasma Glucose (mmol/L)	Participant Glucose value (mmol/L)	Recommendation
Healthy (Normal)	3.9–5.5		No action needed
Pre-diabetic	5.6–7.0		Seek medical advice
Diabetic	7.0 and more		Seek medical advice

Cholesterol measurement:

Measuring of the two different types of cholesterols, LDL and HDL was important. Cholesterol plays an essential role in the prevention of cardiovascular diseases. The

healthy levels of the two different types of cholesterol present in the blood are found in the table below.

Type of cholesterol	Healthy concentration	Participant Value	Recommendation
LDL (bad)	< 3.5 mmol/L		
HDL (good)	> 1.0 mmol/L males		
	> 1.3 mmol/L females		

Low density lipoprotein (LDL), high density lipoprotein (HDL)

If you have any questions, feel free to contact:

Mr S Hanser, Department of Physiology and Environmental Health, University of Limpopo, Q-Block, first floor, Phone Number: 015 268 4189, Email address: sidney.hanser@ul.ac.za

Kind Regards

APPENDIX D: THE LIST OF ALL STANDARD OPERATIONAL PROCEDURES.

General laboratory requirement:

Staff members and postgraduate students

- Individuals should follow the procedural requirements given in this SOP and the relevant legislation.
- Individuals must give their cooperation in all health and safety matters in the research laboratories.

Hazard identification

- A health risk assessment must be conducted in each research laboratory at least once a semester to identify and characterise any hazards present.

Housekeeping

- All passageways and exits must be kept clear of items that may cause occupants of laboratories to trip and fall.
- Keep floors clean and dry to avoid the risk of slips. Provide a warning sign for wet floor areas.
- Any spills of potentially infectious material must be cleaned immediately using an appropriate cleaning product. All laboratory work surfaces must be cleaned with an appropriate disinfectant at the end of the day's work activities
- No samples or reagents may be left on workbenches after procedures have been completed or when occupants have left the laboratory at the end of the day.
- All waste, general and otherwise, must be placed in the specified waste containers for the type of waste.

Training

- All individuals that work in research laboratories must receive training regarding the following before commencing work.

Prohibitions

- No eating, drinking or smoking in the laboratory at any time.
- Food and drink may not be stored in the laboratory.

Accidents

- All accidents must be cleaned up as soon as possible using a suitable cleaning product.
- All accidents or near accidents must be reported to the Laboratory Supervisor and recorded using the DPEH-01 form.

Waste disposal

- A reputable waste contractor must be used to remove hazardous chemical substances and biological hazardous material.
- Waste must be deposited in the correct corresponding waste container.
- The type of waste that may be deposited in each waste container must be clearly indicated on the container.
- Hazardous chemical substances may not be disposed of by pouring it in the sink.
- Different classes of waste hazardous chemical substances such as for example an acid and an alkali, must not be put in the same container.

Working with human blood inside or outside the laboratories:

Training

A. All individuals that work in the laboratory must receive training regarding the following:

- Potential health hazards related to exposure to human blood, blood borne pathogens and other hazardous biological agents.
- Correct use of engineering controls installed in the laboratories
- Correct way to wash hands to remove blood borne pathogens.
- Importance of good housekeeping in laboratories.
- Correct use of the appropriate personal protective equipment such as gloves.
- Safe disposal of waste such as sharps and contaminated material.

- The procedures to be followed in the event of exposure to blood borne pathogens, spillage, injury or any similar emergency situation.
- The duties of persons who might be exposed to hazardous biological agents such as blood borne pathogens in terms of the Occupational Health and Safety Act 85 of 1993.
- The measures that should be taken by the employer to protect an employee against risk of being exposed to biological agents such as blood borne pathogens in terms of the Occupational Health and Safety Act 85 of 1993.

B. Training must be done by a knowledgeable individual that may be an employee of the University of Limpopo or a contracted individual from outside the university.
C. Attendees of the training must sign a document where they acknowledge that they have received the training.
D. All researchers working with human blood that may contain blood borne pathogens must have been vaccinated against Hepatitis B before commencing with work.

Handling and storage of blood samples

A. Human blood samples that may be potentially infectious must be transported in leak-proof, sealed containers that are properly marked and packed. Avoid contamination of the outside surface of the container and make sure the lid is closed tightly.
B. Avoid any hand to mouth or hand to eye contact when working in the laboratory.
C. Procedures using human blood samples must be conducted carefully to prevent the formation of airborne droplets or aerosols.
D. Gloves must be worn for all procedures where there is a possibility of contact with potentially infected human blood samples

Handling of needles and other sharp objects

A. The use of needles and syringes should be restricted as much as possible when working with potentially infectious material.
B. Never recap, bend or break a used needle before disposal.

C. Discard syringes and needles as a unit.
D. Areas where needles and other sharp objects may be present should not be cleaned without hand protection.

Accidents

A. General accidents

- Any accidents or near accidents must be reported to the Laboratory Supervisor or the Departmental SHE representative using form DPEH-01.
- Injuries that require first aid may be treated using the first aid kit that will be placed in the laboratory.

B. Needle prick injuries

- The Needle Prick Injury Policy must be followed (SOP 2015/05).

Personal Protective Equipment (PPE)

- Personal protective equipment, emergency equipment and the first aid kit will be applied wherever necessary.

Waste disposal

A. The users of needles and other sharp instruments or gloves, as well as researcher working with potentially infected human blood or other material are responsible for the disposal of these items.
B. Potentially infectious material must be deposited in the yellow container marked General.
C. Needles and other sharp instruments must be deposited in the yellow container marked Needles. These containers must be available at the point of use.
D. Gloves must be deposited in the yellow container marked gloves.
E. All yellow containers must be emptied and the contents taken away by a reputable waste contractor that is authorised to dispose of biological hazard waste.

Recordkeeping

A. Records of all accidents will be kept using the Accident Reporting form.

B. Results from any risk assessments or exposure monitoring will be kept as records.

A. Records must be kept for a minimum period of 40 years.

Needle prick injury procedure:

Training:

- The Needle Prick Injury Procedure should be discussed with all individual in the Department of Physiology and Environmental Health as part of the general induction to research.

Needle prick injury response:

The exposed individual will be given post-exposure prophylactic treatment as soon as possible in the first six hours by a qualified medical practitioner at the clinic or hospital. This will be accompanied with pre-testing counselling.

APPENDIX E: LETTER OF APPROVAL FROM FACULTY OF SCIENCE AND AGRICULTURE (UNIVERSITY OF LIMPOPO)

07/06/2016

NAME OF STUDENT:	Hanser S
STUDENT NUMBER:	201533352
DEPARTMENT:	Physiology and Environmental Health
SCHOOL:	Molecular and Life Science
QUALIFICATION:	DHSB01

Dear Mr Hanser

FACULTY APPROVAL OF PROPOSAL (PROPOSAL NO.11 OF 2016)

I have pleasure in informing you that your doctoral proposal served at the Faculty Higher Degrees Committee meeting on **07 May 2015** and your title was approved as follows:

"Investigating the effects of HAART on early markers of cardiovascular disease among HIV positive patients in the Mankweng district, Limpopo Province"

Note the following: The study

Ethical Clearance	**Tick One**
Requires no ethical clearance Proceed with the study	
Requires ethical clearance (Human) (TREC) (apply online) Proceed with the study only after receipt of ethical clearance certificate	✓
Requires ethical clearance (Animal) (AREC) Proceed with the study only after receipt of ethical clearance certificate	

Yours faithfully

Prof P Masoko
Secretariat: Faculty Higher Degrees Committee

UNIVERSITY OF LIMPOPO
FACULTY OF SCIENCE
AGRICULTURE
2016 -06- 07
TURFLOOP CAMPUS
PRIVATE BAG X1106
SOVENGA 0727

CC: Dr M van Staden
Prof LJ Mampuru

APPENDIX F: TURFLOOP RESEARCH ETHICS COMMITTEE CLEARANCE CERTIFICATE.

University of Limpopo
Department of Research Administration and Development
Private Bag X1106, Sovenga, 0727, South Africa
Tel: (015) 268 2212, Fax: (015) 268 2306, Email:noko.monene@ul.ac.za

TURFLOOP RESEARCH ETHICS COMMITTEE CLEARANCE CERTIFICATE

MEETING:	**05 July 2016**
PROJECT NUMBER:	**TREC/119/2016: PG**
PROJECT:	
Title:	Investigating the effects of Haart on early markers of cardiovascular disease among HIV-Positive patients in the Mankweng District, Limpopo Province
Researcher:	Mr S Hanser
Supervisor:	Dr M Van Staden
Co-Supervisor:	Dr ELJ Erasmus
	Prof MM Moraba
School:	Molecular and Life Sciences
Degree:	PhD in Physiology and Environmental Health

PROF TAB MASHEGO
CHAIRPERSON: TURFLOOP RESEARCH ETHICS COMMITTEE

The Turfloop Research Ethics Committee (TREC) is registered with the National Health Research Ethics Council, Registration Number: **REC-0310111-031**

Note:
i) **Should any departure be contemplated from the research procedure as approved, the researcher(s) must re-submit the protocol to the committee.**
ii) **The budget for the research will be considered separately from the protocol.**
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES.

Finding solutions for Africa

APPENDIX G: LETTER OF APPROVAL FROM THE DEPARTMENT OF HEALTH (POLOKWANE).

LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH

Enquiries: Stols M.L (015 293 6169) Ref:4/2/2

Hanser S
University of Limpopo

Greetings,

RE: Investigating the effects of HAART on early markers of Cardiovascular Disease among HIV-Positive Patients in the Mankweng District, Limpopo Province

The above matter refers.

1. Permission to conduct the above mentioned study is hereby granted.
2. Kindly be informed that:-
   - Research must be loaded on the NHRD site (http://nhrd.hst.org.za) by the researcher.
   - Further arrangement should be made with the targeted institutions, after consultation with the District Executive Manager.
   - In the course of your study there should be no action that disrupts the services.
   - After completion of the study, it is mandatory that the findings should be submitted to the Department to serve as a resource.
   - The researcher should be prepared to assist in the interpretation and implementation of the study recommendation where possible.
   - The above approval is valid for a 3 year period.
   - If the proposal has been amended, a new approval should be sought from the Department of Health.
   - Kindly note, that the Department can withdraw the approval at any time.

Your cooperation will be highly appreciated.

Head of Department

17/05/17
Date

18 College Street, Polokwane, 0700, Private Bag x9302, POLOKWANE, 0700
Tel: (015) 293 6000, Fax: (015) 293 6211/20 Website: http/www.limpopo.gov.za

The Heartland of Southern Africa - development is about people

APPENDIX H: LETTER OF APPROVAL FROM THE HEALTH CARE COUNCIL (POLOKWANE).

LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH
CAPRICORN DISTRICT

Enq : **Malema DMM**

Tel : **015 290 9266**

From : **Primary Health Care**

Date : **29 June 2017**

To : **Hanser S**
University of Limpopo

Subject : **Investigating the effects of HAART on early markers of Cardiovascular Disease among HIV-Positive patients in Mankweng, Limpopo Province**

The above matter refers

1. Permission to conduct the above mentioned study is hereby granted.
2. Kindly be informed that :
    - In the course of your study there should be no action that disrupts the services.
    - After completion of the study, it is mandatory that the findings should be submitted to the Department to serve as a resource.
    - The researcher should be prepared to assist in the interpretation and implementation of the study recommendation where possible.
    - Kindly note, that the Department can withdraw the approval at any time.

Your cooperation will be highly appreciated.

Thathen
PP Acting Director PHC

2017/06/29
Date

APPENDIX I: LIST OF REFERENCE RANGES

International Classification of adult underweight, overweight and obesity according to BMI*

Classification	BMI (kg/m^2): Principal Cut-Off Points
Underweight	<18.5
Normal healthy weight	18.5–24.9
Class 1 obesity, overweight	25.0–29.9
Class 2 obesity, obesity	30.0–39.9
Class 3 obesity, morbid obesity	>40.0

* Adapted from (WHO 1995; 2000; 2004).

WHO guidelines for defining and diagnose diabetes mellitus and intermediate hyperglycaemia

Classification	Reference
Normal	3.3–5.9
Pre-diabetic	6.0–6.9
Diabetic	≥ 7.0

Adopted from World Health Organisation guidelines (2006)

Classification of Dyslipidaemia in terms of LDL-C, TC, HDL-C and TG.

Classification	Reference (mmol/l)
LDL-C	< 3.0
TC	< 5.0
TG	< 1.7
HDL:	
Female	> 1.3
Male	> 1.1

Adapted from the South African Dyslipidaemia Guideline Consensus Statement (2014)

Expected reference ranges of all biomarkers of interest.

Analyte	Reference range (ng/ml)
D-dimmer	3.2–6.6
sICAM-1	1.2–2.4
P-selectin	2.8–5.8
sVCAM-1	1.7–3.5
CRP	0.17–0.35
Fibrinogen	0.34–0.71
L-selectin	0.05–0.11
VWF	3.6–7.4