Investigating the effects of first line and second line antiretroviral drugs on HIV exposed endothelial function - A clinical study, supported by a mechanistic in-vitro approach

by
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Declaration

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Abstract

Background: There is an interaction between HIV, antiretroviral treatment (ART) and endothelial dysfunction; furthermore, HIV-infected individuals (± ART) show an increased incidence of cardiovascular risk factors. However, the evidence stems mainly from studies in developed countries with a paucity of data on these interactions in the South African context.

Aims: To investigate the effects of first line and second line ART on HIV exposed vascular endothelial function, in a clinical and *in vitro* setting.

Methods: In the clinical study**,** participants were recruited in Worcester and allocated to one of four study groups: HIV-negative, HIV-positive ART naïve, HIV-positive first line ART and HIV-positive second line ART. Data were collected via health questionnaires, anthropometric assessments, blood pressure measurements, brachial artery flow mediated dilatation (FMD) and blood chemistry analyses (C-reactive protein (CRP), fasting glucose, HbA1c, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, as well as CD4 count and viral load in HIV-infected individuals). For the *in vitro* sub-study, a conditioned growth medium was developed containing HIV-related proteins in which aortic endothelial cells (AECs) were incubated. Additionally, AECs were treated with first and second ART drugs. End-points were nitric oxide (NO) production, cell viability and ROS production measured by flow cytometric analysis.

Results: There were no inter-group differences with regard to FMD. The median BMI and waist circumference measurements were lower in the HIV-positive groups versus HIV-negative ($p < 0.05$). Median total cholesterol levels were lower ($p < 0.05$) in the HIV-positive ART groups versus HIV-negative, and higher in the HIV-positive first line ART group versus the ART naïve group ($p< 0.05$). Furthermore, mean LDL-cholesterol levels were lower in all HIV-positive groups versus HIV-negative ($p< 0.05$). Median HBA1C% values were lower in the HIV-positive second line ART versus ART-naïve group ($p < 0.05$). Regression analyses showed that smoking in first line ART, and CRP and CD4 levels in second line ART were negatively associated with FMD%. In the *in vitro* sub-study, the HIV-1 gp160 protein was identified in the HIV-conditioned medium. No effects were observed in HIV-conditioned medium treated AECs. In the first and second line ART dose-response investigations, it was found that double first line drug concentration and normal second line drug concentration exerted no harmful effects on AECs.

Discussion and Conclusion: Clinical data suggested that the cardiovascular risk profile appeared to be more favourable in HIV-positive groups versus HIV-negative. There were no inter-group differences in terms of endothelial function (FMD). CD4 and CRP, as well as female gender were

independent predictors of vascular endothelial function in the HIV-positive second line ART group. Furthermore, smoking was found to be a negative independent predictor of endothelial function in the HIV-positive first line ART group. The *in vitro* findings showed that the HIV-conditioned medium protocol successfully resulted in the expression of the HIV-1 gp160 protein; however, the conditioned medium failed to induce injury. In the ART dose-response investigations, the double drug concentration for first line ART and normal drug concentration for second line ART, could be considered a safe concentration to use in future investigations.

Abstrak

Agtergrond: Daar bestaan ‘n interaksie tussen MIV, antiretrovirale terapie (ART) en endoteeldisfunksie; verder toon MIV-geïnfekteerde persone (±ART) ‘n hoër insidensie van kardiovaskulêre risikofaktore. Hierdie verwantskappe is hoofsaaklik bepaal in populasies van ontwikkelde lande en daar is ‘n tekort aan data in die Suid-Afrikaanse konteks.

Doelwitte: Om die effekte van eerste en tweede linie ART op MIV-blootgestelde vaskulêre endoteelfunksie in ‘n kliniese en *in vitro* omgewing te ondersoek.

Metodes: In die kliniese studie was deelnemers in Worcester gewerf en in een van vier studiegroepe ingedeel: MIV-negatief, MIV-positief sonder ART, MIV-positief eerste linie ART en MIV-positief tweede linie ART. Data is ingesamel via vraelyste, antropometriese metings, bloeddruk bepalings, bragiale arterie vloei-gemedieerde dilatasie (FMD) en bloed biochemiese analises (C-reaktiewe proteïen (CRP), vastende glukose, HbA1C, totale cholesterol, LDL-cholesterol, HDL-cholesterol, trigliseriede, asook CD4 telling en virale lading in MIV-geïnfekteerde individue). Vir die *in vitro* sub-studie was ‘n gekondisioneerde groeimedium ontwikkel wat MIV-verwante proteïene bevat waarin aorta endoteelselle (AECs) geïnkubeer was. Verder was AECs met eerste en tweede linie ART middels behandel. Eindpunte was stikstofoksied (NO) produksie, sellewensvatbaarheid en ROS produksie soos gemeet met vloeisitometriese analise.

Resultate: Daar was geen inter-groep verskille t.o.v. FMD nie. Die mediaan BMI en middellyf omtrek metings was laer in die MIV-positiewe groepe versus MIV-negatief ($p<0.05$). Mediaan totale cholesterol vlakke was laer ($p<0.05$) in die MIV-positiewe ART groepe versus MIV-negatief, en hoër in die MIV-positief eerste linie ART groep versus geen ART ($p<0.05$). Verder was die gemiddelde LDL-cholesterol vlakke laer in alle MIV-positiewe groepe versus die MIV-negatiewe groep ($p<0.05$). Mediaan HbA1c% waardes was laer in die MIV-positiewe tweede linie ART groep versus MIV-positief sonder ART ($p<0.05$). Regressie analises het getoon dat rook in eerste linie ART, en CRP en CD4 vlakke in tweede linie ART negatief met FMD% geassosieer het. In die *in vitro* sub-studie was die MIV-1 gp160 proteïen geïdentifiseer in die MIV-gekondisioneerde medium. Geen effekte was in AECs wat aan MIV-gekondisioneerde medium blootgestel was, waargeneem nie. In die eerste en tweede linie ART dosis-respons eksperimente, het die dubbel dosis eerste linie ART en normale dosis tweede linie ART geen nadelige effekte op die AECs uitgeoefen nie.

Bespreking en Slotsom: Die kliniese data toon ‘n meer gunstige kardiovaskulêre risiko profiel in die MIV-positiewe groepe versus MIV-negatiewe groep. Daar was geen inter-groep verskille t.o.v. endoteelfunksie (FMD) nie. CD4 en CRP, sowel as vroulike geslag was onafhanklike voorspellers

van vaskulêre endoteelfunksie in die MIV-positiewe tweede linie ART groep. Verder was rook ‘n negatiewe onafhanklike voorspeller van endoteelfunksie in die MIV-positiewe eerste linie ART groep. Die *in vitro* data het getoon dat die MIV-gekondisioneerde protokol op suksesvolle wyse tot die uitdrukking van die MIV-1 gp160 proteïen gelei het, hoewel die gekondisioneerde medium nie daarin kon slaag om skade te veroorsaak nie. In die ART dosis-respons eksperimente is aangetoon dat die dubbel dosis eerste linie ART en normale dosis tweede linie ART ‘n veilige konsentrasie is wat in toekomstige studies gebruik kan word.

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Dedicated to my Late Grandmother (Dadi),

Shirin Amir Ali Charania

(1945- 2016)

You are always in my heart.

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List of Abbreviations

ABC	Abacavir
AC	Absolute control
Ach	Acetylcholine
ADC	Analog-to-Digital Conversion
ADP	Adenosine diphosphate
AECs	Aortic endothelial cells
AIDS	Acquired immunodeficiency syndrome
AMI	Acute myocardial infarction
Ang II	Angiotensin II
ANOVA `	Analysis of Variance
ART	Antiretroviral therapy
ATV	Atazanavir
AZT	Zidovudine
BH4	tetrahydrobioptrerin
BMI	Body mass index
BP	Blood pressure
cART	Combination ART
Ca^{2+}	Calcium
CCR	Co-Receptors
CD4	Cluster of differentiation 4
cGMP	Cyclic guanosine monophosphate
CI	Confidence interval
CIMT	Carotid intima media thickness
CM	Conditioned medium
COX	Cyclooxygenase

COX-1	Cyclooxygenase-1
CPGR	Centre for proteomics and genomics research
CRABP1	Cytoplasmic retinoic acid-binding protein type 1
CRP	C-reactive protein
CVD	Cardiovascular disease
CVS	Cardiovascular system
DAF-2/DA	Diaminofluorescein-2/diacetate
DCF	Dichlorofluorescein
ddC	Zalcitabine
DdI	Didanosine
DEA/NO	Diethylamine NONOate
DHFR	Dihydrofolate reductase
DLV	Delavirdine
DBP	Diastolic blood pressure
DMEM	Dulbecco's modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DRV	Darunavir
DTG	Dolutagravil
d4T	Stavudine
ECE	Endothelin converting enzyme
ECs	Endothelial cells
ED	Endothelial dysfunction
EDCF	Endothelial-derived constricting factors
EDHF	Endothelial-derived hyperpolarization factor
EDRF	Endothelium-derived relaxing factors
EFV	Efavirenz
EGM-2	Endothelial cell growth medium

EMNQ	2,3-Dimethoxy-1,4-naphthoquinone
ENF	Enfuvirtide
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
ETR	Etravirine
EXG	Elvitegravir
FBS	Foetal bovine serum
FDA	Food and drug administration
FDC	Fixed dose combination
FL1-H	Flow channel 1
FL2-H	Flow channel 2
FL3-H	Flow channel 3
FMD	Flow mediated dilatation
FSC	Forward-scattered light
FVP	Fosamprenavir
FTC	Emtricitabine
GCP	Good clinical practice
GLUT4	Glucose transporter type 4
GTPCH	Guanosine triphosphate cyclohydrolase
HAART	Highly active ART
HbA1c	Glycated hemoglobin
HC	Hip circumference
HDL	High density lipoprotein
HDL-C	High-density lipoprotein cholesterol
HIV	Human immunodeficiency virus
HIV-1	HIV- type 1
HIV-2	HIV- type 2

HPCSA	Health Professions Council of South Africa
HREC	Health Research Ethics Committee
HUVECs	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule 1
IDV	Indinavir
IHD	Ischaemic Heart Disease
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
InSTIs	Integrase strand transfer inhibitors
LDL	Low density lipoproteins
LDL-C	Low-density lipoprotein cholesterol
LPV/r	Lopinavir/ritonavir
MCFS	Macrophage colony stimulating factor
MCM-1	Macrophage-chemotactic protein-1
MMP	Matrix metalloproteinases
MCP-1	Monocyte chemotactic protein 1
MVC	Maraviroc
NF-κB	Nuclear factor kappa-B
NHLS	National Health Laboratory Services
NM	Normal AEC medium
NFV	Nelfinavir
NO	Nitric oxide
NOS	Nitric oxide synthase
NRTIs	Nucleotide reverse transcriptase inhibitors
NVP	Nevirapine
PG	Prostaglandin

PI	Protease inhibitors
PIT	Pathological intimal thickening
PBS	Phosphate buffer saline
PRF	Pulse repetition frequency
RAL	Raltegravir
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
RPV	Rilpivirin
RT	Reverse transcriptase
RTV	Ritonavir
SA	South Africa
SBP	Systolic blood pressure
SQV	Saquinavir
SSA	Sub-Saharan Africa
SSC	Side-scattered light
SRA	Scavenger Receptor A
SREBP	Sterol regulatory element binding proteins
TC	Total cholesterol
TG	Triglyceride
TDF	Tenofovir DF
TNF-α	Tumour necrosis factor-α
TP	Thromboxane prostanoid
TPV	Tipranavir
TXA2	Thromboxane A
VCAM-1	Vascular cell-adhesion molecule-1
VSMC	Vascular smooth muscle cell
vWF	Von Willebrand factor

WC	Waist circumference
WHO	World Health Organization
WHR	Waist-hip ratio
2-ME	2-mercaptothanol
3TC	Lamivudine

List of Symbols

%	Percentage
°C	Degrees Celsius
G	Gram
kDa	Kilo Dalton
kg	Kilogram
L	Litre
m	Meter
M	Molar
mg	Milligram
min	Minute
ml	millilitre
mM	Milimolar
mmHg	Pressure
mW	Miliwatt
ng	Nanogram
nM	Nanomolar
v	Volume
α	Alpha
β	Beta
µ	Micro
µl	Microliter
µM	Micromolar

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Chapter 1 : Literature Review

1.1 Cardiovascular Disease

1.1.1 Introduction and Epidemiology

Cardiovascular disease (CVD) represents diseases of the heart, blood vessels, and vascular diseases of the brain (Mendis, Puska et al. 2011). There are several underlying mechanisms of CVDs. For the purpose of the current study, the focus will be on CVDs due to atherosclerosis.

Currently, CVD is the most common and the leading cause of death worldwide (Lin, Zhang et al. 2013, Townsend, Nichols et al. 2015). According to the world health organization (WHO), CVD mortality will continue to increase in the decades ahead. In 2012, an estimated 17.5 million people died from CVDs, which represents 31% of all deaths globally. Furthermore, of these deaths, an estimated 7.4 million were as a result of coronary artery disease and 6.5 million were due to stroke (WHO 2010, WHO 2015). However, interestingly, for the past two decades, there has been a decline in deaths from CVD in high income countries contrasted by a rapid increase in low- and middle income countries (Mendis, Puska et al. 2011, Cappuccio & Miller 2016, Mendis 2014). In fact, 80% of CVD-related deaths occur in low-and middle income countries (Cappuccio & Miller 2016).

As with other low- and middle income regions, sub-Saharan Africa (SSA) also has a rising epidemic of non-communicable diseases, including CVD. CVD has evolved into a major health concern due to its rising mortality and morbidity rates in SSA (Dalal, Beunza et al. 2011). In South Africa (SA), particularly, the WHO estimates the burden of NCD to be two to three times higher than in developed countries, with the poor in the urban settings being increasingly affected disproportionately (Mayosi, Flisher et al. 2009). It has been further projected that there will be a 41% increase in cardiovascular related deaths between the year 2000 to 2030 (Mpe 2010).

1.1.2 Atherosclerosis pathophysiology

The underlying process in the blood vessels that results in ischaemic heart disease (IHD) and cerebrovascular disease (stroke) is known as atherosclerosis. It accounts for a large proportion of CVDs. In fact, IHD has the largest contribution to CVD mortality worldwide. The pathological process of atherosclerosis is complex and involves the development of an arterial plaque due to the cholesterol rich fraction of the blood, low density lipoproteins (LDL) (Naseem 2005). Plaque development is a long-term process over several years in the walls of the arteries. The process

involves all the structural elements of the arterial wall, including circulating cells such as platelets, leukocytes and inflammatory cells such as monocytes and macrophages. Briefly, in atherosclerosis, cholesterol rich fractions are continuously deposited inside the lumen of the arteries which progressively lead to narrowing of the lumen diameter and eventually restricting arterial blood flow (Mendis, Puska et al. 2011, Naseem 2005, Sakakura, Nakano et al. 2013). Furthermore, there are many factors that promote the process of atherosclerosis and CVDs which are described in the section below.

More precisely, the initiation of atherosclerosis, is when LDL passively diffuses into the arterial wall where it is trapped as a result of its interaction with apolipoprotein B100 (protein moiety of LDL) and matrix proteoglycans. Through prolonged exposure to reactive oxygen species (ROS) and reactive nitrogen species (which are present in an attempt to repair localized damage), LDL is oxidatively modified. This oxidised LDL leads to a proinflammatory response in the surrounding cells and stimulates the production of chemokines such as chemotactic protein- 1 and growth factors (which attract monocytes to the area). (Barbaro 2003, Naseem 2005). In addition, the oxidised LDL leads to the expression of adhesion molecules such as P-selectin, vascular adhesion molecule-1 and intercellular adhesion molecule-1 on the endothelium's surface (Naseem 2005, Barbaro 2003). This enables the entry of recruited monocytes into the arterial wall (Naseem 2005). Eventually, this process leads to the formation of lipid-laden foam cells and a necrotic core of oxidized lipids, which is highly thrombotic in nature (Naseem 2005). Ultimately, the highly thrombotic core of the plaque ruptures to form platelet-rich thrombi (Naseem 2005).

Atherosclerosis is triggered by hypercholesterolaemia (**refer to figure 1.1** for the process atherosclerotic changes in the arterial wall). Expression of vascular cell-adhesion molecule-1 (VCAM-1), and selectins facilitates the monocyte rolling and attachment to endothelium. The secretion of macrophage-chemotactic protein 1 (MCP-1) is elicited by alterations and oxidation of LDL. Monocytes present in the arterial intima mature into macrophages which express scavenger receptors such as Scavenger Receptor A (SRA) and CD36. These receptors enable the uptake of the altered and oxidised LDL and convert them into foamy macrophages which are rich in cholesterol esters and free fatty acids. Macrophages increase due to the present MCP-1 and macrophage colony stimulating factor (MCSF). T cells undergo antigen specific activation, after which they enter the intima and amplify the inflammatory response and sustain it by secreting interferon-γ which send signals to aid in the process. The presence of reactive oxygen species allows an enhanced release of matrix metalloproteinases (MMPs) by the macrophages. This facilitates the breakdown of collagen to allow migration of cells within the plaque. Foamy macrophages (foam cells) infiltrate the

pathological intimal thickening (PIT) lesion and the lipid pool areas that are formed by smooth muscle cell apoptosis, and proteoglycan aggregation. Accumulation of oxidised LDL and macrophage infiltration elicits the conversion of the lipid pool to a necrotic core. Once in the necrotic core, oxidised LDL and macrophage infiltration further elicits macrophage death and defective efferocytosis (removal of apoptotic and necrotic cells by phagocytic cells). (Sakakura, Nakano et al. 2013).

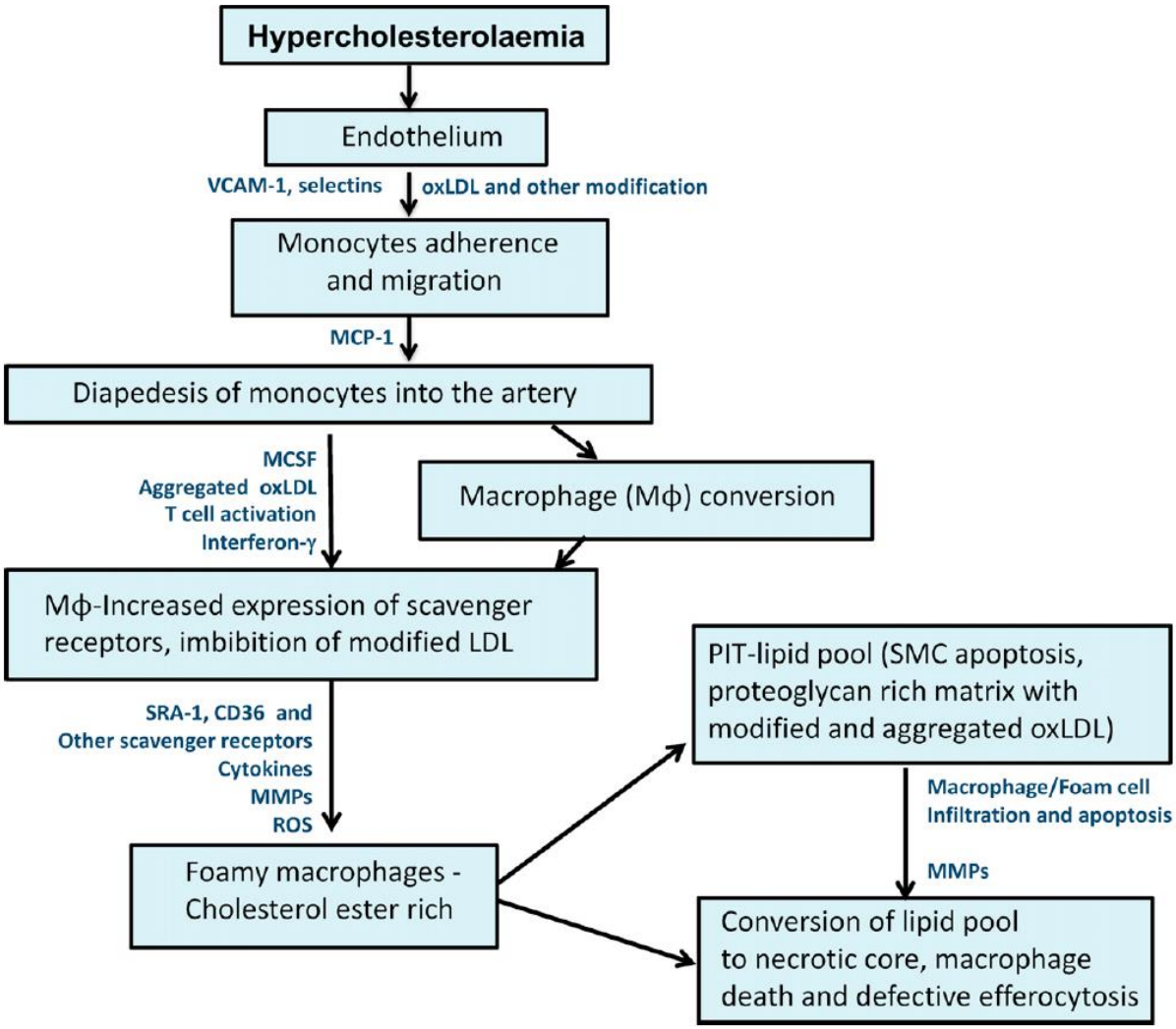

Figure 1.1: Sequence of events that lead to pro-atherosclerotic changes in the arterial wall (Sakakura, Nakano et al. 2013).

1.1.3 Cardiovascular Risk factors

A risk factor is defined as a variable that increases the likelihood of developing a disease or infection (Fedele, Bruno et al. 2011). The Framingham Heart Study introduced the concept of risk factors for future cardiovascular diseases (Kannel, Dawber et al. 1961, Mensah 2013). These factors are predominantly the same in SSA in comparison to the rest of the world. The risk factors include high blood pressure (BP), tobacco use, including exposure to second hand smoke, harmful use of alcohol, high total blood cholesterol, high fasting plasma glucose, high body mass index (BMI), high intake of dietary sodium, low dietary intake of fruits and vegetables, low physical activity, and household and ambient air pollution (Mensah 2013). These mentioned risk factors explain the majority of the population burden of CVD in SSA (Mensah 2013). Furthermore, the risk factors can be divided into modifiable and non-modifiable risk factors. **Refer to table 1.1**.

Table 1.1: *Modifiable and non-modifiable risk factors (Adapted from Fedele, Bruno et al. 2011, Mensah 2013).*

Modifiable Risk factors	**Non-modifiable Risk factors**
Lack of exercise	Age
Visceral obesity	Sex
Left ventricular hypertrophy	Ethnic origin
Tobacco use, smoking, second hand smoke	Family history of CVD
High blood pressure	
High LDL cholesterol	
Low HDL cholesterol	
Diabetes and glucose intolerance.	
Harmful use of alcohol	
High intake of dietary salt	
BMI	
Low dietary intake of fruit and vegetables	

Abbreviations: LDL: Low-Density Lipoprotein; HDL: High-Density Lipoproteins; BMI: Body mass index; CVD: Cardiovascular disease.

1.2 The Vascular Endothelium and its function

The endothelium represents the inner most layer of all blood vessels. It consists of a continuous monolayer of endothelial cells (ECs). The ECs are known to represent a total surface area of approximately 4000 to 7000m^2 (Flammer & Luscher , 2011). The endothelium forms an interface between the circulating blood in the lumen and the rest of the vessel wall, thereby playing a critical role in vascular homeostasis (Van den Oever, Raterman et al. 2010). It actively controls the vascular tone and permeability, and regulates the exchange of molecules in response to environmental and molecular signals (Flammer & Luscher , 2011). It essentially maintains the balance between coagulation and fibrinolysis, inflammatory activity, and cell proliferation. A healthy endothelium not

only is crucial in preventing thrombotic events but further exerts anticoagulant, antiplatelet, and fibrinolytic properties (Davignon, Ganz 2004, Flammer & Luscher , 2011) **Refer to table 1.2** for an overview of the effects of healthy endothelium.

Table 1.2: *List of favourable and atheroprotective effects of healthy endothelium (Adapted from Bonetti, Lerman et al. 2003)*

Favourable and atheroprotective effects of the healthy endothelium
Promotion of vasodilation
Antioxidant effects
Anti-inflammatory effects
Inhibition of leukocyte adhesion and migration
Inhibition of smooth muscle cell proliferation and migration
Inhibition of platelet aggregation and adhesion
Anticoagulant effects
Profibrinolytic effects

The highly complex endothelium has mechanosensors in its cells which give them the ability to sense mechanical stimuli such as pressure and shear stress, as well as receptors for hormonal and other vasoactive substances; to which the endothelium responds to by releasing agents that regulate vasomotor function, trigger inflammatory processes, and affect homeostasis (Endemann & Schiffrin 2004, Limaye & Vadas 2007). The endothelium releases endothelium-derived relaxing factors (EDRF) such as nitric oxide (NO), bradykinin, prostacyclin (PGI2) and endothelial-derived hyperpolarisation factor (EDHF), mostly in response to an increase in intracellular calcium. The endothelium also produces many endothelial-derived constricting factors (EDCF) such as endothelin-1 (ET-1), thromboxane A (TXA2) and angiotensin 11 (Mudau, Genis et al. 2012, Flammer & Luscher 2011).

1.2.1 The endothelium-derived factors and their role in endothelial function

As discussed above, the metabolically active endothelium plays a vital role in maintaining vascular homeostasis by releasing a range of vasoactive factors that can either dilate or constrict the blood vessels in response to a stimulus (Mudau, Genis et al. 2012).

1.2.1.1 Endothelium-derived relaxing factors (EDRFs)

The vasodilatory state of the endothelium is maintained by EDRFs such as nitric oxide (NO), bradykinin, prostacyclin (PGI2) and endothelial-derived hyperpolarisation factors (EDHF).

1.2.1.1.1 Nitric oxide (NO)

The most prevalent and important of the endothelium derived relaxing factors is NO (Hall & Gyton 2015). NO, initially loosely termed EDRF, was discovered and characterised in 1987 (Ignarro, Buga et al. 1987). This molecule was shown to relax vascular smooth muscle cells (Flammer & Luscher 2011). NO is a simple diatomic gas and a free radical which makes it highly reactive in signalling pathways and easily transportable between tissues and cells (Strijdom, Chamane et al. 2009). It is produced in endothelial cells by a family of enzymes called NO synthases (NOS) (Fitridge &Thompson 2011). NOS can be found in many tissues throughout the body, especially in the nervous and cardiovascular system. (Strijdom, Chamane et al. 2009) The NOS family has 3 isoforms, namely neuronal NOS (nNOS, NOS-1), inducible NOS (iNOS, NOS-2) and endothelial NOS (eNOS, NOS-3) (Mudau, Genis et al. 2012). Physiologically, eNOS and nNOS are known to be calcium-dependent. However, eNOS can also be calcium independent and is known to be the primary enzyme responsible for the generation of NO in ECs under physiological conditions (Mudau, Genis et al. 2012). eNOS specifically, is the endothelial isoform that catalyses NO biosynthesis via a two-step oxidation of L arginine to L-citrulline (Andrew & Mayer 1999) (**refer to figure 1.1**).

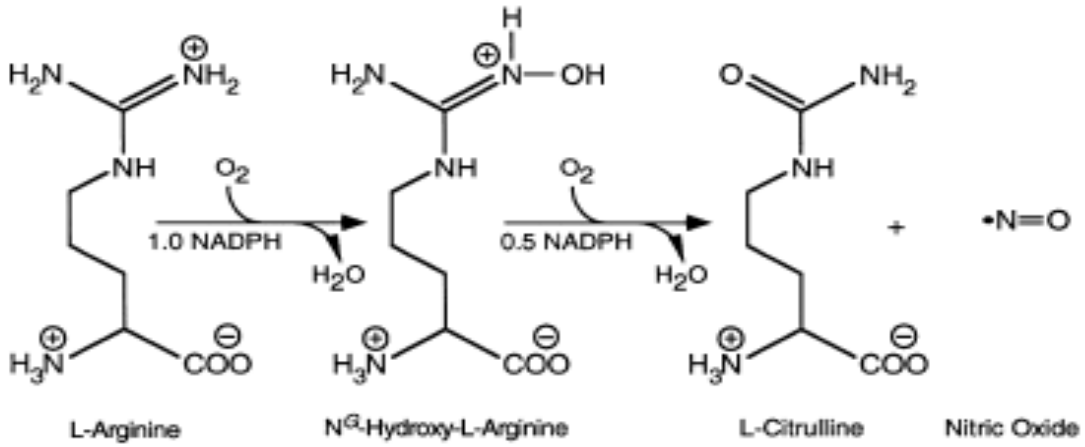

Figure 1.2: NO synthesis via the NOS enzyme (Andrew, Mayer 1999).

The release of NO is mainly in response to shear stress, the primary activator of eNOS. Furthermore, circulating hormones (catecholamines, vasopressin, aldosterone), plasma constituents (thrombin, sphingosine-1-phosphate), platelet products (serotonin, adenosine diphosphate [ADP]), and autacoids (histamine, bradykinin, prostaglandin) can also elicit the release of NO (Lüscher & Vanhoutte, 1990; Vanhoutte et al., 2009; Michel & Vanhoutte 2010). Once NO is synthesized, it diffuses towards the luminal surface of the endothelium and the underlying vascular smooth muscle cells and activates guanylate cyclase which produces the second messenger, cyclic guanosine monophosphate (cGMP). cGMP activates two specific cGMP-dependent protein kinases (PKGI and PKG II) (Gewaltig & Kojda 2002). Of the two, PKGI is the primary kinase that mediates vasodilation and inhibition of platelet aggregation (Flammer & Luscher 2011, Gewaltig & Kojda 2002, Deanfield, Halcox et al. 2007).

The most important role of NO is that it promotes a vasodilatory effect on the vasculature (Endemann 2004, Strijdom, Chamane et al., 2009). It also inhibits smooth muscle cell proliferation and prevents platelet adhesion and aggregation as well as leukocyte adhesion and migration into the arterial wall (Flammer & Luscher 2011). Therefore, NO not only acts as a vasodilator but also has anti-inflammatory and anti-thrombotic effects on the vasculature and thus is a key role player in maintaining vascular homeostasis and endothelial function. (Endemann 2004, Strijdom, Chamane et al., 2009). **Refer to figure 1.3**.

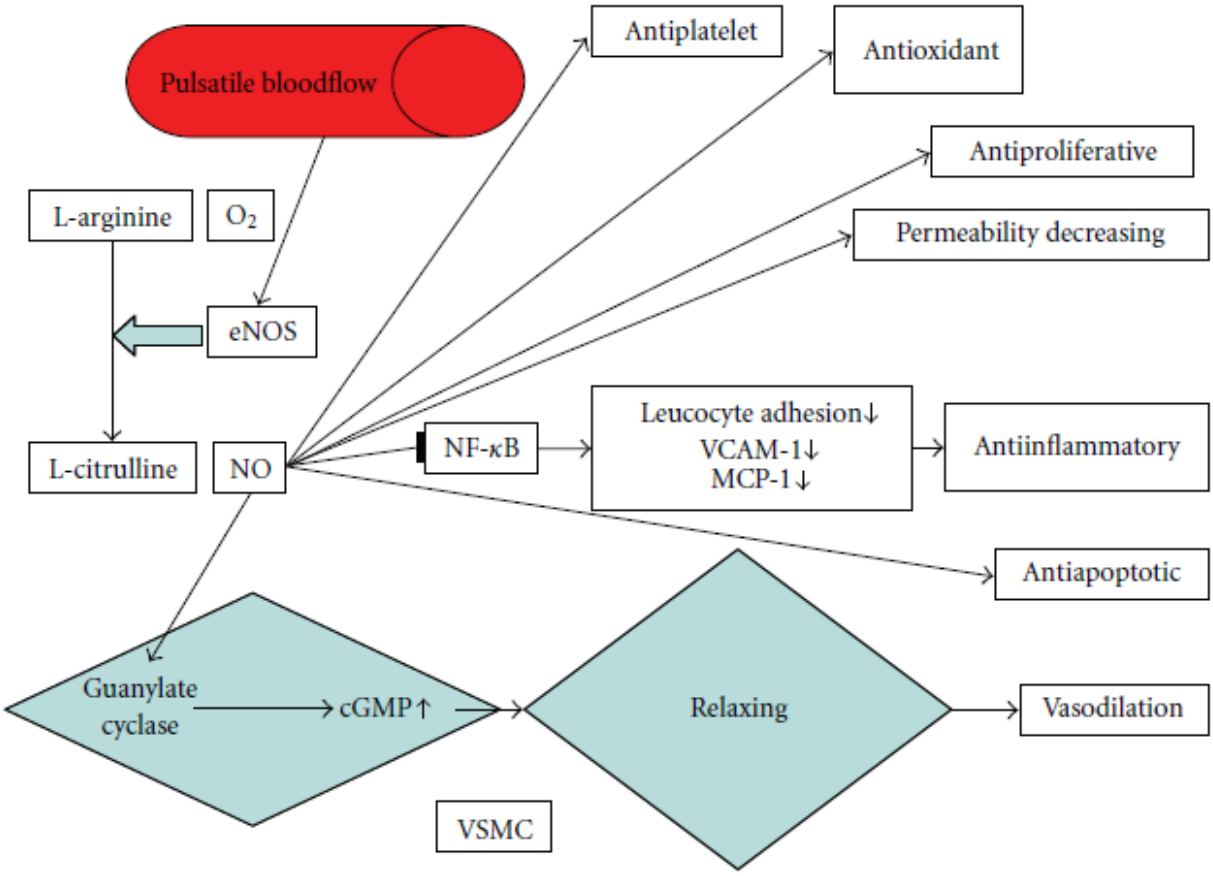

Figure 1.3: Synthesis and properties of NO as an important factor in endothelial function (Van den Oever, Raterman et al. 2010).

Furthermore, NO has several other effects in the cardiovascular system. In the myocardium it is known to be anti-hypertrophic, anti-apoptotic, and cardioprotective against ischaemic injury. In addition, NO is found to play a role in cardiac cell contractility, generation and proliferation. In contrast, excessive amounts of NO in cardiomyocytes are found to be harmful, pro-apoptotic and pro-necrotic (Strijdom, Chamane et al., 2009).

1.2.1.1.2 Prostacyclin (PGI2)

Prostacyclin (PGI2) is an eicosanoid which is synthesised by cyclooxygenase-1 (COX-1) from arachidonic acid and increases cAMP in smooth muscle cells as well as in platelets (Flammer & Luscher 2011). Unlike NO, which is continuously released by agonists, PGI2 is released only in a transient manner. It is partly released in response to shear stress but rather produced when there are disturbances in the endothelial function. Upon release it binds to a specific receptor on platelets and vascular smooth muscle cells to limit vasoconstriction (Fitridge & Thompson 2011). PGI2 aids in the

release of NO by endothelial cells and vice versa, the action of PGI2 in the vascular smooth muscle is also potentiated by NO, which prolongs its half-life (Flammer & Luscher 2011).

1.2.1.1.3 Endothelium-derived hyperpolarisation factors (EDHF)

Non- NO and non- prostaglandin meditated endothelium dependent vasodilation has been partly attributed to EDHF (Ozkor & Quyyumi 2011). These factors are mostly site and species specific and are ultimately responsible for vascular smooth muscle hyperpolarisation and eventually vasodilation (Ozkor & Quyyumi 2011, Taddei, Ghiadoni et al. 1999). Studies suggest that the EDRFs response persists and increases when the vessel size decreases, and in presence of combined inhibition of NO and PG12 (Ozkor & Quyyumi 2011, Flammer & Luscher , 2011, Taddei, Ghiadoni et al. 1999). These factors are also thought to play a compensatory role in endothelium dependent vasodilation during reduced NO availability (Ozkor & Quyyumi 2011, Flammer & Luscher , 2011, Taddei, Ghiadoni et al. 1999).

EDHFs mainly act by increasing potassium (K^+) conductance which subsequently results in the propagation of depolarization of vascular smooth muscle cells and relaxation. Acetylcholine is known to be responsible for hyperpolarization of vascular smooth muscle cells of healthy blood vessels. The action of hyperpolarization is simulated by certain K^+ agonists which are unaffected by inhibitors of nitric oxide synthase or cyclooxygenase (Ozkor & Quyyumi 2011). EDHFs allow smooth muscle cell K^+ channels to open, permitting K^+ efflux along its chemical gradient, resulting in membrane hyperpolarization (Ozkor & Quyyumi 2011). There are many identified EDHFs. In 2011, hydrogen sulphide was recognized as a major EDHF that acts by activating ATP-sensitive, intermediate conductance and small conductance potassium channels through cysteine S-sulfhydration (Mustafa, Sikka et al. 2011). Other EDHFs include K^+, cytochrome P450 metabolites (epoxyeicosatrienoic acids- arachidonic acids) and hydrogen peroxide (also activates calcium dependent potassium channels) (Ozkor & Quyyumi 2011). Furthermore, lipoxygenase products, NO, cyclic adenosine monophosphate, C type natriuretic peptide, and electrical coupling through myoendothelial gap junctions are also identified as EDHF. (Flammer & Luscher 2011, Mustafa, Sikka et al. 2011, Ozkor & Quyyumi 2011).

1.2.1.1.4 Interactions between NO, PG12 and EDHF

NO, PGI2 and EDHFs are the three main mediators of endothelium-dependent vasodilation. They do not appear to be mutually exclusive, in fact, they act in a synergistically complex way to maintain a healthy vasculature (Ozkor & Quyyumi 2011) **(Refer to figure 1.4)**. NO dominates in the conduit arteries, whereas EDHFs appear to contribute more in the resistance vessels of the microcirculations (Shimokawa, Yasutake et al. 1996). Studies have shown that NO could inhibit EDHF while further research demonstrates that EDHF responds once NO and PGI2 production is inhibited (Ozkor & Quyyumi 2011).

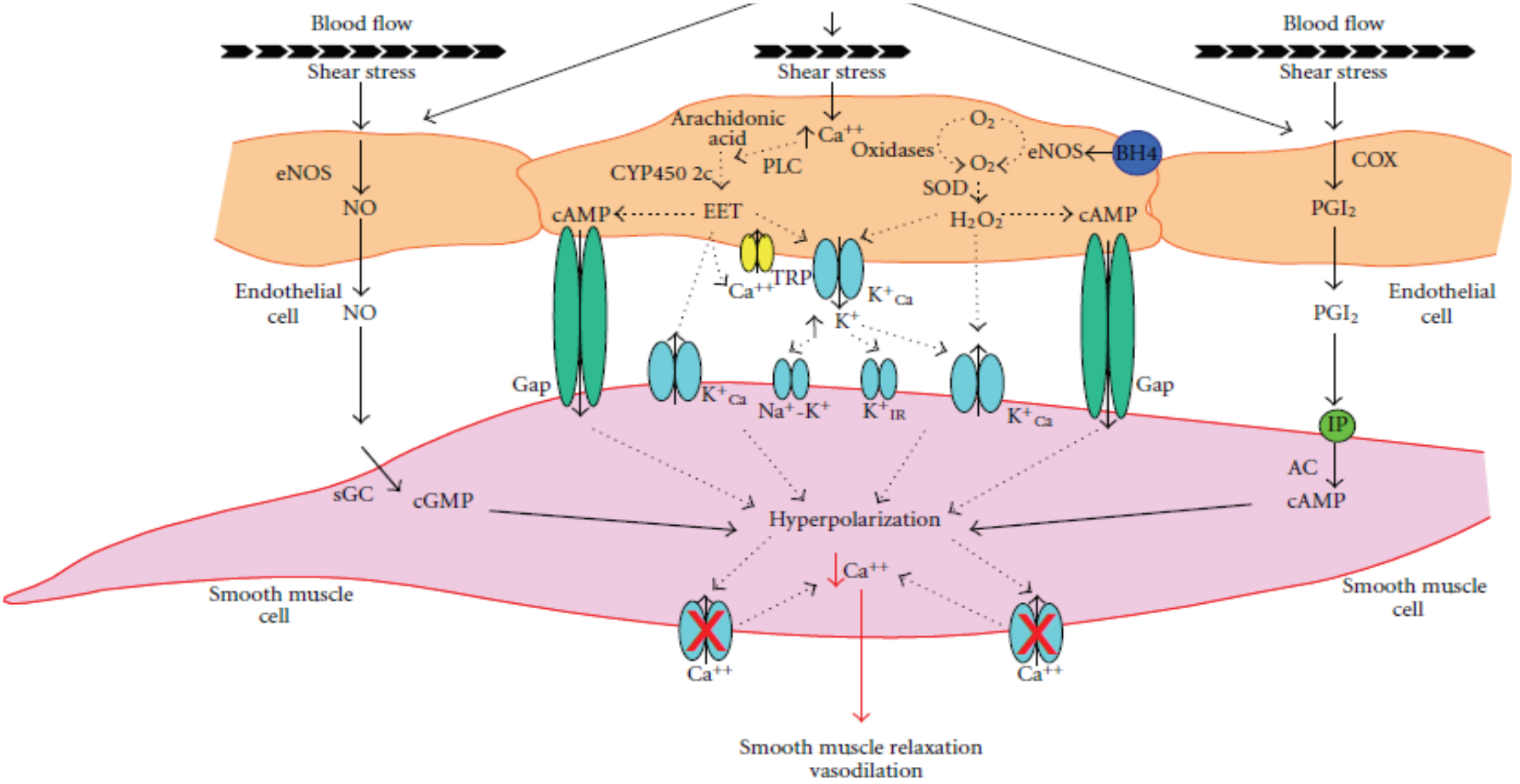

Figure 1.4: Mechanisms for endothelial cell mediated relaxation. Agonist (bradykinin/acetylcholine/substance P) or shear stress increases the activity of endothelial NO synthase (eNOS) and cyclooxygenase (COX), providing nitric oxide (NO) and prostacyclin(PGI2) (Ozkor & Quyyumi 2011)

1.2.1.2 Endothelium-derived constricting factors (EDCFs)

The vasoconstrictory state is maintained by EDCFs such as endothelin-1 (ET-1), thromboxane A (TXA2) and angiotensin 11.

1.2.1.2.1 Endothelin 1 (ET-1)

ET-1 is a potent vasoconstrictor which was discovered a few years after NO. The ETs belong to a family of 21 amino acid peptides, of which there are three isoforms (ET-1, ET-2, and ET-3) (Fitridge & Thompson 2011). These isoforms are basically converted by the endothelin converting enzyme (ECE) from their precursors known as 'big endothelin' originating from pre-proendothelin peptides cleaved by endopeptidases (Flammer & Luscher , 2011). ET-1 is more dominant in the cardiovascular system and is mostly synthesized by vascular endothelial cells; however, ET-1 can also be synthesized in vascular smooth muscle cells, extravascular tissues and glomerular and endothelial cells in the kidney (Bourque, Davidge et al. 2011). ET-1 can exert opposite effects to NO, and there are many factors playing a role in its production and release (Luscher, Yang et al. 1990). These factors include, hypoxia, shear stress, angiotensin II, thrombin, adrenaline, inflammatory cytokines and oxidized low density lipoproteins (Fitridge & Thompson 2011, Flammer & Luscher , 2011).

There are known receptors for ET-1 such as ET_A and ET_B, and ET-1 acts predominantly by binding to these G-protein coupled receptors. Both the receptor isoforms are found in vascular smooth muscle cells, however, ET_B is additionally located in the endothelium (Bourque, Davidge et al. 2011). ET_A receptors are mainly responsible for vasoconstriction, whereas ET_B receptors promote NO and prostacyclin production which ultimately results in reduced ET-1 production (Bourque, Davidge et al. 2011).

1.2.1.2.2 Thromboxane A

Similar to the prostaglandin family, thromboxane A_2 (TXA_2) is a product of the cyclooxygenase (COX) pathway (Smith, Tan et al. 2010). However, in contrast to PGI2, TXA_2 is a proatherogenic prostanoid that can induce vasoconstriction, platelet activation and adhesion (Smith, Tan et al. 2010). TXA_2 mediated platelet activation is a key event in thrombus formation (Ellinsworth, Shukla et al. 2014). Therefore, the relative concentrations of TXA_2, PGI2 and NO in the circulation and microenvironment are critical for normal cardiovascular and endothelial function (Smith, Tan et al. 2010).

TXA_2 is produced following the metabolism of prostaglandin H_2 (PGH_2) by a TXA_2 synthase, a member of the cytochrome P450 epoxygenase (CYP 5) family, and is the preferred ligand for TXA_2 receptors known as thromboxane prostanoid (TP) receptors (Ellinsworth, Shukla et al. 2014). TXA_2 essentially acts as an agonist after it is converted by COX and TXA_2 synthase (Capra, Bäck et al. 2014) (**Refer to figure 1.5)**. Activation of the platelet TP receptor triggers platelet activation, secretion, and aggregation, which play important roles in the formation of both hemostatic plugs and pathological thrombi, particularly at high arterial wall shear rates (Roald, Barstad et al. 1994). A normal response to TP receptor activation supports normal hemostasis (Capra, Bäck et al. 2014).

Furthermore, other prostanoids such as PGI2 and hydroxyeicosatetraenoic acids (HETEs), can also activate TP receptors. The receptors are expressed in both vascular smooth muscle cells (VSMCs) and ECs, indicating both paracrine and autocrine actions of TXA_2. (**Refer to figure 1.5)** Once TXA_2 occupies the VSMC TP receptors, contraction is initiated through coupling of either Gq/11 or, to a greater extent, G12/13 (Ellinsworth, Shukla et al. 2014). Coupling with the latter evokes the biosynthesis of inositol 1,4,5-trisphophate (IP3) and activation of specific RhoA guanine nucleotide exchange factors that in turn activates Rho kinase. This is achieved by G12/13 coupling to phospholipase C (PLC). Ultimately, Rho kinase alters the phosphorylation status of threonine residues within the MYPT1 subunit of myosin light chain phosphatase (MLCP) and the MLCP inhibitory protein CPI-17, thus eliciting contraction (Ellinsworth, Shukla et al. 2014).

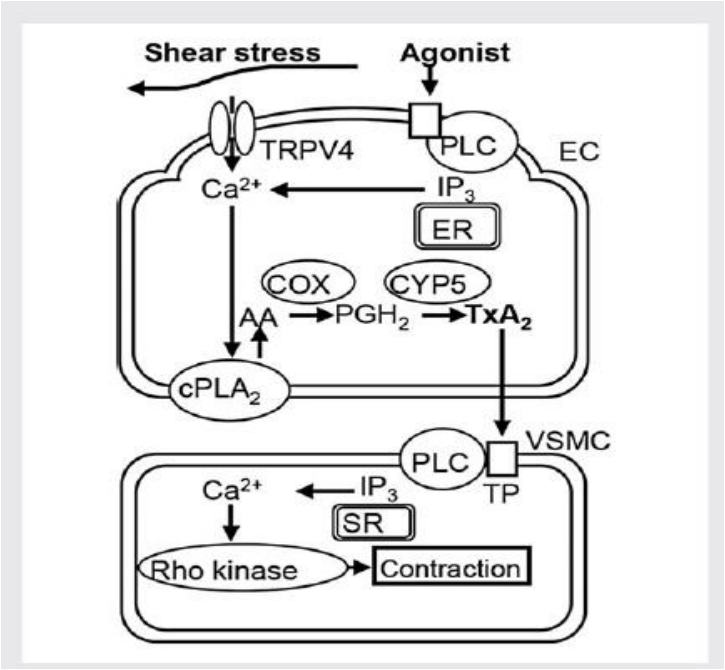

Figure 1.5: Mechanisms of endothelial cell (EC)-derived thromboxaneA2 (TxA2) biosynthesis and direct TxA2-induced vascular smooth muscle cell (VSMC) contraction. AA, arachidonic acid; COX, cyclooxygenase; cPLA2, constitutive phospholipase A2; CYP, cytochromeP450; IP3, inositol 1,4,5-trisphosphate; ER, endoplasmic reticulum; PGH2, prostaglandin H2; PLC, phospholipase C; SR, sarcoplasmic reticulum; TP, thromboxane/prostaglandin receptor; TRPV4, transient receptor potential vanilloid type 4 channel (Ellinsworth, Shukla et al. 2014).

1.2.1.2.3 Angiotensin II

Angiotensin II (Ang II) is a pleiotropic hormone that influences the function of many cell types and regulates many organ systems. In the cardiovascular system it promotes vascular inflammation and atherogenesis (Doughan, Harrison et al. 2008). It is a potent vasoconstrictor that increases peripheral vascular resistance and elevates arterial blood pressure. It is known for its pathophysiological role in CVDs such as hypertension, heart failure and atherosclerosis. Studies have additionally shown that Ang II promotes endothelial oxidative stress by redox activation of xanthine oxidase synthase (Landmesser, Spiekermann et al. 2007).

Ang II essentially is a bioactive peptide of the renin-angiotensin system (RAS). The diverse actions of Ang II are mediated via AT1 and AT2 receptors, which couple to many signalling molecules, including small G proteins, phospholipases, mitogen-activated protein (MAP) kinases, phosphatases, tyrosine kinases, NADPH oxidase, and transcription factors (Cat & Touyz 2011). In general, acute Ang II stimulation induces vasoconstriction through changes in the intracellular free calcium

concentration [$Ca2^{+}$] whereas long-term stimulation leads to cell proliferation and proinflammatory responses (Cat & Touyz 2011).

1.3 Endothelial dysfunction

1.3.1 Introduction

Endothelial dysfunction (ED) is a term generally used to describe any abnormality in the endothelium, especially that of impaired endothelium-dependent vasorelaxation, as well as altered anticoagulant and anti-inflammatory properties (Cai & Harrison 2000). ED is essentially characterized by an imbalance in endothelium-derived factors that can negatively affect vascular function (Flammer & Luscher 2011). ED is associated with many CVDs such as atherosclerosis, hypertension, coronary artery disease, diabetes and severe viral infections such as HIV.

1.3.2 Pathophysiology of ED

The pathophysiology of ED is complex and involves many mechanisms, however the hallmark of ED is impaired NO bioavailability largely due to increased oxidative stress and inflammation. The reduced NO availability is either due to decreased NO production or through loss of NO biological activity (Van den Oever, Raterman et al. 2010). The production of NO is halted in cells that are exposed to oxidative stress, which is caused by either an increase in oxidant production, or a reduction in antioxidant protection or a failure in repairing oxidative damage (Van den Oever, Raterman et al. 2010). Several cardiovascular risk factors such as hyperglycaemia, insulin resistance, dyslipidaemia, inflammation and cigarette smoking may induce oxidative stress (Van den Oever, Raterman et al. 2010). Cell damage is induced by ROS, which under normal conditions are scavenged by intra and extra cellular mechanisms but due to increased oxidative stress, these mechanisms are unable to cope with ROS (Van den Oever, Raterman et al. 2010). These free radicals can disrupt the balance of NO and leave the endothelium overtly permeable, permitting toxins to pass through as well as allowing cells that should remain in the blood to pass through blood vessels and into adjacent tissues (Rubanyi & Vanhoutte 1986, Rajendran, Rengarajan et al. 2013). Some of these include proteins such as C reactive protein (CRP), which is produced by the liver and is responsible for inflammation (Rajendran, Rengarajan et al. 2013). Inflammation is also known to decrease NO bioavailability and CRP is shown to lower eNOS activity (Verma, Wang et al. 2002, Endemann 2004)

In some cases, NO can react with some superoxide to form peroxynitrite (a highly reactive and harmful species), that leads to the degradation of the eNOS cofactor tetrahydrobioptrerin (BH4), followed by oxidative stress permitting uncoupling of eNOS (**refer to figure 1.6**). The reductase function of eNOS is activated and thus more ROS is produced instead of NO (Endemann 2004, Van den Oever, Raterman et al. 2010). Oxidative stress is linked to the proinflammatory state of the vessel wall. (Verma, Wang et al. 2002, Endemann 2004). Furthermore, oxidative stress can also lead to cell death, specifically programmed cell death or apoptosis (Van den Oever, Raterman et al. 2010).

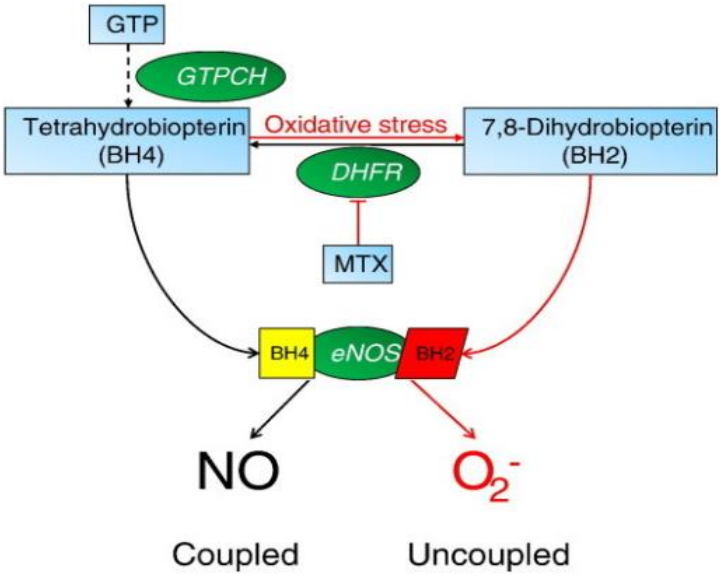

Figure 1.6: Schematic representation of the tetrahydrobiopterin (BH4) recycling pathway and eNOS coupling. BH4 is synthesized de novo from GTP via a series of reactions involving GTP cyclo hydrolase (GTPCH), 6- pyruvoyltetrahydropterin synthase, and sepiapterin reductase. Dygyrdofolate reductase (DHFR) can be inhibited by methotrexate (MTX) and can regenerate BH4 from BH2 as part of the recycling pathway. Both BH4 and BH2 bind eNOS with equal affinity, however, BH4-bound eNOS produces NO, whereas BH2- bound eNOS promotes uncoupling and eNOS-derived superoxide rather than NO (Crabtree, Hale et al. 2011)

1.4 Assessment of Endothelial Function

In 1986, ED was demonstrated for the first time by intracoronary infusion of acetylcholine and quantitative coronary angiography in atherosclerotic coronary arteries (Ludmer, Selwyn et al. 1986). Since then, several less invasive and even non-invasive techniques have been developed to measure endothelial function. **Refer to table 1.3** for some of the techniques available to assess endothelial function. The principle behind all of these techniques relates to large conduit arteries such as the brachial or radial artery dilating in response to reactive hyperaemia or as a result of intra-arterial infusion of pro-vasodilatory agents such as acetylcholine (Ach), bradykinin or serotonin in presence of an endothelium that is functionally capable of releasing NO and other vasodilatory factors (Flammer, Anderson et al. 2012, Flammer & Luscher , 2011, Flammer & Lüscher 2010).

***Table 1.3**:Various techniques for the measurement of endothelial function (Adapted from Mudau, Genis et al. 2012)*

Method	Brief description
Flow-dependent dilation of the brachial artery	This method employs a high-resolution ultrasound to quantify flow-mediated dilation of the brachial artery. The technique relies on endothelium-dependent release of NO and other EDRF's in response to reactive hyperaemia.
Forearm plethysmography	Involves intra-brachial infusion of endothelial-dependent vasodilators such as acetylcholine, metacholine, substance P and bradykinin, with subsequent measurement of changes in endothelial function of forearm arterioles. The response to acetylcholine is significantly reduced by intra-arterial infusion of N^G-monomethyl-L-arginine (but not by acetylsalicylic acid), thus demonstrating a key role of NO in this technique.
Finger-pulse plethysmography (ENDO-PAT) hyperaemia.	A non-invasive technique that measures changes of the pulse-wave amplitude during reactive hyperaemia. Low pulse-wave amplitudes are associated with compromised endothelial function and are therefore good predictors of cardiovascular disease. This technique reflects changes in flow and in digital microvessel dilatation and is only partly dependent on NO.
Pulse curve analysis	A non-invasive technique that relies on the measure of arterial stiffness to quantify endothelial function. This technique relies on accurate recording of the radial pressure wave, its calibration against brachial pressure, then generation of the ascending aortic pressure waveform through use of a generalized transfer function in a computerized process.

Quantitative coronary angiography following intracoronary infusion of acetylcholine	An invasive approach of quantifying endothelial function, which involves intracoronary infusion of the endothelium-dependent vasodilator, acetylcholine, and subsequent measurement of the vasomotor response due to relaxing factors.

1.4.1 Flow mediated dilatation (FMD) of the brachial artery

FMD of the brachial artery has become the gold standard non-invasive technique for the measurement of endothelial function in clinical research. This technique was developed in 1992 to evaluate early changes in vascular function of systemic arteries. The technique relies on endothelium-dependent release of NO and other EDRF's in response to reactive hyperaemia (Green, Dawson et al. 2014, Flammer & Lüscher 2010, Flammer & Luscher , 2011). The reactive hyperaemia is induced by a rapid deflation of a pneumatic blood pressure cuff, wrapped around the forearm that has been hyper-inflated to a suprasystolic pressure of 200 mm Hg for five minutes (Deanfield, Halcox et al. 2007). Deflation of the cuff after 5 minutes' occlusion of the brachial artery results in a surge of blood flow (hyperaemia) and a concomitant increase in shear stress along the vessel which activates the mechanoreceptors in ECs to release NO. FMD is then expressed as a percentage change in the brachial artery diameter in response to the increased shear stress induced by the reactive hyperaemia, and this is measured by a high resolution ultra sound technique (**refer to figure1.7**). (Jarrete, Zanesco et al. 2016, Flammer, Anderson et al. 2012). The technique is relatively complicated and requires intensive training of the operators as well as standardization of the protocols (Flammer & Lüscher 2010, Flammer & Luscher , 2011, Flammer, Anderson et al. 2012).

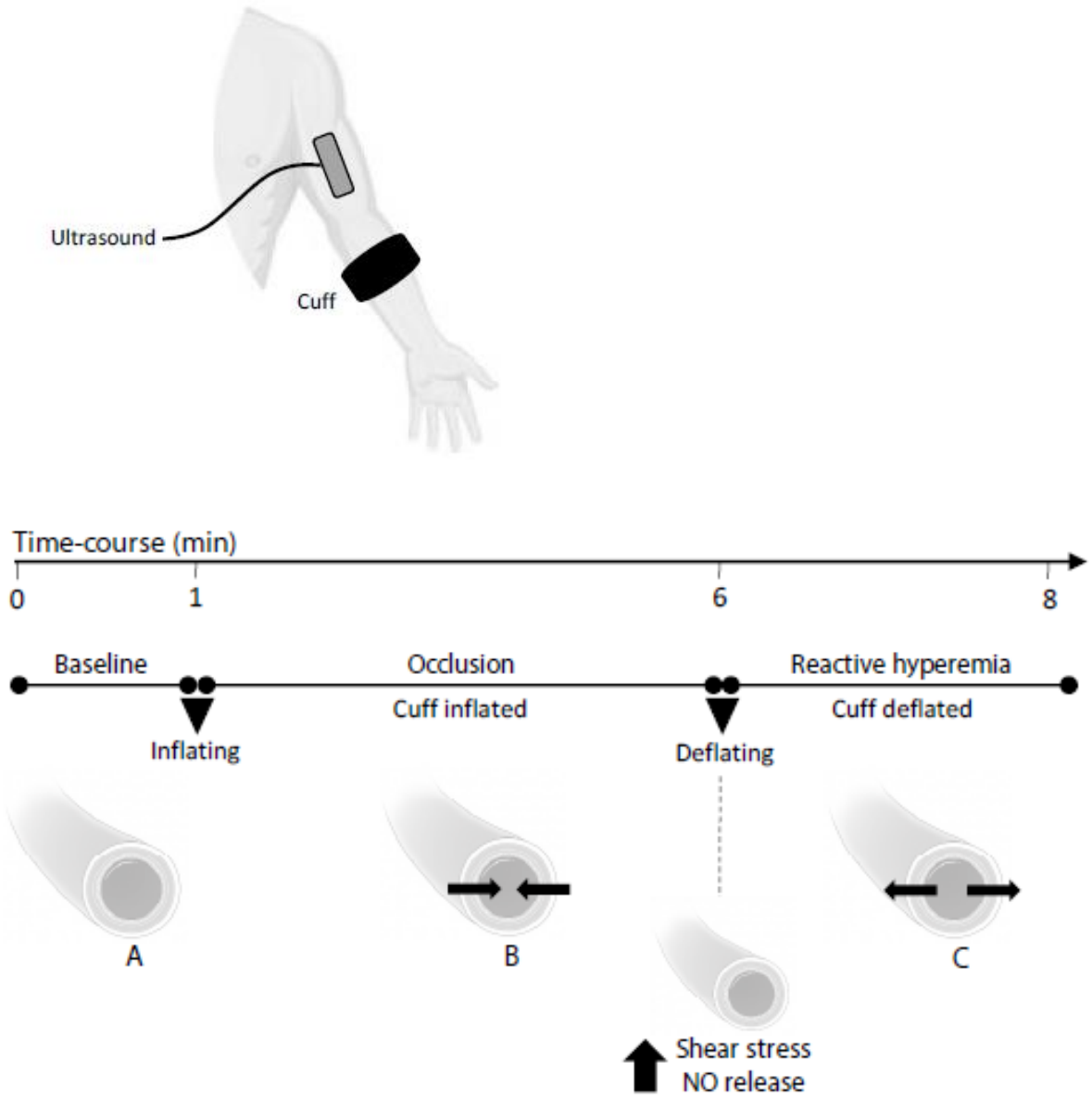

Figure 1.7: The FMD procedure consists of three phases.

A. Baseline arterial diameter is measured. B. Occlusion phase. The blood pressure cuff is hyper-inflated to approximately 50 mm Hg above systolic blood pressure value, or 200 mm Hg, for five minutes. C. The reactive hyperaemia phase (post-occlusion). Measurement of the maximum arterial diameter after the cuff release (peak of the response occurs between 45 to 60 seconds), characterizing the hyperaemic period (Jarrete, Zanesco et al. 2016). **Abbreviation:** NO: nitric oxide.

1.5 ED, risk factors and cardiovascular disease

Long term exposure of the vascular endothelium to cardiovascular risk factors leads to the loss of its defence mechanisms. This can result in endothelial activation and dysfunction. Current literature refers to ED as one of the major links between exposure to cardiovascular risk factors and the development of atherosclerotic disease (Sampson, Engelgau et al. 2015, Bonetti, Lerman et al. 2003, Mudau, Genis et al. 2012). **Refer to figure 1.8** below.

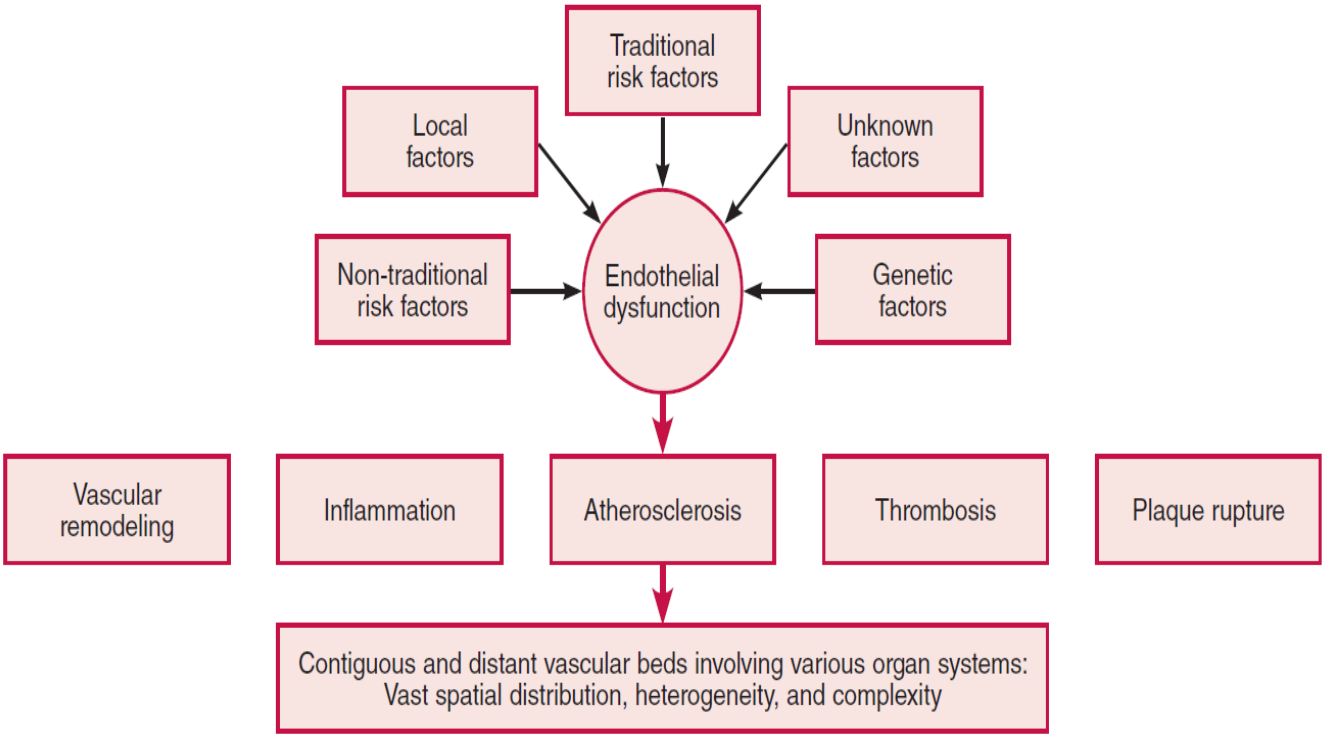

Figure 1.8: The link between endothelial dysfunction, cardiovascular risk-factors and atherosclerosis. Traditional and non-traditional risk factors, local factors (e.g. shear stress), and genetic factors determine the status of endothelial function. Sustained exposure to harmful factors can lead to pathophysiological changes in the endothelium, i.e. endothelial activation and dysfunction which can progress to atherosclerosis, the underlying mechanism of many CVDs such as IHD (Sampson, Engelgau et al. 2015).

1.6 Human immunodeficiency virus and acquired immunodeficiency syndrome (HIV/AIDS)

1.6.1 Introduction and Epidemiology of HIV /AIDS

HIV/AIDS was first described in 1981, and two years later, the HI virus was confirmed to be the aetiological agent for AIDS. Initially it was believed to be on a slow rise, today, HIV is one of the most devastating and widespread infectious agents known to mankind (Delpech 2013). The response to the HIV pandemic for the first two decades was poor and only began to improve once successful antiretroviral therapy (ART) was introduced (Delpech 2013, Deeks, Lewin et al. 2013). Since its introduction in 1996, ART has become a major milestone in revolutionising the clinical management of HIV/AIDS (De Cock, Jaffe et al. 2012). Even though HIV is a disease without cure, today it is managed as a chronic rather than acute disease with over 30 different approved ART drugs (Ruelas & Greene 2013).

Since the beginning of the HIV epidemic, more than 70 million people have been infected with HIV and approximately 35 million people have died of HIV/AIDS worldwide (WHO 2016). Globally, by the end of 2015, approximately 36.7 million people were living with HIV. The burden of HIV varies significantly between different countries and regions; however, HIV is largely concentrated in SSA, with nearly 1 in every 25 adults (4.4%) living with HIV which accounts for nearly 70% of the people living with HIV worldwide. It is noteworthy, however, that the number of newly infected people with HIV is declining worldwide, with sharp declines in the Caribbean and the SSA region (O'Cofaigh & Lewthwaite 2013). Declining transmission can be attributed to a combination of testing, prevention counselling and treatment (O'Cofaigh & Lewthwaite 2013). However, the global prevalence of HIV is still on the rise as people on anti-retroviral therapy are living longer (Maartens, Connie, et al., 2014).

1.6.2 Pathophysiology of HIV

Currently, HIV isolates are grouped into two types, HIV- type 1 (HIV-1) and HIV-type 2 (HIV-2). Globally, HIV-1 is mainly responsible of AIDS, whereas HIV-2 is largely restricted to parts of western and central Africa. Genetically, HIV is a member of the *Lentivirus* genus of the *Retroviridae* family. Infections with lentiviruses, generally prescribe to a chronic course in the disease progression, with a long period of clinical latency and persistent viral replication (Luciw 1996, Fanales-Belasio, Raimondo et al. 2010). HIV, a retrovirus, comprises of two identical copies of RNA molecules. The

virus is characterized by the presence of structural genes such as *gag*, *pol*, *env* as well as other regulatory and accessory genes. The gag gene encodes for the structural proteins of the core such as (p24, p7, p6) and matrix (p17). The *env* gene encodes glycoprotein gp160 which forms a homodimer and is cleaved into the viral envelope glycoproteins gp120 and gp41, responsible for the recognition of the cell surface receptors and the *pol* gene encodes for enzymes crucial for viral replication, which are the reverse transcriptase (known to convert viral RNA into DNA), the integrase that incorporates the viral DNA into host chromosomal DNA (the provirus), and the protease that cleaves large *Gag* and *Pol* protein precursors into their components. **Refer to figure 1.9** for the structure of HIV-1 particle.

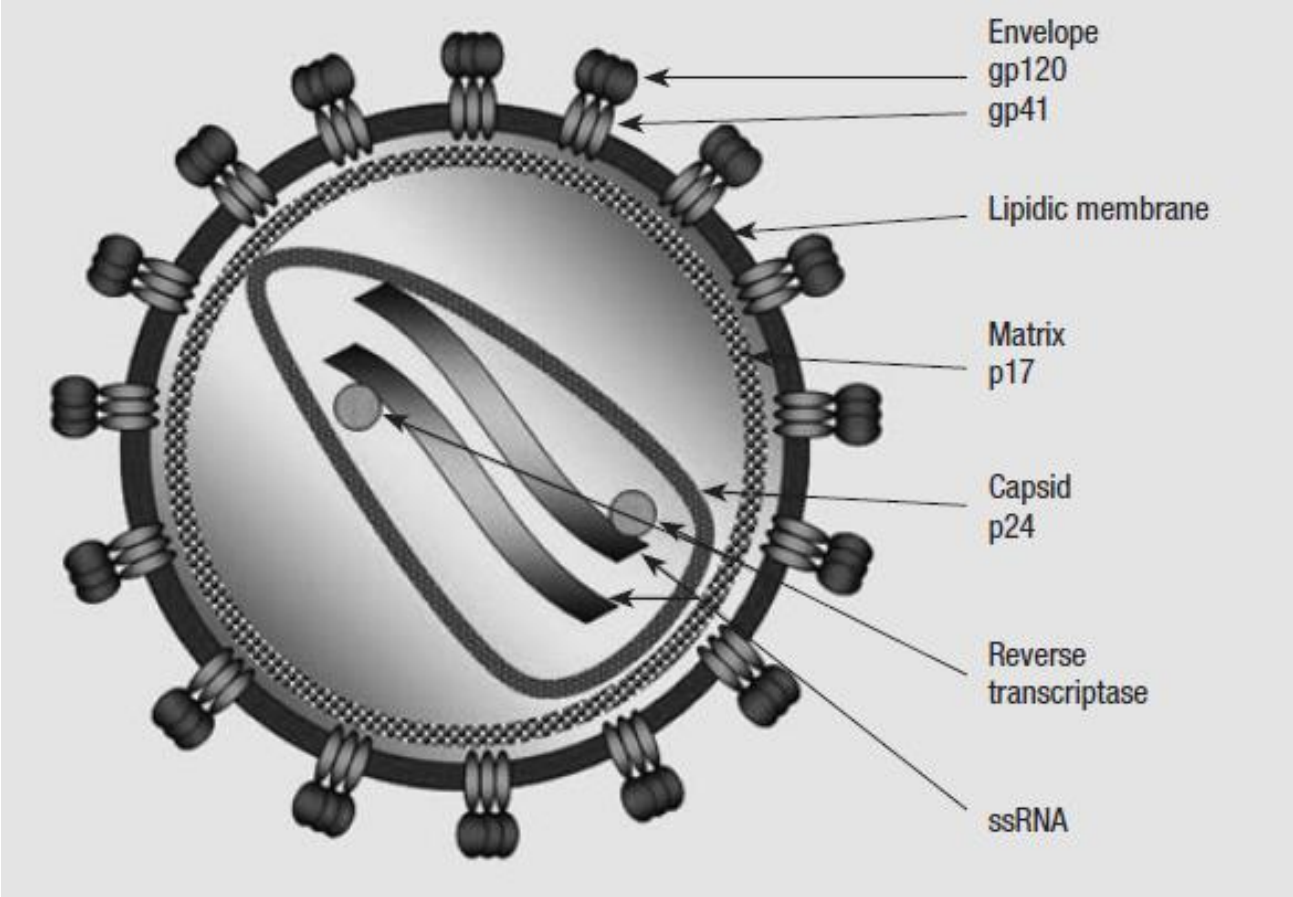

Figure 1.9: Structure of HIV-1 particle (Fanales-Belasio, Raimondo et al. 2010).

1.6.3 HIV life cycle

Refer to figure 1.10 for a schematic representation of the HIV replication cycle. The main target of HIV is activated CD4- T lymphocytes (Maartens, Celum, et al., 2014). Entry of the virus is via its interaction with CD4 and Chemokine Co-Receptors (CCR) and is divided into three major events: virus binding, activation and fusion (Fanales-Belasio, Raimondo et al. 2010). The viral envelope is a trimeric complex, comprising of the heterodimer proteins gp120 and gp41, which are vital for virus recognition and entry into target cells. The HIV gp120 binds a 58 kDa monomeric glycoprotein (considered as CD4) which is found on 60% of circulating T lymphocytes cell surfaces. Gp120 binding with the CD4 protein leads to a structural change in the virus envelope complex, exposing a specific domain in the gp120 permitting binding with the chemokine receptors on the cell membrane (Weiss 1993, Fanales-Belasio, Raimondo et al. 2010). The double binding of gp120 to both the CD4 and chemokine receptor permits a stable attachment of the virus which allows the N terminal fusion protein gp41 to penetrate the cell membrane and cause a collapse of the extracellular portion into a hairpin bringing the virus and cell membrane close together leading to fusion and subsequent entrance in the viral capsid (Fanales-Belasio, Raimondo et al. 2010, Kirchhoff 2013).

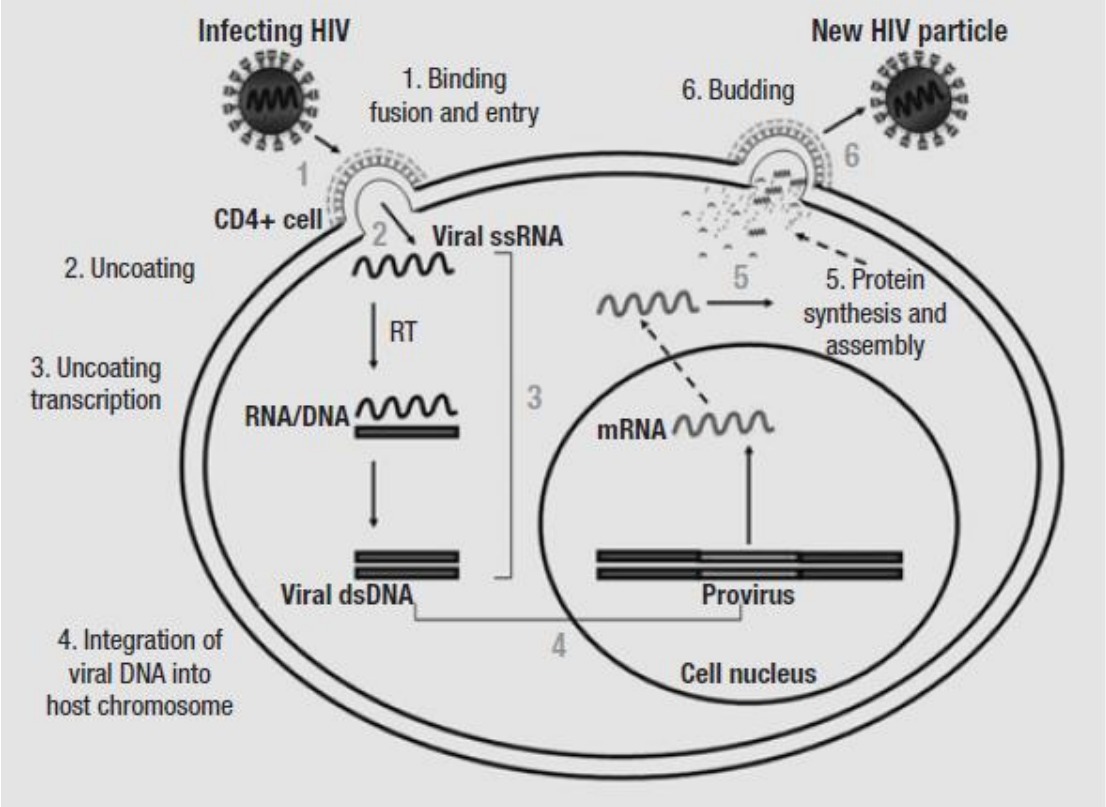

Figure 1.10: HIV replication cycle. RT: reverse transcriptase; dsDNA: double strand DNA (Kirchhoff 2013, Fanales-Belasio, Raimondo et al. 2010)

After the membrane fusion, the virus core uncoats into the cytoplasm of the target cell, freeing its viral RNA. The action of reverse transcriptase and integrase enzymes converts viral RNA to proviral DNA. The integration of proviral DNA and the expression of the provirus require that the target cell is in an activated state. Upon cell activation, transcription of proviral DNA into a messenger RNA occurs **(Figure1.10)**. The transcription process initially results in the early synthesis of regulatory HIV-1 proteins such as Tat and Rev (Fanales-Belasio, Raimondo et al. 2010, Kirchhoff 2013). Viral messenger RNA coding for long fragments migrates into the cytoplasm, where structural proteins of the new virus are synthesized **(Figure 1.10)**. Large gp160 precursor molecules are cleaved by the HIV-1 protease into gp120 and gp41. The Gag and Pol proteins are also derived from a large 160 kD precursor molecule, from which the HIV protease cleaves the p24, p17, p9 and p7 Gag final products and the Pol proteins (Miceli & Parnes 1993, Fanales-Belasio, Raimondo et al. 2010). The cleavage of the precursor molecules by the HIV-1 protease is necessary for the generation of infectious viral particles. The formation of new viral particles is a stepwise process: two viral RNA strands associate together with replication enzymes, while core proteins assemble over them forming the virus capsid. This immature particle migrates towards the cell surface (Fanales-Belasio, Raimondo et al. 2010). The large precursor molecules are then cleaved by the HIV-1 protease, resulting in new infectious viral particles. Finally, the newly formed HIV particle buds off through the host cell membrane (Figure 1.10), thus acquiring a new envelope. The surface of the newly formed HIV particle becomes studded with HIV glycoproteins (protein/sugar combinations) that are used to bind to CD4 cell co-receptors to infect other cells (Kirchhoff 2013, Fanales-Belasio, Raimondo et al. 2010).

1.6.4 Antiretroviral therapy (ART)

The management of HIV/ AIDS was significantly revolutionized with the development of ART in the 1990s (Ruelas, Greene 2013). Soon after the initial development of ART, approved for monotherapy, combination ART (cART), also known as highly active ART (HAART), was approved which transformed HIV from a progressive fatal illness to a chronic manageable disease (Maartens, Celum, et al., 2014, O'Cofaigh & Lewthwaite 2013). The use of HAART is known to dramatically suppress the overall burden of HIV, maintaining adequate immune function as well as preventing fatal opportunistic infections (Moore, Chaisson 1999, Maartens, Gary, Celum, Connie, Lewin,Sharon R, 2014). Today, successful use of HAART has decreased the progression of HIV to AIDS in many parts of the world (Fauci & Folkers 2012).

Antiretroviral agents predominantly act on various stages of the HIV life cycle to inhibit key steps in viral replication. At the moment, there are six classes of antiretroviral agents classified according to their molecular mechanism and their target viral enzymes. (Arts & Hazuda 2012, Soriano, Mendoza 2002, O'Cofaigh & Lewthwaite 2013). **Refer to figure 1.11** for a schematic representation. **Refer to table 1.4** for examples of drugs available in each class. Nucleoside/ nucleotide reverse transcriptase inhibitors (NRTIs) were the first class of agents approved by the Food and Drug Administration (FDA) (Arts & Hazuda 2012). NRTIs play an integral role in the HIV treatment and remain part of the current standard care, even though they are known to be less potent than the other classes of drugs (Shen, Peterson et al. 2008, Cox, Aperia et al. 1994). They are often administered as prodrugs, which can elicit antiviral effects upon cell entry and phosphorylation by cellular kinases. NRTI's competitively inhibit reverse transcriptase (RT) and cause a termination of the DNA chain (Weller, Williams 2001, Arts & Hazuda 2012). The structural similarity of NRTIs to the DNA nucleoside bases, permits integration into the proviral DNA chain, preventing other nucleoside incorporation and thus terminating the proviral DNA formation. As with all ARTs, treatment with NRTIs can result in resistance, often mediated by one of the two mechanisms: 1) impaired incorporation into the proviral DNA chain or 2) removal of the NRTIs from the proviral DNA chain (Arts & Hazuda 2012, Clavel & Hance 2004, Soriano, Mendoza 2002)

A few years later, non-nucleoside/ nucleotide reverse transcriptase inhibitors (NNRTIs) were introduced in 1996 which act by non-competitively inhibiting reverse transcriptases by binding to their allosteric site. NNRTIs have an impact in the handling of substrate (nucleotides) by reverse transcriptase via binding near the active site (Arts & Hazuda 2012). The agents are further classified in 1st generation and 2nd generation NNRTIs. Resistance in this class generally results from mutations in the RT that alter the ability of NNRTIs to bind to the binding site (Clavel, Hance 2004). Interestingly HIV-2 is naturally resistant to the agents in this class (Arts & Hazuda 2012, Soriano & Mendoza 2002). Another important class of ARTs are the protease inhibitors (PIs). They act by blocking the viral protease enzymes which are responsible for the production of mature virions after budding from the membrane. Agents from this class specifically inhibit the cleavage of gag and gag/pol precursor proteins (Kim & Baxter 2008, Arts & Hazuda 2012). Surviving viral particles that are formed in the presence of the PIs are flawed and mostly non-infectious (Flexner 1998). Cross resistance in this class is mostly observed with primary resistance mutations occurring near the active site of the enzyme, located at the inhibitor binding site (Arts & Hazuda 2012, Clavel & Hance 2004, Flexner 1998).

Furthermore, some of the relatively recent classes of ART include fusion inhibitors and integrase inhibitors. Integrase inhibitors target the viral enzyme integrase responsible for integration of viral DNA into the DNA of the infected cell. All agents in this class competitively inhibit the strand transfer reaction and thus can also be referred to as integrase strand transfer inhibitors (InSTIs) (Arts & Hazuda 2012). Fusion inhibitors are also known as entry inhibitors, and they target the HIV cycle extracellularly by inhibiting the fusion of HIV with the CD4 of the target cell. Lastly, similar to fusion inhibitors, there is one novel drug available today that acts particularly on CCR5, a chemokine receptor antagonist, which inhibits fusion between the cell membranes (Arts & Hazuda 2012, Bai, Xue et al. 2013).

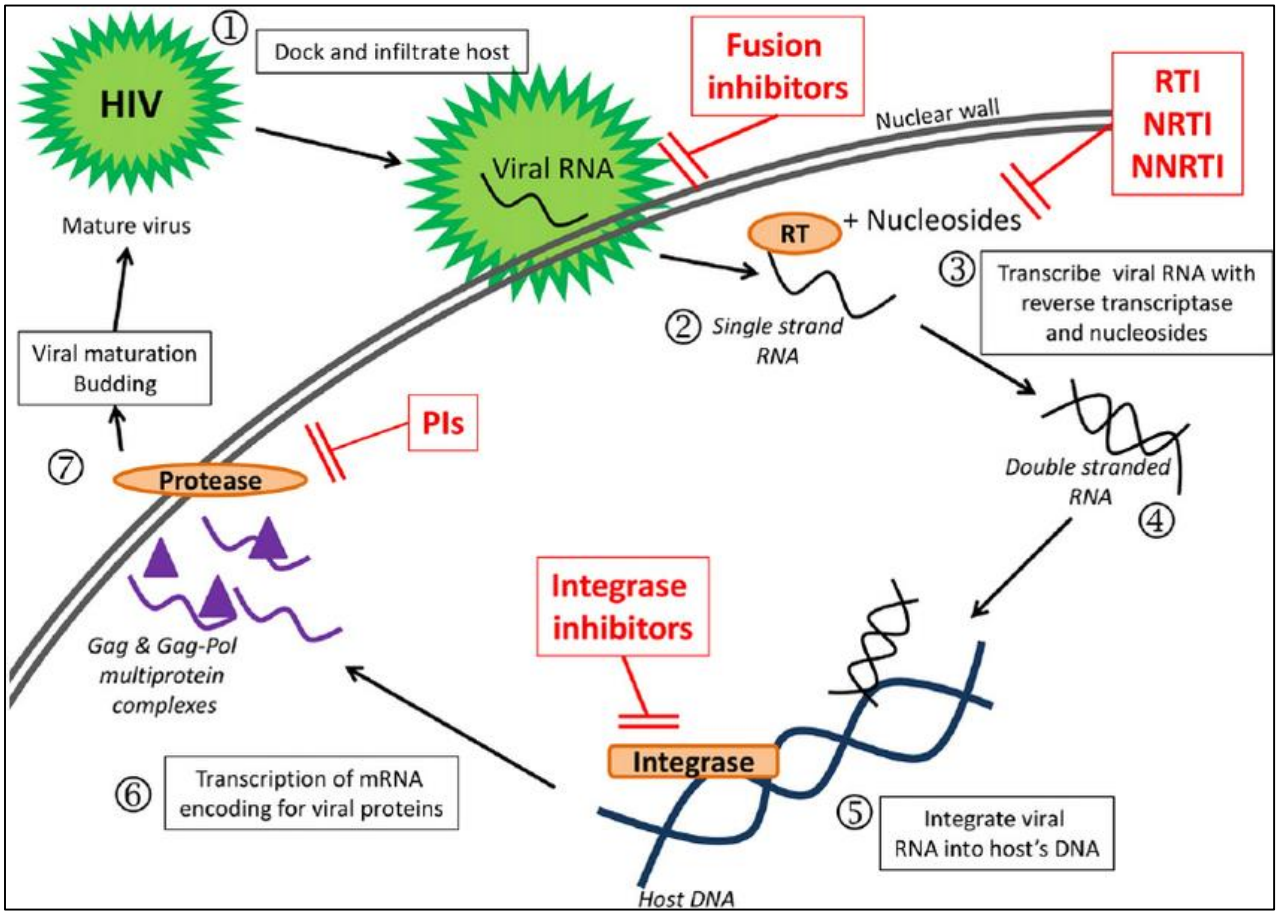

Figure 1.11: Schematic representation of the HIV life cycle and the sites of action of the various classes of ART (Reyskens, Essop 2014).

Table 1.4: *ARV drugs available in each of the ART classes.*

NRTIs	**NNRTIs**	**PIs**	**Integrase Inhibitors**	**Fusion Inhibitors**	**CCR5 antagonists**
Abacavir (ABC) Didanosine (ddI) Emtricitabine (FTC) Lamivudine (3TC) Stavudine (d4T) Tenofovir DF (TDF) Zalcitabine (ddC) Zidovudine (AZT)	Delavirdine (DLV) Efavirenz (EFV) Nevirapine (NVP) Etravirine (ETR) Rilpivirin (RPV)	Atazanavir (ATV) Darunavir (DRV) Fosamprenavir (FPV) Indinavir (IDV) Lopinavir/ritonavir (LPV/r) Nelfinavir (NFV) Saquinavir (SQV) Tipranavir (TPV)	Elvitegravir (EVG) Raltegravir (RAL) Dolutagravil (DTG)	Enfuvirtide (ENF)	Maraviroc (MVC)

1.7 HIV and ART- A South African perspective

1.7.1 Introduction

In South Africa (SA), the HIV/AIDS epidemic began in the 1980s. At present, South Africa has the highest prevalence of people living with HIV/AIDS in the world. In 2015, approximately 7 million people were living with HIV and about 380, 000 were newly infected with HIV. Furthermore, in the same year, 180 000 South Africans died from AIDS related illnesses. ART for the treatment of HIV was introduced in 1996 in SA, however the national HIV ART treatment programme only officially began in 2003. Interestingly, globally, SA today has the largest government sponsored ART rollout programme (**Refer to table 1.5** for a detailed overview of some of the ART drugs available in SA). SA has followed the WHO recommendations on initiating treatment based on the individuals CD4 count. Furthermore, South Africa changed the CD4 level at which people could start ART from 200 to 350 in 2013 and then later to 500 by the end of 2014, making more people eligible for treatment. Recently in 2015, WHO released guidelines recommending ART treatment should now be initiated immediately after HIV diagnosis and SA has begun this implementation in 2016.

1.7.2 Fixed dose combination (FDC) drug regimens

In April 2013, a fixed dose combination (FDC) tablet (trade name: Atripla®, or Odimune®) was introduced in SA, which contains 300mg of Tenofovir, 200 mg of Emtricitbine and, 600mg of Efavirenz (Davies 2013). **Refer to table 1.5** for a summary and classification of the drugs. In adults, the tablet is taken once daily on an empty stomach. Dosing at bedtime is recommended to improve tolerability of nervous system symptoms. The tablet is not recommended for patients under 18 years of age.

This initiation of a triple FDC tablet was a significant advancement in the SA's national ART programme. This substantially simplified the prescription of ART drugs, dispensing and stock management due to a reduction from three separate drugs to one combined tablet (Davies 2013). This further improved treatment adherence and the burden of patients subjected to multiple drug regimens (Meintjes, Conradie et al. 2014) The efficacy of the FDC ART has previously been proven in randomized control trials (Davies 2013, Gallant, Staszewski et al. 2004, Gallant, DeJesus et al. 2006).

1.7.3 First line ART in SA

First line treatment for HIV infected individuals over the age of 15, includes a combination of Tenofovir, Emtricitabine or Lamividune and Efavirenz (Davies, 2013). These drugs are provided as FDC. Alternative first line treatment for individuals in whom any of the abovementioned drugs are contraindicated, comprises of replacing the contraindicated drug with another recommended drug, for instance Efavirenz can be replaced with Nevarapine and Tenofovir can be replaced with Abacavir. If the contraindication is to both Efavirenz and Nevarapine then those drugs can be replaced with Lopinavir together with Ritonavir (Davies 2013).

1.7.4 Second line ART in SA

For patients failing first line treatment, the WHO recommends switching from NRTI/NNRTI regimens to PI based regimens (Ramadhani, Bartlett et al. 2014). According to the South African antiretroviral guidelines (Adult) 2015, second line regimens for HIV individuals include a combination of Zidovudine, Lamivudine and, Lopinavir with Ritonavir. For an alternative treatment, Tenofovir can be replaced with Zidovudine if the above mentioned combination fails.

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Table 1.5: *Detailed overview of ART drugs approved in SA.*

Drug	Recommended dose and Molecular mass	Class	Mechanism of Action	Adverse events
Emtricitabine (FTC)	300mg/ day; 247.2g/ mol	NRTI	Cellular enzymes phosphorylate FTC to FTC-5'-triphosphate which in turn competes with the natural substrate, deoxycytidine 5'- triphosphate and is subsequently incorporated into the nascent viral DNA and leads to chain termination It lowers the patient's viral load and increases CD4 count.	Common side effects include headache, diarrhoea, nausea, fatigue, dizziness, depression, insomnia, abnormal dreams, rash, abdominal pain, asthenia, increased cough, and rhinitis. Skin hyperpigmentation is very common in paediatric patients.
Tenofovir Disoproxial Fumarate (TDF)	300 mg/ day; 287.2g/mol	NRTI	TDF diphosphate inhibits HIV reverse transcriptase through competition with the natural substance deoxyadenosine 5'- triphosphate and leads to DNA chain termination after incorporation into the viral-DNA.	The most common side effects include nausea, vomiting, diarrhoea, and asthenia. Less frequent side effects include hepatotoxicity, abdominal pain, and flatulence. TDF has also been implicated in causing kidney toxicity, particularly at elevated concentrations.

Table 1.5 continued

Efavirenz (EFV)	300 mg/ day; 315.7g/mol	NNRTI	EFV is selective for HIV-1 and diffuses into the host cell and binds next to the active site of the reverse transcriptase enzyme which leads to a conformation change and inhibits the enzyme's function. It is further a non-competitive inhibitor of HIV-1 reverse transcriptase with respect to template, primer or nucleoside triphosphates	Common side effects include diarrhoea, dizziness, drowsiness, headache, increased sweating, poor concentration, trouble sleeping, depression and skin rash.
Lamivudine (3TC)	300 mg/ day; 229.26 g/mol	NRTI	3 TC is an analogue of cytidine. It can inhibit both types (1 and 2) of HIV reverse transcriptase and also the reverse transcriptase of hepatitis B virus. It is phosphorylated to active metabolites that compete for incorporation into viral DNA. It has a synergistic effect when given with AZT	Common side effects included headache, nausea, malaise, fatigue, nasal signs and symptoms, respiratory tract infections, throat and tonsil discomfort, abdominal discomfort and pain, vomiting, diarrhoea, and cough**.**
Zidovudine (AZT)	300 mg twice daily; 267.2 g/mol	NRTI	AZT is a thymidine analogue. AZT works by selectively inhibiting HIV's reverse transcriptase, the enzyme that the virus uses to make a DNA copy of its RNA.	Serious side effects include lactic acidosis, liver problems, muscle weakness and blood disorders such as severe anaemia or neutropenia.

Table 1.5 continued

Lopinavir (LPV)	400 mg twice daily; 628.8 g/mol	PI	Lopinavir inhibits the HIV viral protease enzyme. This prevents cleavage of the gag-pol polyprotein and results in an inadequate viral assembly. This subsequently results in non-infectious, immature viral particles.	Common side effects include an increase in serum cholesterol and triglycerides.
Ritonavir (RPV)	100 mg twice daily;	PI	Ritonavir was originally developed as an inhibitor of HIV protease; however, it is rarely used for its own anti-viral activity. It remains widely used as a booster of other protease inhibitors. More specifically, ritonavir is used to inhibit a particular liver enzyme that normally metabolizes protease inhibitors, cytochrome P450-3A4 (CYP3A4). This drug is mostly given in combination with lopinavir.	Common side effects include asthenia, malaise, diarrhoea, nausea and vomiting, abdominal pain, dizziness, insomnia, sweating, taste abnormality, metabolic hypercholesterolemia, hypertriglyceridemia, elevated transaminases and elevated creatine phosphokinase(CPK) An interesting side effects of this drug is hyperglycaemia. It appears that ritonavir directly inhibits the GLUT4 insulin-regulated transporter, keeping glucose from entering fat and muscle cells. This can lead to insulin resistance and cause problems for people with type II diabetes.

1.8 Cardiovascular disease and risk factors in HIV infected individuals

1.8.1 Introduction

Successful management of HIV-infection and HIV/AIDS with ART has resulted in CVD to become one of the top causes of death in the HIV infected population, partially due to increased lifespan of these individuals as well as due to direct effects of ART drugs. Understanding the risk of CVD in HIV infected individuals is complex (de Gaetano Donati, Cauda et al. 2010). The prevalence of cardiovascular risk factors in HIV infected individuals is known to be high and may precede HIV infection (Fedele, Bruno et al. 2011). Several studies have also shown a high prevalence of smoking and saturated fat rich diets in HIV infected individuals when compared to the general population (Fedele, Bruno et al. 2011). In addition, some studies have shown low physical activity and a greater incidence of overweight / obesity present in HIV infected individuals (Mashinya, Alberts et al. 2015, Paik & Kotler 2011). All factors such as the host's genetics, traditional risk factors, adverse effects of ART as well as the inflammatory state of HIV may be considered as potential risk factors for CVD (de Gaetano Donati, Cauda et al. 2010, Aberg 2009) **(Refer to figure 1.12)**.

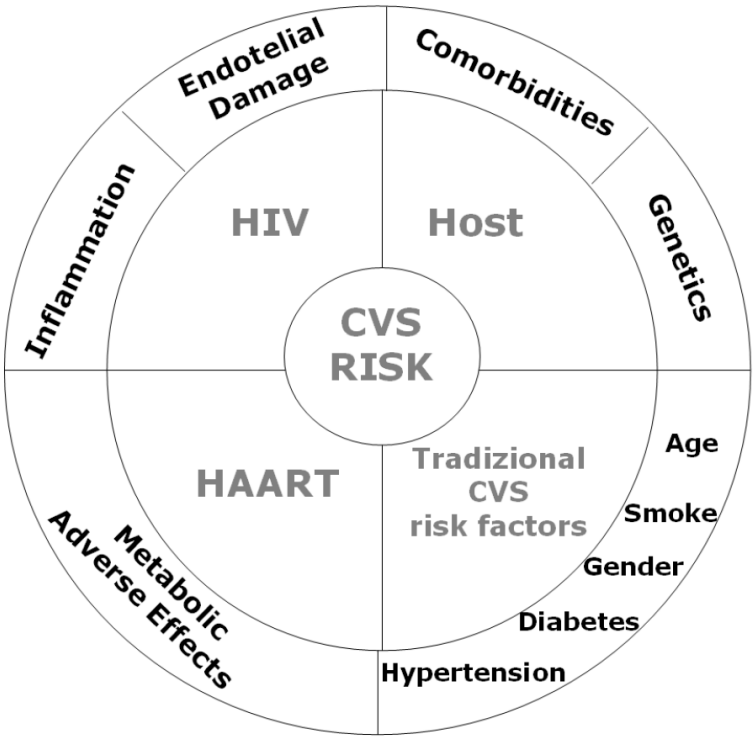


Figure 1.12: Risk factors of CVD in HIV. Cardiovascular risk in HIV-infected individuals can be attributed to the host's genetics, traditional risk factors, adverse effects from antiretroviral therapy or the inflammatory state associated with HIV itself (De Gaetano Donati, Cauda et al. 2010).

1.8.2 Effects of HIV infection on cardiovascular disease and risk

It has been found that HIV infected individuals are at greater risk of developing CVD both in the presence and absence of ART (Wang, Yi et al. 2015). Furthermore, studies have demonstrated a higher rate of acute myocardial infarction (AMI) and cardiovascular risk factors in HIV infected individuals compared to the controls (Wang, Yi et al. 2015). Several studies have investigated the mechanisms and nature of atherosclerosis in HIV infected populations. For example, a study found an increased presence of plaque and non-calcified plaque in HIV infected participants compared to the control population. This study ultimately demonstrated that HIV-infected individuals without significant metabolic abnormalities may still develop non-calcified plaque and thus have an increased risk of IHD (Fitch, Lo et al. 2010, Wang, Yi et al. 2015)

An increased risk of CVD in HIV-infected individuals remains incompletely understood. One theory relates to HIV induced hyperlipidaemia and hypercholesterolemia which promote the pathogenesis of atherosclerosis. It has been found that HIV-infection and some ART drug classes, especially agents from the PI class can cause dyslipidaemia in the HIV infected population, thus contributing to an increased risk of CVD (Wang, Yi et al. 2015, Stein, Currier et al. 2014, Brown & Glesby 2012). In fact, unmanaged HIV replication has been found to be an independent risk factor for changes in the individual's lipid profile (Fedele, Bruno et al. 2011). Two studies in particular have shown that HIV infected individuals have a higher total LDL and HDL cholesterol as well as serum triglyceride levels compared to the control groups entailing that these infected individuals are at a much higher risk of developing CVD (Kaplan, Kingsley et al. 2007, Fedele, Bruno et al. 2011). The effect of these risk factors is found to be similar in both HIV infected and non-infected individuals, suggesting that these factors contribute to the risk in a comparable way regardless of the HIV status (Joy, Keogh et al. 2007, Fedele, Bruno et al. 2011). Furthermore, current literature suggests that the events progressing to CVD overlap and intertwine, and do not necessarily occur in a distinct manner in HIV infected individuals (Fedele, Bruno et al. 2011).

It is debatable as to whether HIV or ART has a greater contribution to cardiovascular risk. However, untreated HIV has been associated with an increased level of systemic inflammatory markers, such as IL-6, proinflammatory cytokines and CRP. Increased IL-6 levels are found to be a strong predictor of future cardiovascular events and overall mortality in ART naïve and ART treated HIV infected patients (Kuller, Tracy et al. 2008, Wang, Yi et al. 2015). Several studies have demonstrated that the continuing HIV replication and immune depletion significantly contributes to an increased prevalence of elevated biomarkers of inflammation, altered coagulation, and monocyte activation, and this contribution is independent of the substantial contribution from comorbid conditions (Sandler, Wand

et al. 2011, Wang, Yi et al. 2015). A study found that HIV infection correlates with premature atherosclerosis even in absence of detectable viremia and ART, thus appearing to be an independent risk factor (Hsue, Hunt et al. 2009). High rates of cardiovascular events when ART is halted further suggests that HIV infection in itself may elevate cardiovascular risk (Fedele, Bruno et al. 2011, Wang, Yi et al. 2015).

1.8.3 Effects of ART on cardiovascular disease and risk

The relationship between ART and cardiovascular risk is not well understood. However, as mentioned, the literature suggests that HIV individuals regardless of ART generally do present with underlying traditional cardiovascular risk factors, which complicates the demonstration of the risk associated with the type of ART or specific ART drugs. In fact, recently, data have emerged suggesting that ART may improve factors associated with increased CVD risk (Fedele, Bruno et al. 2011).

Although there are many benefits of ART, upon its introduction, several reports of MI and premature atherosclerosis emerged in individuals receiving ART (Maggi, Lillo et al. 2004, Fedele, Bruno et al. 2011). ART induced hyperlipidaemia and hypercholesterolemia is also found to promote the pathogenesis of atherosclerosis. It has been further found that a few ART drugs, especially agents from the PI class can cause dyslipidaemia in HIV infected population, thus contributing to an increased risk of CVD (Wang, Yi et al. 2015, Stein, Currier et al. 2014, Brown & Glesby 2012). Other studies have specifically found NRTIs and PIs to increase the risk of developing IHD (Steyn, Sliwa et al. 2005, Skowyra, Zdziechowicz et al. 2012). Many studies have found a strong association between the type, duration, and current use or non-use of ART and the severity of metabolic disorders such as hyperlipidaemia, insulin resistance, and lipodystrophy. In the ongoing Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) study, it was found that the relative risk of CVD increased as the duration of ART increased, however the absolute risk of cardiovascular disease remained low for most patients, except those with multiple other cardiovascular risk factors (Caron, Auclair et al. 2001, Fedele, Bruno et al. 2011). However as soon as it was established that uncontrolled HIV viremia and the presence of risk factors similar to the general population may cause CVD amongst HIV infected individuals, it was suggested that discontinuation or interruption in ART may be of little benefit in lowering cardiovascular risk (Wang, Yi et al. 2015). Data from a study demonstrated that patients in the CD4-guided intermittent ART group were at increased risk of myocardial damage in comparison to individuals who received ART (Fedele, Bruno et al. 2011, Ananworanich, Gayet-Ageron et al. 2006). Furthermore, the study found that, IL-6 and D-dimer

levels increased after discontinuation of ART, and this increase was associated with increases in HIV RNA levels (Phillips, Carr et al. 2008, Fedele, Bruno et al. 2011).

1.9 Endothelial dysfunction in HIV-infected individuals

1.9.1 Introduction

HIV is capable of infecting endothelial cells which may directly lead to endothelial dysfunction (Skowyra, Zdziechowicz et al. 2012). The exact underlying mechanism by which the virus induces ED is not fully understood. However, several possible mechanisms which have been proposed **(refer to table 1.6)** and are currently the focus of research (Subbarao, Lowe et al. 2011, Monsuez, Charniot et al. 2009).

Table 1.6: Proposed mechanisms of HIV induced ED (Adapted from Monsuez, Charniot et al. 2009).

Proposed pathogenesis of HIV induced ED
Direct endothelial injury from the HIV-1 virus and the component proteins of HIV-1
HIV-induced chronic inflammation
HIV-induced dyslipidaemia and metabolic syndrome
Direct endothelial injury from antiretroviral therapy
ART-induced dyslipidaemia and metabolic syndrome

1.9.2 HIV infection and ED

The untreated state of HIV is associated with ED. HIV is able to enter ECs via several ways such as through the CCR-3 and CCR-4, cluster of differentiation 4 (CD4) or galactosyl-ceramide receptors (Wang, Yi et al. 2015, Skowyra, Zdziechowicz et al. 2012). Entry of the virus also occurs though GP120 and tat protein as well as participation of cytokines secreted by HIV infected mononuclear and adventitial cells. The HI virus can injure the endothelium in a few ways. It can cause direct cell death through apoptosis or necrosis (Wang, Yi et al. 2015). In addition, the virus can infect and decrease endothelial progenitor cells (the circulating colony forming unit endothelial cells) which are essential for the endothelial damage repair (Teofili, Iachininoto et al. 2010). Furthermore, HIV

proteins, such as Gp120, are responsible for apoptosis of the endothelial cells and stimulation of macrophages to produce excessive amounts of NO, which can progress to direct endothelial damage (Wang, Yi et al. 2015). Interestingly it has been found that exposure to cigarette smoke and HIV gp120 causes a synergistic increase in endothelial cell death (Green, Yi et al. 2014, Wang, Yi et al. 2015). Moreover, the HIV TAT protein can activate inflammatory pathways via mononuclear cells that are known to produce tumour necrosis factor-α (TNF-α) and the pro-inflammatory transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Tat protein also elicits the expression of adhesion molecules E-selectin, vascular cell adhesion protein 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), which alter endothelial permeability (Willerson & Ridker 2004, Wang, Yi et al. 2015).

It has been found that HIV infected individuals have an increased levels of endothelial micro-particles (released particularly during apoptosis), which may be seen as a marker of EC injury. Furthermore, it has been established that HIV infected individuals have higher levels of cellular adhesion molecules in comparison to the control individuals (Francisci, Giannini et al. 2009, Wang, Melancon et al. 2013). The individuals also appear to have increased levels of plasma proinflammatory cytokines such as TNF- α, interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), monocyte chemotactic protein 1 (MCP-1), and VCAM-1, ICAM-1, P-selectin, and von Willebrand factor (vWF). All of the above mentioned inflammatory biomarkers appeared to be elevated irrespective of the antiretroviral therapy. In addition, a study has shown that ED is more pronounced when HIV infection is severe and more advanced (Wang, Yi et al. 2015, Subbarao, Lowe et al. 2011). Furthermore, impaired endothelial function was demonstrated by means of FMD in HIV infected individuals compared to the control (Skowyra, Zdziechowicz et al. 2012, Solages, Vita et al. 2006).

1.9.3 ART and ED

Several studies have suggested that ART may result in the development of ED. Particularly NRTIs and PIs seem to play a major role in ED (Skowyra, Zdziechowicz et al. 2012, Subbarao, Lowe et al. 2011). HAART can play a role in the aetiology of ED in several ways. Firstly, it has been found to cause a significant increase in total cholesterol (TC) and triglyceride (TG) levels. Secondly, HAART in some individuals has led to insulin resistance or lipodystrophy (Mooser 2003, Skowyra, Zdziechowicz et al. 2012). Most notably ART drugs from the PI class are known to cause adverse effects on lipid profile. Studies have also found an increased level of P-selectin, tissue plasminogen activator and plasminogen activator inhibitor in treated HIV individuals when compared to the ART naive group. In addition, higher levels of cholesterol co-exists with a high concentration of P-selectin,

t-PA and ICAM-1 (whereas triglycerides correlate with plasminogen activator inhibitor 1) (De Gaetano Donati, Rabagliati et al. 2003, Skowyra, Zdziechowicz et al. 2012). Furthermore, HAART has been shown to result in decreased NO levels while increasing ROS and cholesterol, which could accelerate foam cell formation (as seen in atherosclerosis) (Chan, Sviridov et al. 2009, Skowyra, Zdziechowicz et al. 2012).

While analysing the literature, most studies investigating the effects of HAART on ED were found to be performed on two groups of drugs: PIs and NRTIs. The PIs are found to directly induce ED. They are thought to be responsible for mitochondrial DNA damage in ECs which can lead to total destruction of the endothelium independent of the individual's lipid profile (Zhong, Lu et al. 2002a, Skowyra, Zdziechowicz et al. 2012). It has been found that HIV infected individuals on PIs have a significantly lower FMD values and higher carotid intima media thickness (CIMT), a method to detect early atherosclerotic vascular disease. However, during analysis of specific drugs, there are some contradicting results. For instance, dramatic endothelial impairment is found with the drug indinavir, whereas the newer PI drugs such as lopinavir and ritonavir have not been shown to induce ED in HIV infected individuals (Dube, Shen et al. 2008, Grubb, Dejam et al. 2006, Skowyra, Zdziechowicz et al. 2012).

Interestingly, NRTIs are also found to increase total cholesterol, LDL-cholesterol and TG. Individuals on drugs from this class are found to be more prone to develop vascular complications as these drugs are associated with endothelial damage. In addition, NRTIs lead to an increased level of the vasoconstrictor endothelin-1, cell proliferation and cause the appearance of intracellular gaps in endothelial surface (Hebert, Crenshaw et al. 2004a, Skowyra, Zdziechowicz et al. 2012) . Moreover, a significant negative influence on the aortic endothelium-dependent relaxation is observed (Skowyra, Zdziechowicz et al. 2012). Furthermore, drugs from this class are suspected to increase the production of ROS. The drug abacavir (ABC) is particularly known to play a role in ED. It has been found that individuals treated with ABC present with a more severe form of ED in comparison to other drugs in this class. In general, NRTI intake was also associated with higher levels of proinflammatory cytokines and CRP (DAD Study Group 2007, Skowyra, Zdziechowicz et al. 2012).

1.9.4 HIV infection *in vitro* studies

In vitro infection of human umbilical vein endothelial cells (HUVECs) with HIV-1 has previously been demonstrated (Kline & Sutliff 2008). The method used in the study involved inoculation of the cells with the different isolates of HIV-1 infection (Corbeil, Evans et al. 1995). It was found that HIV

infection in HUVECs significantly enhanced viral protein synthesis and up regulates the production of interleukin-6 (IL-6), and granulocyte colony-stimulating factor, which are cytokines that stimulate HIV-1 synthesis (Conaldi, Serra et al. 1995, Kline & Sutliff 2008).

There are various other *in vitro* HIV infection models that can be used for experiments. One such method is the use of HL2/ 3 cells. These cells have been used by many studies to demonstrate the HIV infection in *in vitro* models. The cells are derived from the Hela cell line and are transfected with a clone of the HXB2 strain that lack the reverse transcriptase coding sequence. This results in a high level production of the HIV-1 gag, Env (particularly gp120), Tat, Rev and Nef proteins without shedding of infectious viral particles (Ciminale & Felber et al. 1990, Klug, Ashkenazi et al. 2014, Africa 2014). For this very reason the HL2/3 cell line can be used as an ideal research tool for the investigation of the inflammatory viral proteins or as a HIV cell model to test the effects of the various ARV drugs on cells exposed to HIV related proteins *in vitro*.

1.9.5 ART and ED in *in vitro* studies

In comparison to studies investigating the role of ART in ED in humans, *in vitro* studies are scarce. Studies focus mostly on cell toxicity. However, it has been found that ART can lead to direct microvascular changes. It has been found that drugs from the class of NRTIs have a role in ED (Sutliff, Dikalov et al. 2002). Notably, Zidovudine (AZT), seems to be a major role player in inducing cytotoxicity in cultured endothelial cells without the induction of apoptosis. Zidovudine has also been found to promote the release of vasoconstrictor and mitogen endothelin 1 and enhance proliferation of vascular smooth muscle cells and HUVECs (Hebert, Crenshaw et al. 2004). In addition, cytotoxic effects of AZT have been found, where intercellular gaps between adjacent human coronary artery endothelial cells are damaged and hence promoting a platform for the inflammatory cascade (Jiang, Hebert et al. 2007, Fiala, Murphy et al. 2004). Furthermore, cell culture experiments show an enhanced endothelial ROS production, in response to AZT (Kline & Sutliff 2008).

The drugs from the class of PI, have been of great interest in *in vitro* experiments. Many studies have demonstrated PI-induced endothelial dysfunction (Wang, Chai et al. 2007). PI treatment in *in vitro* experiments have demonstrated serious mitochondrial disturbances by reduced cellular respiration and ATP production. The drugs in this class are further implicated to increase production of ROS in experiments (Kline & Sutliff 2008). They have been found to also increase endothelial cell permeability and leukocyte adhesion in cell culture models (Mondal, Pradhan et al. 2004, Kline & Sutliff 2008). Notably, the drug ritonavir has been found to directly cause endothelial mitochondrial DNA damage and cell death, especially via the necrosis pathway (Zhong, Lu et al. 2002, Subbarao,

Lowe et al. 2011). Furthermore, ritonavir has been found to decrease eNOS mRNA and protein levels in cultured human coronary endothelial cells (Fu, Chai et al. 2005, Subbarao, Lowe et al. 2011).

1.10 Concluding Remarks

The introduction of ART has substantially decreased HIV/AIDS related mortality. HIV infected people are living longer with a better quality of life, however infected people are riddled with earlier unrecognized complications such as endothelial dysfunction and cardiovascular diseases (Skowyra, Zdziechowicz et al. 2012, Gresele, Falcinelli et al. 2012). The underlying mechanisms of these complications are unclear. As discussed, there are many reports indicating that HIV infected individuals on ART are at a greater risk of developing CVD. ED is an initiating event in atherogenesis and may contribute to HIV associated atherosclerosis. We are now aware, that the virus interacts with the cardiovascular system (CVS), especially the endothelium and platelets (Subbarao, Lowe et al. 2011). In fact, the virus itself may lead to ED. However, it is not well understood whether HIV or the ART is more responsible for ED. Furthermore, it is unclear as to which ARV drugs or regimens are more associated with cardiovascular risk and ED. Therefore, further exploration in the matter is warranted.

1.11 Problem Identification & Aims of the Study

1.11.1 Problem identification

Very few studies have assessed cardiovascular risk factors in HIV infected individuals in low income countries (Mashinya, Alberts et al. 2015). Most studies on cardiovascular risk factors in HIV individuals are performed in Western countries (Mashinya, Alberts et al. 2015). Furthermore, there is a paucity of studies on cardiovascular risk factors in the HIV population in South Africa (Fourie, Van Rooyen et al. 2010, Malaza, Mossong et al. 2012, Mashinya, Alberts et al. 2015). A recent study described the association of ART and CVD risk in the rural individuals in the Mpumalanga Province of SA. However, the use of ART was self-reported in the study and according to the authors, the CVD risk may have been underestimated due to the stigma associated with HIV (Clark, Gómez-Olivé et al. 2015, Mashinya, Alberts et al. 2015).

Moreover, very few studies have investigated the association between HIV/ ART and ED in SA. A recent study in the Northwest confirmed the link between endothelial activation and HIV (Fourie, Schutte et al. 2015), however, very little has been investigated on the SA approved first (combination of efavirenz, emtricitibine and tenofivir) and second (lopinavir and ritonavir) line treatment on ED in adult South Africans and to the best of our knowledge nothing has been reported in Western Cape populations. In addition, very little is known about which type of treatment regimen is more prone to be associated with ED. Furthermore, *in vitro* studies investigating the effects of these particular drugs on ECs are scarce.

Therefore, the overall aim of the study was to investigate the association between HIV and the South African approved first line and second line ART drugs, endothelial function and cardiovascular risk. The epidemiological (main) study was underpinned by an *in vitro* sub-study.

1.11.2 Main aim of the study

To investigate the effects of first line and second line antiretroviral drugs on HIV exposed vascular endothelial function and cardiovascular risk.

1.11.2.1 Specific aims of the Epidemiological (main) Study

i. To investigate endothelial function in a cohort of HIV positive, ART naïve; HIV positive on first line ART treatment; HIV positive on second line treatment, and HIV negative participants. Endothelial function was assessed using non-invasive flow-mediated dilation (FMD) of the brachial artery, by Doppler technology.

ii. To investigate the possible association between the type of ART treatment (i.e. first line and second line), and changes in endothelial function in the participants.

iii. To investigate the possible association between the cardiovascular risk profile (dyslipidaemia, hypertension, obesity, diabetes mellitus and smoking) and endothelial function (as assessed by FMD) in the cohort, using data from comprehensive health questionnaires, anthropometric measurements, and clinical screening and serum analyses.

1.11.2.2 Specific aims of the *in vitro* sub-study

i. To develop an *in vitro* model of HIV-infection by exposing aortic endothelial cells (AECs) to growth medium containing HIV-related proteins.

ii. To investigate the effects of first line and second line ART drugs administered to AECs exposed to HIV-related proteins in culture on cell viability, NO production and oxidative stress status.

Chapter 2 : Methods (clinical study)

This chapter describes the experimental design and methods used for the epidemiological studies. All data was collected in partnership with an assigned post-doctoral fellow at the Worcester Community Health Care Centre.

2.1 Ethics approval

Ethics approval was received from the Health Research Ethics Committee (HREC) of the University of Stellenbosch (ethics reference: N12/12/086). Good Clinical practice (GCP) was maintained throughout the study and adhered to the South African GCP guidelines. Furthermore, research was conducted in line with the standard operating procedures and guidelines of the HREC of the University of Stellenbosch.

2.2 Study design and participants

This was a sub-study embedded in a larger, international study called 'EndoAfrica'. The study was of a cross sectional nature and data were collected from May 2015 to July 2016. The study was in collaboration with the Winelands HAART to HEART study lead by Prof Faadiel Essop from the Department of Physiological Sciences of Stellenbosch University. The participants were recruited from Worcester and surrounding areas of the Western Cape Province by a Health Professions Council of South Africa (HPCSA) registered nurse. **Refer to table 2.1** for the inclusion and exclusion criteria for the participants of this study.

Table 2.1: *Inclusion and exclusion criteria used for the participants of the study*

Inclusion criteria	Exclusion Criteria
Participants ≥18 years of age	Participants < 18 years of age
Not pregnant	Pregnant
≥ 3 months' post-partum	< 3 months' post-partum
HIV-positive participants' not on treatment	Terminally ill
HIV-positive participants on first line ART	Active TB
HIV-positive participants on second line ART	

Abbreviations: ART, antiretroviral therapy

The recruitment commenced a few days before the assessments, where the participants were informed about the study and requested to fast for at least 10 hours prior to the assessments. Assessments commenced at 08h00 in the morning. This also ensured that the participants had some time to make an informed and a well thought decision before volunteering to be part of the study. The research nurse had access to the clinic patient files and was able to recruit participants based on their HIV status. Furthermore, HIV testing for participants with unknown status was performed with the Quality advance HIV virus, rapid confirmation test by Abon, China. The participants were also pre and post counselled by the registered nurse who had training in HIV counselling. The study consisted of four groups of participants, stratified according to their HIV status and ART (**refer to figure 2.1**). Informed consent was obtained from the participants prior to any assessments (**refer to appendix A** for the informed consent document)

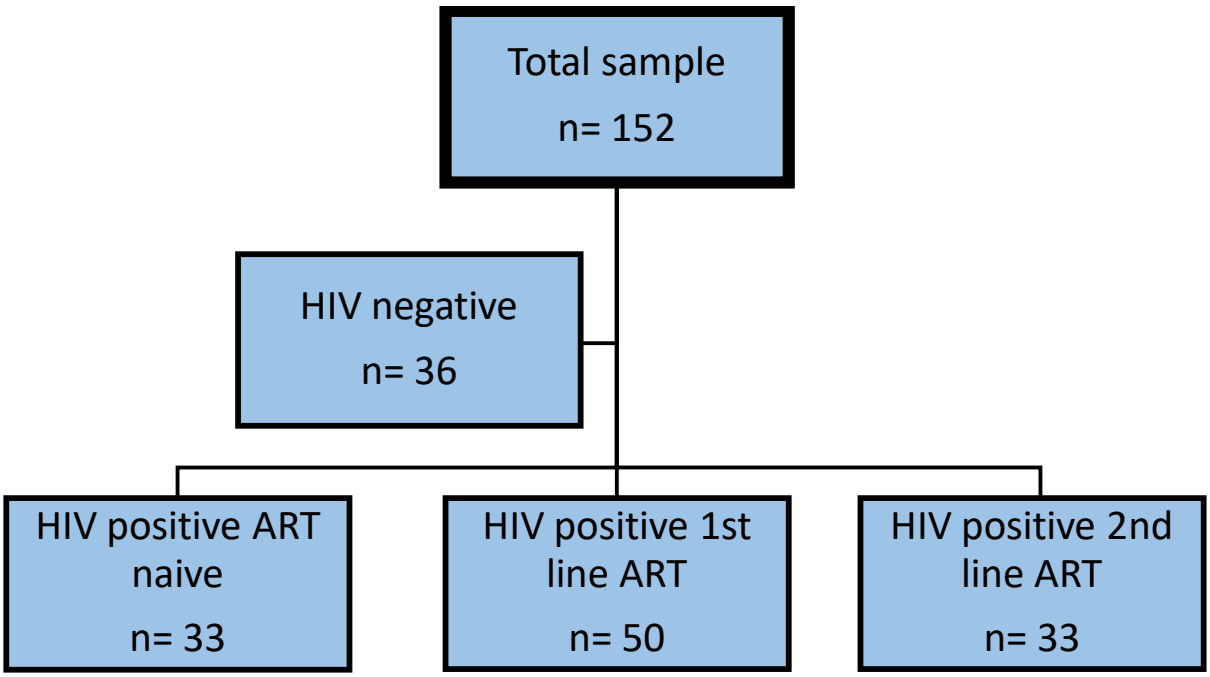


Figure 2.1: A schematic representation of the four study groups and their respective sample sizes.

The HIV negative participants were recruited in the same manner as those with HIV. They predominantly belonged to the same socio economic status and ethnicity as the HIV positive participants. They were individuals from the community who attended the clinic as out-patients, or worked at the clinic, and were recruited by the research nurse based on the study's inclusion criteria. This recruitment process enabled the study to consider the HIV negative group as a relevant control group for the HIV positive groups.

2.3 Qualitative assessments (Medical History and Lifestyle questionnaires)

Qualitative data was collected by means of an interview and questionnaire. Medical history and lifestyle parameters including smoking and drinking (alcohol consumption) status were assessed. **Refer to appendix B** for the questionnaire used in the study. The participants were asked the questions objectively in either English, Afrikaans or Xhosa language based on their language preference. In addition, the patient clinic file was used to retrieve and confirm medical and treatment history data of all the participants.

2.4 Clinical investigations

2.4.1 Biochemical assessments

Fasting whole blood samples were collected by the research nurse and prepared according to standardized methods. Serum C-reactive protein (CRP), whole blood fasting glucose and glycated haemoglobin (HbA1c), as well as serum triglycerides, high density lipoprotein (HDL), low density lipoproteins (LDL), and total cholesterol were analysed by the National Health Laboratory Services (NHLS), Worcester, Western Cape which was located at the Worcester Hospital, next to the clinic (field site). In addition, CD4 and viral load levels were determined by the NHLS, Green Point, Western Cape, for the HIV-infected participants. These samples were transported to the NHLS by the research nurse or the clinic porters, immediately after collection of blood from all the participants on the day.

2.4.2 Anthropometric measurements

The anthropometric measurements included: body mass index (BMI), waist and hip circumference, and waist-hip ratio (WHR). Before the body mass was recorded, a digital Electric Platform scale (Adult, 200kg, 0.02 increments) was set to zero, after which the participant was instructed to stand on the centre of the scale without any support. The measurement was recorded to the nearest 0.1 kg. Height was measured using a stadiometer to the nearest 0.1cm. The participant was requested to stand on the centre of the base with their back to the stadiometer. They were requested to keep their feet together and move back until their heels, buttocks and upper part of the back has touched the stadiometer. BMI was calculated by dividing the body mass by height squared. Waist circumference (WC) and hip circumference (HC) were measured using a measuring tape. A cross hand technique was used and participants were requested to stand in upright position with their abdomen relaxed, arms at sides and feet together. For WC, the measuring tape was placed around the participant's waist between the 10^{th} lower costal rib and the superior iliac crest at the narrowest plane of the abdomen. For HC, the measuring tape was placed around the largest area of the buttocks around the gluteal muscles of the participant with their feet together. Both WC and HC measurements were taken to the nearest 0.1cm. Waist to hip ratio was calculated by dividing WC by HC.

2.4.3 Blood pressure measurements

Blood pressure and heart rate measurements were recorded using a digital OMRON® sphygmomanometer. The participants were requested to sit in a relaxed position before the measurement was taken. All blood pressure and heart rate measurements were taken in triplicate to ensure accuracy and minimise white coat hypertension and possible anxiety. There was a 15 to 20 min period of rest between each of the three measurements.

2.4.4 Measurement of endothelial function- Flow mediated dilatation (FMD)

As described earlier in chapter 1, section 1.5.1, FMD is a non-invasive technique for the assessment of endothelial function. For this study, FMD was measured on fasted participants in a supine position in a quiet room. A blood pressure cuff was wrapped below the antecubital fossa on the right arm of the participant. The brachial artery of the right arm was located in B mode with an Esaote MyLabTMFive ultrasound, (Maastricht, Netherlands) and 12 MHz linear probe, after application of CLINICA ultrasound gel. The probe was securely fixed on a single axis probe holder from SMT Medical, (Graz, Austria) (**refer to figure 2.2 and 2.3**), ensuring a stable image of the located artery. Doppler mode was used to visualise and locate the brachial artery, with pulse repetition frequency (PRF) set at 6.7 kHz. Once the artery was clearly visualised, depth was increased to 3 cm before switching to pulse wave mode. In pulse wave mode, the angle of insonation was set to 60^0 (**refer to figure 2.4**)**.** Following this, the brachial artery diameter and shear rate were automatically recorded throughout the assessment (with FMD studio and Cardiovascular Suite version 2.8.1. edge detection, QUIPU software, Pisa, Italy) on an Apple MacBook Pro laptop (2010 edition). Baseline brachial artery diameter was recorded for 60 seconds (**refer to figure 2.3. A**). After the baseline recording phase, occlusion of the brachial artery was achieved via inflation of the blood pressure cuff to a supra-systolic pressure of 200 mmHg (or 50 mmHg above the prior systolic pressure measurement) for 5 min (refer to figure 2.3. B). Finally, the cuff was deflated and the post-occlusion artery diameter was recorded for a further 120s **(refer to figure 2.3. C**). FMD was then automatically calculated by the FMD studio and Cardiovascular Suite QUIPU software as the ratio of brachial artery diameter after reactive hyperaemia to the baseline diameter and was expressed as a percentage change. In summary, the basic formula that the softer used to calculate FMD % was:

$$\frac{\textbf{Maximum diameter} - \textbf{Mean Baseline Diameter}}{\textbf{Mean Baseline diameter}} \textbf{ X 100}$$

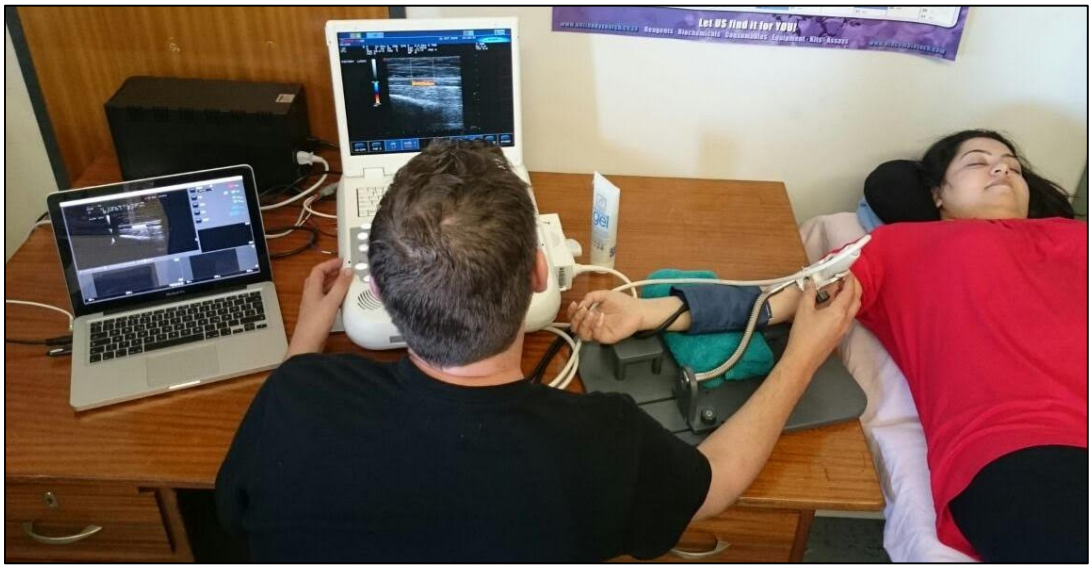


Figure 2.2: A photograph representing a typical FMD assessment with the MyLabTMFive ultrasound and 12 MHz linear probe. On the left, is an Apple Macbook pro laptop with FMD studio and Cardiovascular Suite version 2.8.1. edge detection QUIPU software that records the artery diameter and shear rate throughout the assessment. The study participant is in the supine position with the right arm resting on the probe-holder platform. (This photograph features individuals from the study research team, and consent was given by both individuals to include it in the thesis).

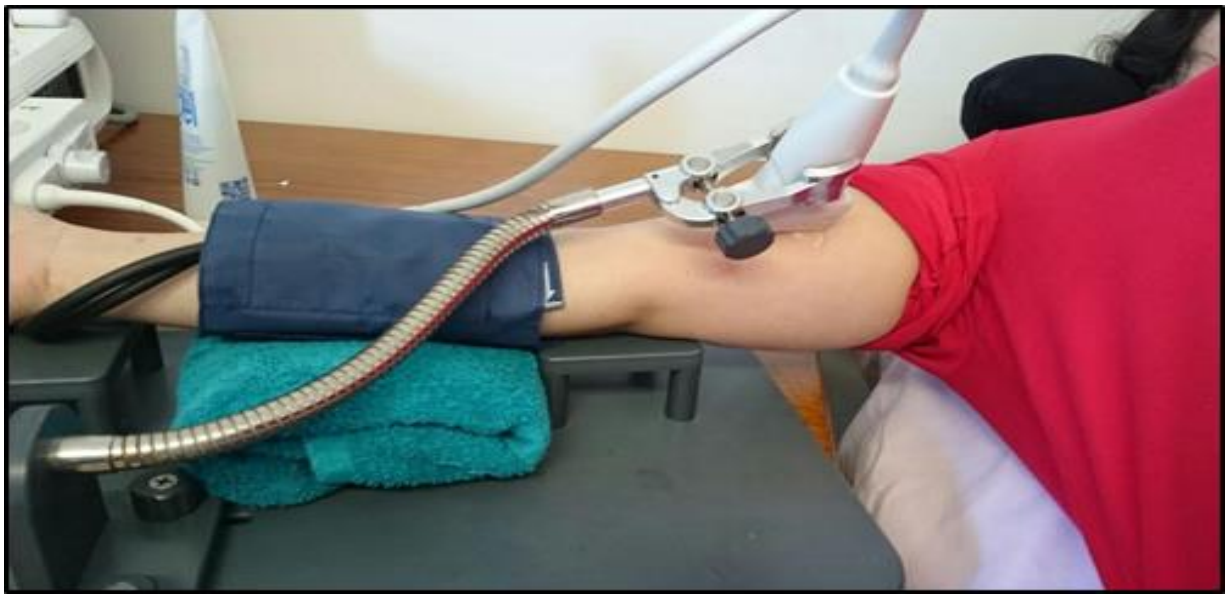


Figure 2.3: A photograph depicting the blood pressure cuff wrapped around the right arm of the study participant and the probe securely fixed in the SMT medical probe holder.

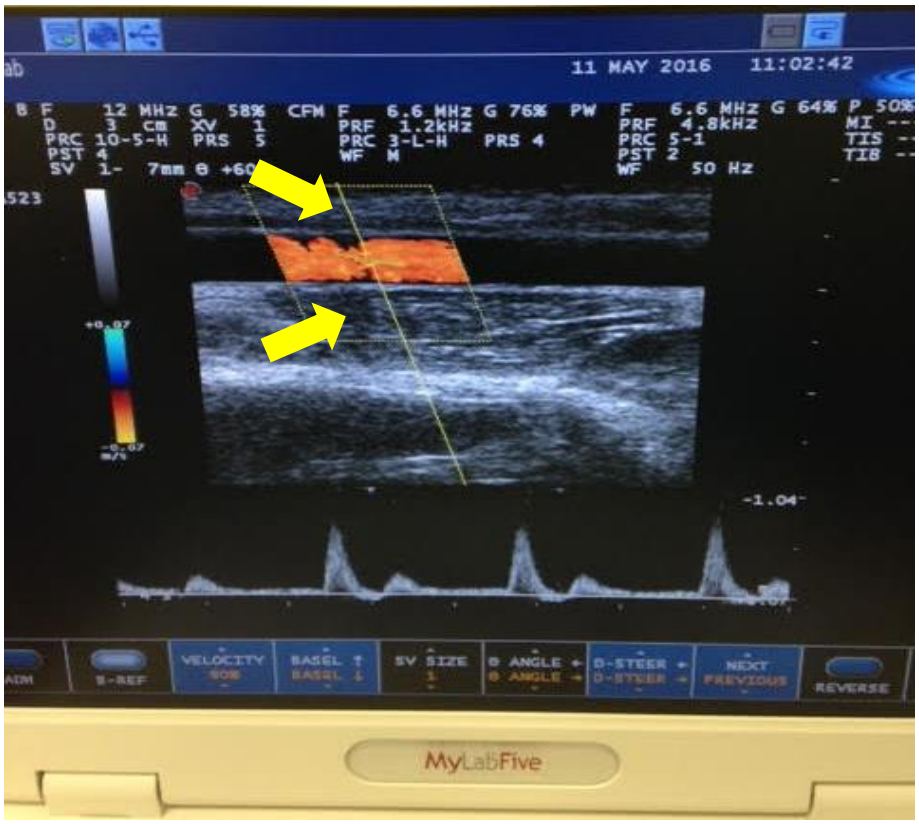
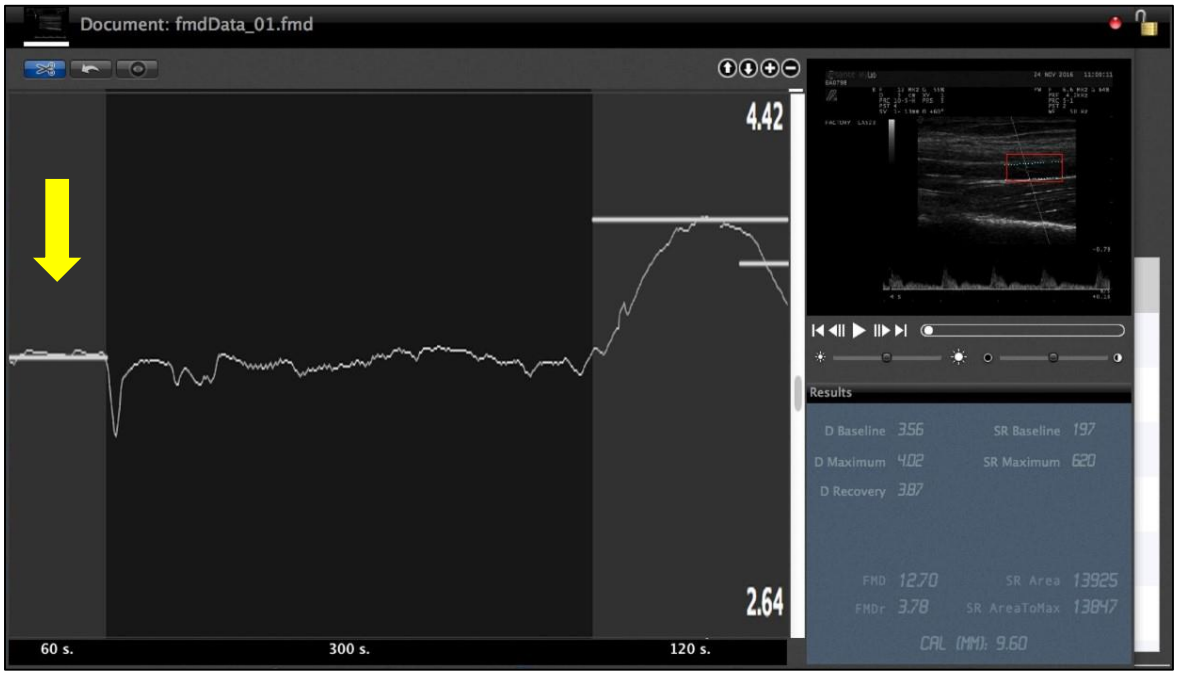


Figure 2.4: An image of the brachial artery on an Esaote MyLabTMFive ultrasound with the correct settings in Doppler mode. The yellow arrows indicate the location of the brachial artery on the image.

A.



B.

C.

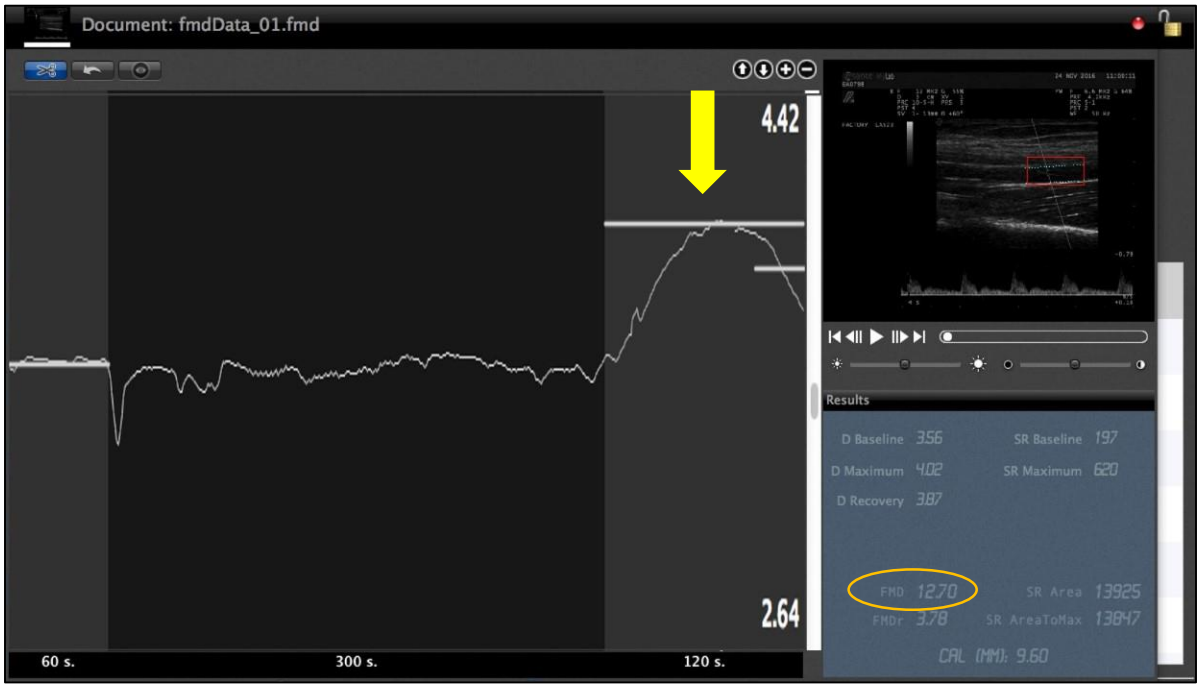


Figure 2.5: An example of Flow mediated dilation (FMD) technique in 3 phases using FMD studio and Cardiovascular Suite version 2.8.1, edge detection software images. The yellow arrows indicate the phase of interest in each image A. Baseline artery diameter is measured for a duration of 60 seconds. The recording is relatively stable indicating no or minimal changes in the artery diameter. B. Inflation of the blood pressure cuff (occlusion) for a total duration of 300 seconds which leads to ischaemia C. Deflation of blood pressure cuff leading to hyperaemia and subsequent shear stress-induced arterial dilation with maximum diameter recording followed by recovery diameter recording (total duration 120 seconds). FMD is then calculated as a percentage change in the arterial diameter from the baseline to maximum diameter. The FMD % is circled in yellow on the image for this particular assessment.

2.5 Experimental protocol

In summary, the assessments were performed in a stepwise manner (**refer to figure 2.6**). Step one included recruitment, scheduling of an appointment obtaining informed consent, performing anthropometric measurements and collection of blood samples from the left arm. Step two comprised of blood pressure measurements and collection of qualitative data via an interview and filling in of a health questionnaire. The final step comprised of the FMD measurement of the participant's brachial artery. Upon completion, participants were offered a small snack as they had arrived fasted for the assessments.

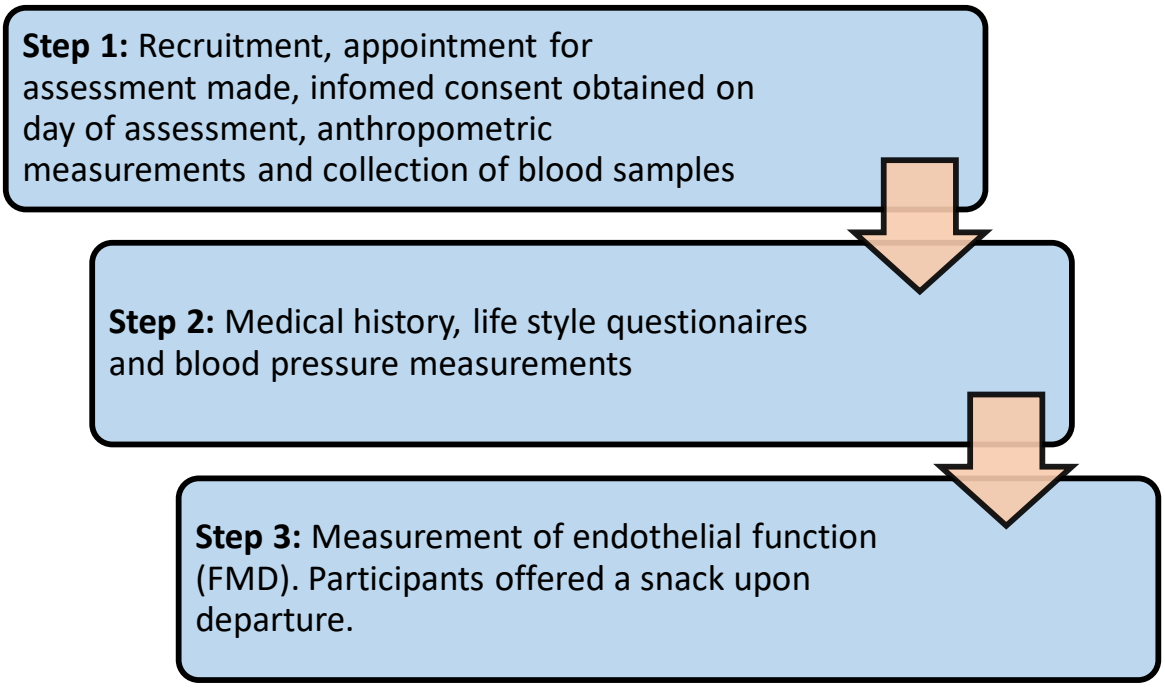


Figure 2.6: A schematic representation of a stepwise experimental protocol.

2.6 Statistical analysis

All collected data was captured in Microsoft Office Excel 2016. Stata® version 14 was used to analyse the data and the level of significance was set at $p < 0.05$.

Descriptive statistics: For normally distributed data, one-way ANOVA was performed with Bonferroni *post hoc* test to determine significant differences between the four groups. Pearson's Chi square analysis was performed for categorical data. In addition, for non-parametric data, a pairwise comparisons of group was done using a one way Kruskal-Wallis ANOVA.

Independent predictors and associations: Multiple linear regression models were used, with entry and exit criteria set at 0.05 and 0.1 respectively, and data were stratified on group for all models. The remaining predictor variables were entered in a stepwise forward fashion. The regression analysis were performed to determine independent predictors and associations in each of the 4 study groups as well as in total HIV positive and the total HIV negative group. In addition, regression analysis was performed on the total population. Baseline artery diameter, maximum artery diameter and FMD% were used as dependent variables in the regression models. Independent variables included HIV status (for total population), gender, age, use of cardiovascular medication, smoking history, waist circumference, mean systolic blood pressure, LDL cholesterol, HDL cholesterol, CRP, and fasting glucose. The confounding variables gender, age, use of cardiovascular medicine, smoking and waist circumference were forced to remain in all regression models. Additionally, CD4 was included as an independent variable in the HIV positive groups. The candidate performed all statistical analyses using Statistica Software version 13 (StatSoft, Inc., USA) the level of significance was set at $p < 0.05$, which were subsequently independently validated by a professional biostatistician from the Biostatistics Unit, Faculty of Medicine and Health Sciences of Stellenbosch University. The results from the biostatistician is included in this thesis.

Chapter 3 : Methods (*in vitro* sub-study)

This chapter describes the materials and methods used for the *in vitro* sub-study.

3.1 General materials

- *Lonza Ltd* (Clonetics, Cambrex Bio Science, Walkersville, USA) (supplied by Whitehead Scientific locally): Endothelial cell growth medium (EGM-2) and Dulbecco's modified Eagle's Medium (DMEM) with 4.5g/L glucose and L Glutamine.
- *Life Technologies* (Carlsbad, California, USA): Attachment factor and trypsin (500 BAEE units' trypsin / 180 µg EDTA•4Naper ml in Dulbecco's PBS)
- *Biochom-Biotech*: Foetal bovine serum (FBS).
- *Calbiochem* (San Diego, CA, USA) (supplied by Merck locally): 4,5-diaminofluorescein-2/diacetate (DAF-2/DA).
- *Sigma-Aldrich* (St Louis, Mo, USA): Dimethyl sulfoxide (DMSO); 2′, 7′-Dichlorofluorescein (DCF); 2,3-Dimethoxy-1,4-naphthoquinone(DMNQ); Diethylamine NONOate diethylammonium salt (DEA/NO) and Penstrep.
- *Bio-Legend* (San Diego, CA, USA) (supplied by Biocom-Biotech locally): Propidium iodide solution and cell staining buffer.
- *VEC Technologies* (University at Albany Foundation, 1 University Pl, Rensselaer, NY 12144, USA): Aortic endothelial cells (rat) (AECs).

3.2 Introduction to cell culture technique

Cell culture is a process whereby cells are grown in a condition outside of their natural environment. All cells were cultured and incubated in a standard CO_2 incubator (Forma Series II, Thermo Electron Corporation, Waltham, MA, USA) at 37^0 C, 40 – 60% humidified and 5% CO_2 atmosphere. The cells were cultured in a sterile environment and passaged (division and growth of cells) from one generation to the next.

3.2.1 The trypsinzing and passaging procedure of AECs

Cells received in a single 75mm^2 flask (VEC Technologies), were passaged as shown in **figure 3.1** and stored in liquid nitrogen for later use. Pellets from these passages were stored in 1ml of "freezing medium" containing 900µl FBS, 50µl AEC medium and 50µl DMSO. For experimental purposes, cells were removed from storage in liquid nitrogen, cultured in 35mm petri dishes and 2ml specialized aortic endothelial growth medium (supplemented with 10% FBS, vascular endothelial growth factor - VEGF, human epidermal growth factor – hEGF, human fibroblastic growth factor hFGF, long chain human insulin-like growth factor - R3-IGF-1, ascorbic acid, hydrocortisone and gentamicin and amphotericin anitbiotics) and passaged according to their "P-status" (P – parent generation) as shown in **figure 3.1**.

All experiments were performed once the AECs reached confluency, which secured cell cycle arrest which basically entails that any further cell proliferation is terminated at the G0 phase due to cell to cell contact. This essentially minimizes possible cell cycle variability when evaluating experimental results. At this stage the cells were still morphologically and functionally viable as well as they yielded the required number of plates for statistically acceptable sample sizes. Petri dishes were randomly assigned to respective control and experimental groups.

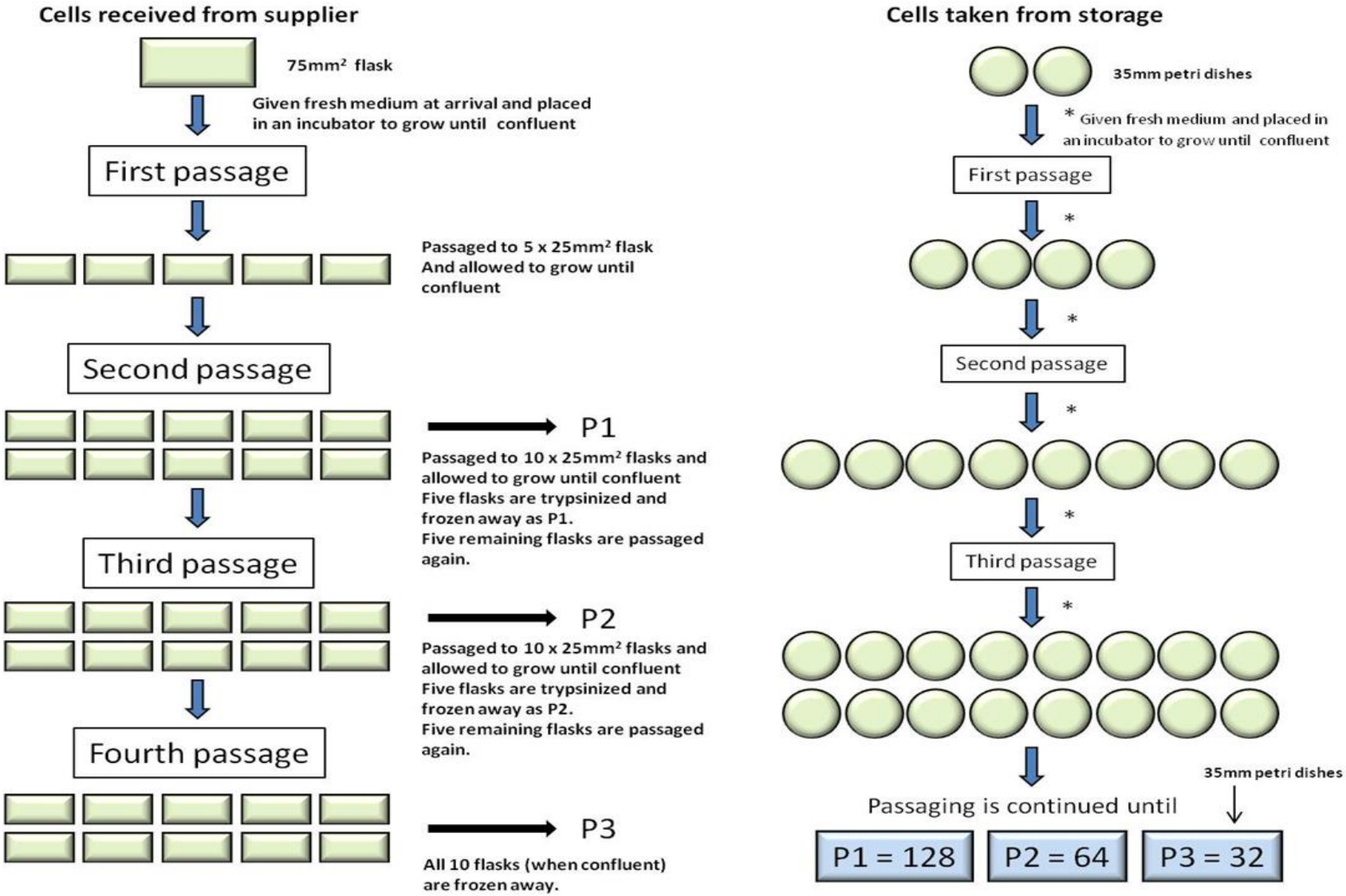


Figure 3.1: Cell culture passaging procedure (Genis; PhD dissertation 2014).

3.2.2 HL2/3 cell cultures HIV- Conditioned *in vitro* model

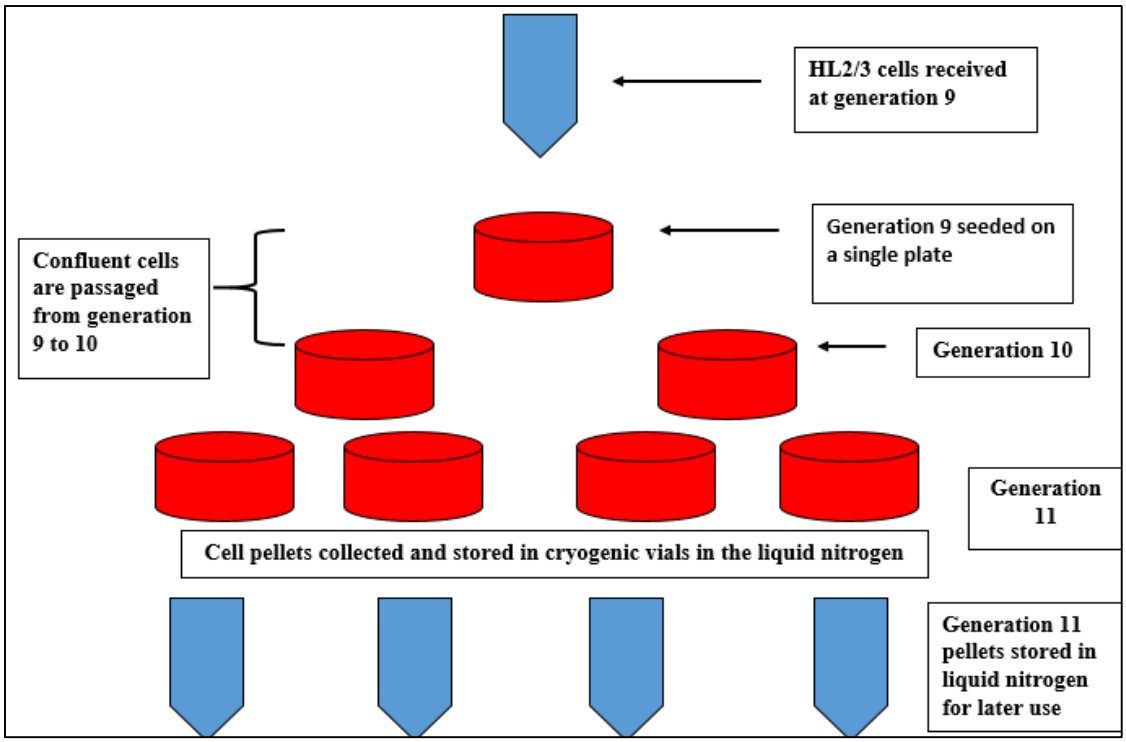
In order to simulate a HIV-infection micro-environment, AECs were incubated in conditioned growth medium that had previously been exposed to HL2/ 3 cells. HL2/3 cells are known to secrete HIV proteins into the growth medium and were kindly donated by Dr Carine Smith from the Department of Physiological Sciences, University of Stellenbosch. The HIV-protein conditioned medium protocol using HL2/3 cells has previously been demonstrated (Africa and Smith 2015).

The HL2/3 cells were received at generation 9 (9^{th} passage) and can be sustained up to generation 15. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5g/L glucose and 4.5g/L L-Glutamine, and supplemented with 10% FBS and 1% penicillin streptomycin ("pen strep"). HL2/3 cells were cultured in 100x 20mm petri dishes and passaged up to generation 11, after which they were stored in liquid nitrogen for future use. **Refer to figure 3.2 A** for a complete schematic of the HL2/3 cells passaging procedure.

3.3 HIV conditioned medium protocol

Approximately two weeks before the intended use of the HIV conditioned medium, the stored HL2/3 cells (generation 11) were taken from storage and cultured. The cells were passaged until generation 14, and upon 75% confluency, the medium was replaced with AEC growth medium. The HL2/3 cells were allowed to grow in this medium for 24 hours, after which the HIV protein-containing conditioned AEC growth medium was removed from the cells and stored at -80 ºC (**refer to figure 3.2 B**).

A



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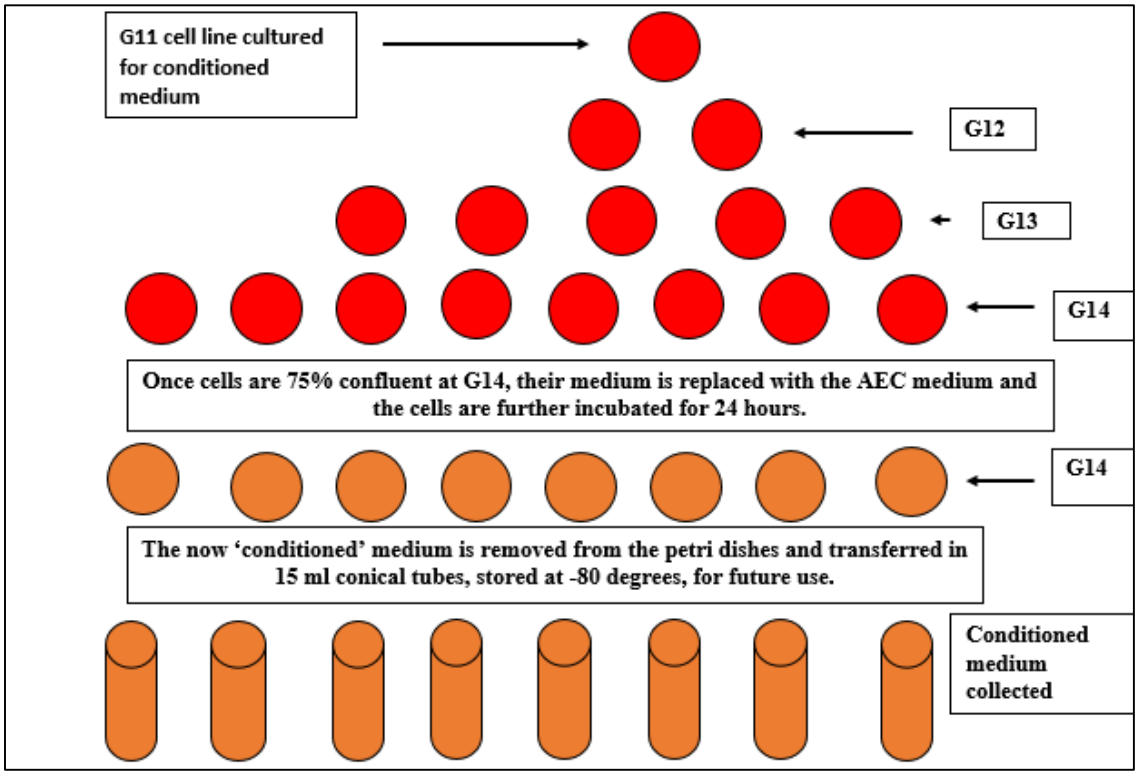


Figure 3.2: Schematic representation of the conditioned medium protocol.

3.4 Protein precipitation and extraction for Proteomics analysis

To identify and confirm that the conditioned growth medium contained HIV proteins, samples of the medium were sent for proteomics analysis at the Centre for Proteomic and Genomic Research (CPGR), Cape Town. For the purpose of proteomics analysis, the HL2/3 cells were incubated for 24 hours in medium containing no FBS, as it might "mask" the presence of other proteins, such as the HIV proteins of interest (Nonnis, Maffioli *et al*. 2016).

3.4.1 Precipitation process

A fixed volume was taken from the conditioned medium. Cold (-20°C) acetone (four times the volume of the medium) was added. The samples were vortexed thoroughly and left to incubate for 60 minutes at -20°C. Following this, the samples were centrifuged at 15000 rpm for 10 minutes. The supernatant was carefully removed (not to dislodge the precipitated protein pellet).

3.4.2 Protein extraction: from pellet with Lysis Buffer

To extract protein, 800 µl lysis buffer consisting of: 20mM Tris-HCl; 1mM EGTA; 1mM EDTA; 150mM NaCl; 1mM β-glycerophosphate; 1mM sodium orthovanadate; 2.5mM tetra-sodium diphosphate; 50µg/ml PMSF; 10µg/ml aprotinin; 10µg/ml leupeptinin and 1 % triton-X100 were added to each pellet. Samples were homogenized by use of a Heidolph Silent Crusher Homogenizer and left at 4 ºC for 20 minutes. After homogenization, the tubes were centrifuged at 15 000 rpm for 20 minutes, the supernatant was collected and the protein content was determined by the Bradford protein assay (Bradford, 1976).

The standard curve was set up by calculating the mean of the duplicate standard values (mean OD values) using Excel. The protein (µg) values were plotted on the X-axis against the mean OD values on the Y-axis. Using the standard curve, the amount of protein, lysis buffer and Laemmli sample buffer (Laemmli, 1970), that must be added to each sample, to ensure that an equal amount of protein per volume unit used was calculated.

850 µl Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptothanol (2-ME), 0.0004% bromophenol blue and 0.125 M Tris-HCl) was diluted with 150µl 2-ME before it was added to the lysates. After the lysates were prepared using the calculated values, the samples were boiled for 5 minutes and the aliquots were stored at -80°C.

The lysates were then loaded onto a gel and sent to the centre of proteomics and genomics research (CPGR) for the identification of the HIV related proteins. The methods used by CPGR included 'in gel digestion' and liquid chromatography–mass spectrometry (LCMS) **refer to appendix C,** analytical report by CPGR proteomics manager, Dr Zac Macdonald).

3.5 Flow cytometric analysis (for assessment of NO, ROS and cell viability)

Flow cytometry was originally developed in the late 1960s. Today it is a popular technique that utilises light to count and profile cells in a heterogeneous fluid mixture. It is a technology that is used to analyse the physical and chemical characteristics of particles in a fluid as it passes through a minimum of one laser. Cell components are fluorescently labelled and then excited by the laser to emit light at varying wavelengths. The fluorescence can be measured to determine various properties of single cells. Up to thousands of particles per second can be analysed as they pass through the liquid stream. A flow cytometer has five components:

1. A flow cell - liquid stream (sheath fluid), which is responsible for the carrying and the alignment of cells ensuring that single file passes through the light beam for sensing.
2. A measuring system - typically used to measure conductivity (measurement of impedance) and optical systems - lamps (mercury, xenon); high-power water-cooled lasers (argon, krypton, dye laser); low-power air-cooled lasers (argon (488 nm), red-HeNe (633 nm), green-HeNe, HeCd (UV)); diode lasers (blue, green, red, violet) resulting in light signals
3. A detector and Analog-to-Digital Conversion (ADC) system - which converts analog measurements of forward-scattered light (FSC) and side-scattered light (SSC) as well as dye-specific fluorescence signals into digital signals that can be processed by a binary computer
4. An amplification system - linear or logarithmic
5. A computer for analysis of the signals.

The process of collecting data from samples using the flow cytometer is termed 'acquisition'. Acquisition is mediated by a computer physically connected to the flow cytometer, and the software which handles the digital interface with the cytometer.

Flow cytometric analyses formed a cornerstone of the cell culture experiments and were conducted on a Becton-Dickinson FACSCalibur flow cytometer (Franklin Lakes, NJ) **(figure 3.3).** located in the Stellenbosch University-BD Flow Cytometry Unit, Department of Biomedical Sciences, Faculty

of Medicine and Health Sciences. Data were analysed by means of the FlowJo version 10.1 software package. For each experimental sample, a total of 5000 - 10 000 “events” (cells) were acquired and visualised on a scatterplot (**figure 3.4 A**). The cell population of a given sample was subsequently gated (**figure 3.4 B**) in order to exclude any debris and non-cellular particles. From the gated populations, the mean fluorescent intensity emitted by the fluorescent probe was determined and depicted on histogram graphs (**figure 3.4 C**). All fluorescence data are expressed relative to control which was adjusted to 100%.

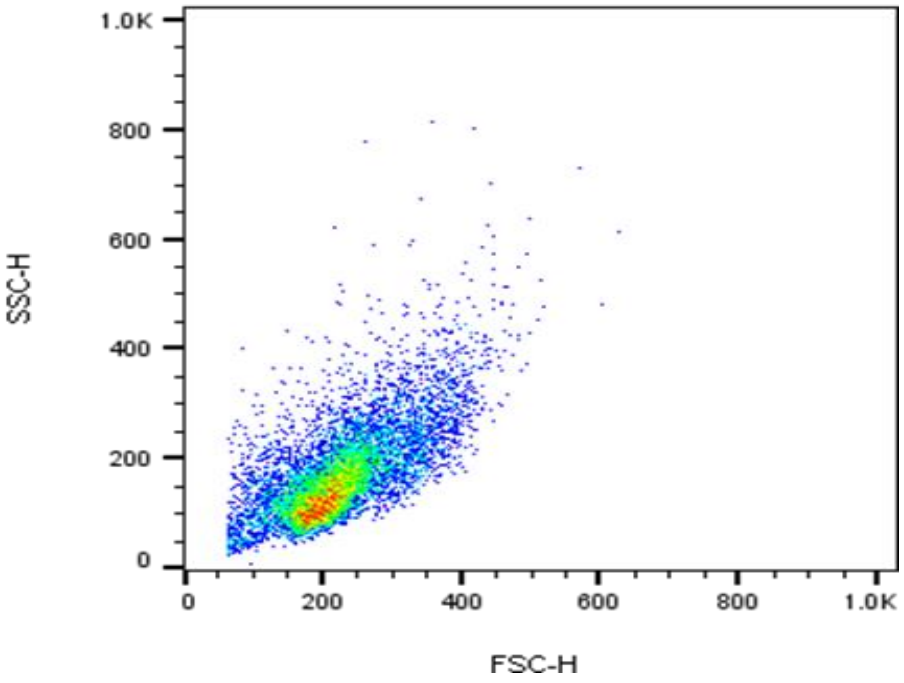
The flow cytometer has different channels under which the various probe experiments can be conducted.

- FL-1H: Excitation: 488 nm / emission: 530 nm (argon laser);
- FL-2H: Excitation: 488 nm / emission: 585 nm (argon laser);
- FL-3H: Excitation: 488 nm / emission: 670 nm (argon laser);
- FL-4H: Excitation: 635 nm / emission: 661 nm (red diode laser).

Each flow cytometry experiment included probe containing control samples and an absolute control (AC) sample without the probe which represented the auto-fluorescence of cells. The experiments also contained a few positive control samples which validated the specificity of the probe and the entire experiment in general. The positive control should demonstrate a significantly higher fluorescence compared to control samples for the experiment and probe to be considered valid.

Figure 3.3: FACS Calibur flow cytometer.

A



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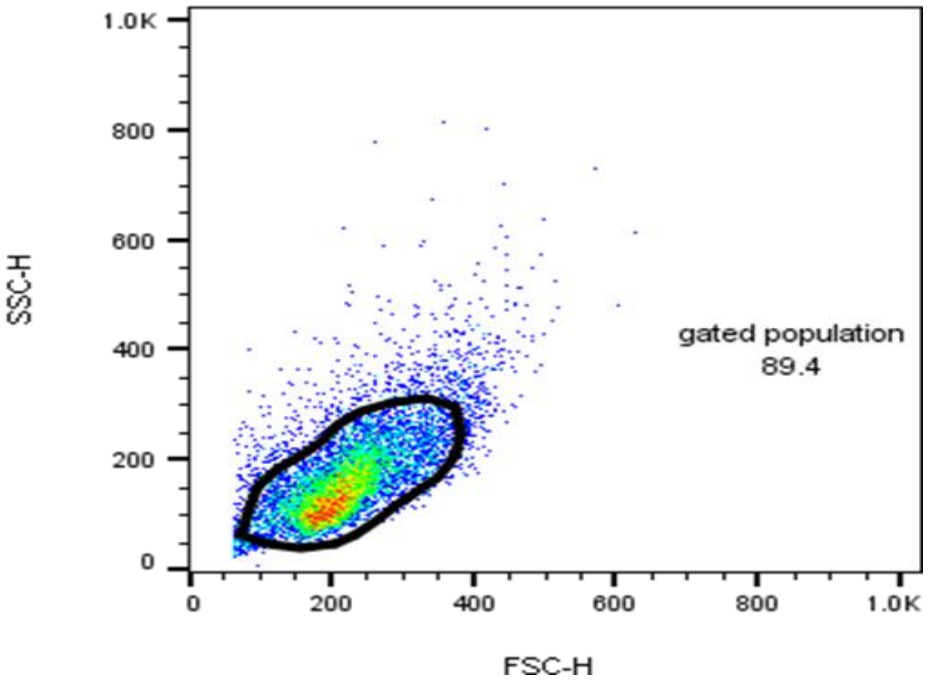

C

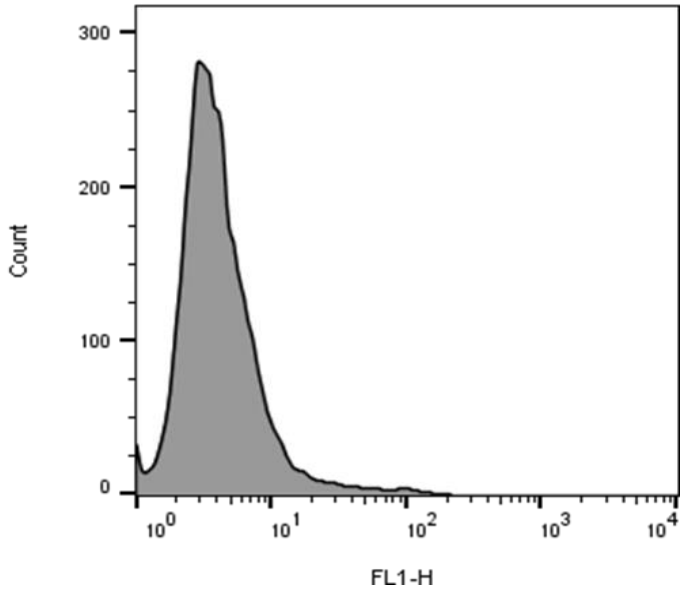

Figure 3.4: A representative scatterplot of an AEC sample indicating the forward scatter (FSCH; X axis), which measures cell size, and side scatter (SSC-H; Y axis) which measures cell granularity. B. The population of interest is gated and only events acquired within the gate will be taken into account in the histogram. C. A representative histogram plot showing the flow channel 1 (FL1-H; X axis) which measures fluorescence intensity and number of events/ counts (Events; Y axis).

3.5.1 NO measurements

3.5.1.1 Diaminofluorescein diacetate (DAF-2 DA)

DAF has been widely used for the measurement of nitric oxide (NO) in living cells and tissues (Zhou, He 2011). DAF-2 DA is a non-fluorescent cell permeable reagent that upon entry in the cell is hydrolysed by cystolic esterases, releasing DAF-2 and sequestering the reagent intracellularly. Upon reacting with NO, DAF- 2/DA is oxidized and emits a green fluorescent colour which can be analysed in flow channel 1 (FL1-H) of the flow cytometer. This 2-hour protocol of NO measurement has been previously established in our laboratory (Strijdom et al., 2004; Strijdom et al., 2006). Please **refer to figure 3.5** for a schematic representation of the protocol used for DAF-2/DA treatment.

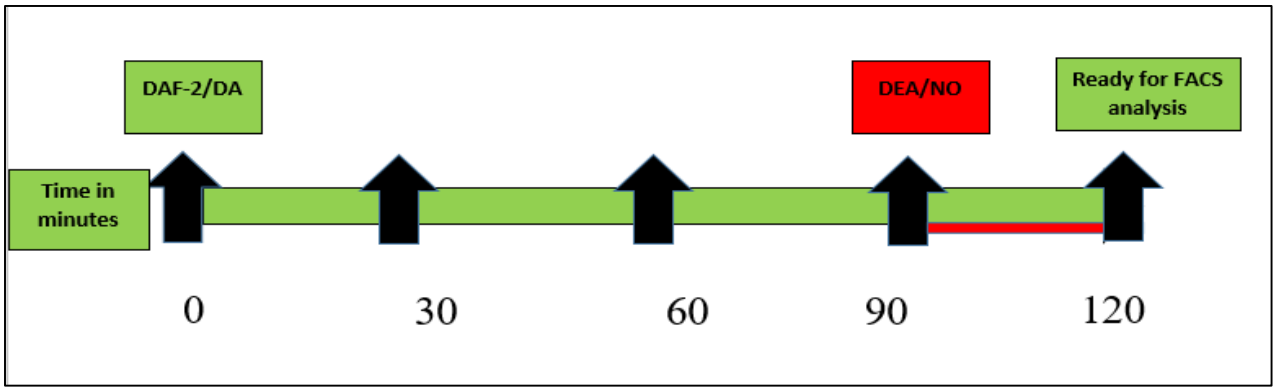

Figure 3.5: Schematic representation of the DAF-2/DA and positive control (DEA/NO) protocol.

3.5.1.1.1 Positive control: Diethylamine NONOate (DEA/NO)

It has been previously shown that DEA/NO, an NO donor is a consistent and reliable positive control for intracellular NO measured by DAF-2/DA (**refer to figure 3.6** for schematic DEA/NO protocol). It is able to significantly increase DAF-2/ DA fluorescence (**refer to figure 3.7**).

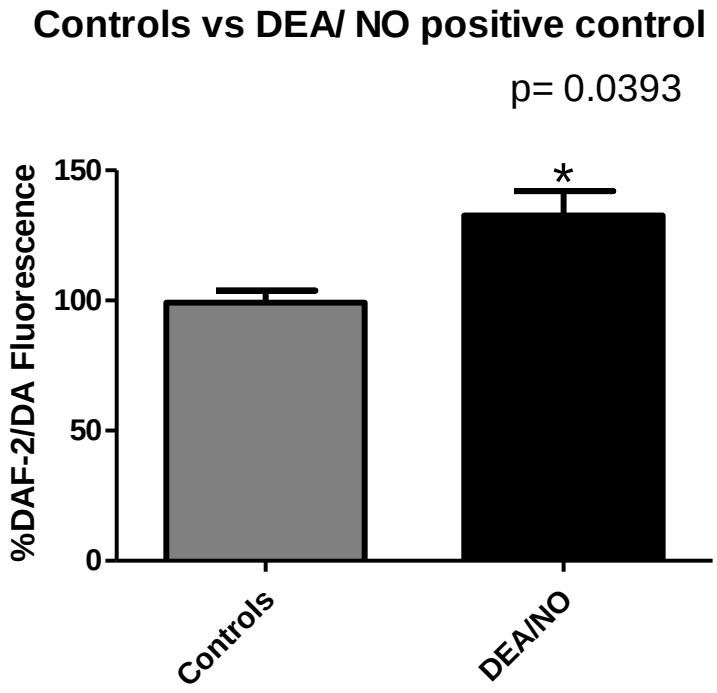

Figure 3.6: DEA/ NO significantly increased mean DAF-2/ DA fluorescence intensity and was included as a positive control in all DAF experiments, n = 11 / group.

3.5.2 ROS measurements

3.5.2.1. 2′,7′-Dichlorofluorescin diacetate (DCF)

DCF is a cell-permeable non-fluorescent probe, widely used to evaluate cellular ROS levels. DCF is chemically reduced and once it passes through the plasma membrane, it is de-esterified intracellularly to a hydrophilic alcohol and may turn into highly fluorescent 2′,7′-dichlorofluorescein due to oxidation that can be considered to involve ROS (Karlsson, Kurz et al. 2010). The oxidation can be as a result of acetate groups removing intracellular esterases within the cell, i.e. oxidation is by ROS. This results in a yellow fluorescent colour being emitted which can be analysed in flow channel 3 (FL3-H) of the flow cytometer. Therefore, cells that show high fluorescence are deemed to have higher ROS intracellularly. Please **refer to figure 3.8** for a schematic representation of the DCF protocol used.

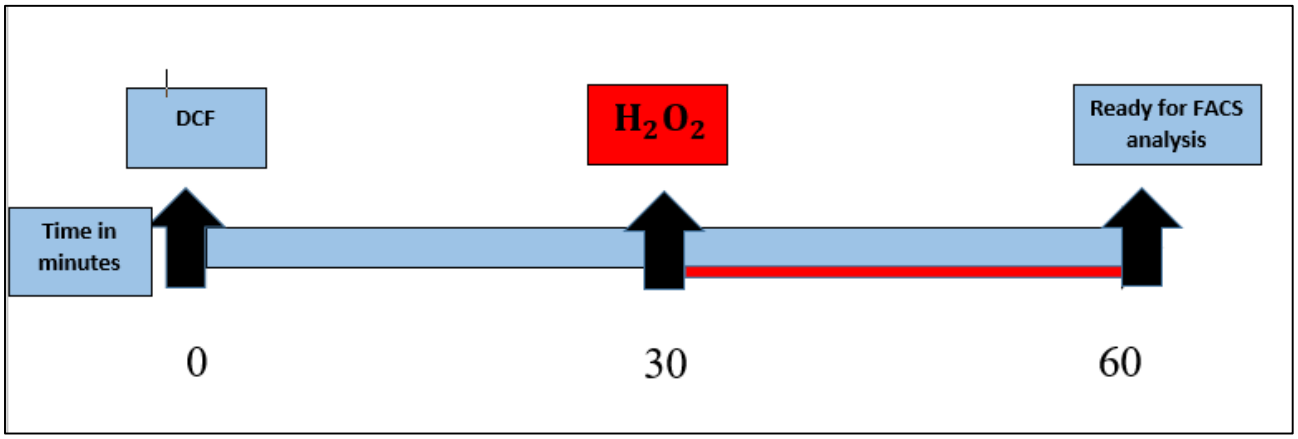

Figure 3.7: Schematic presentation of the DCF and positive control H_2O_2 protocol.

3.5.2.1.1 Positive control: Hydrogen peroxide (H_2O_2)

H_2O_2 was used as a positive control for DCF (**refer to figure 3.8** for a schematic H_2O_2 protocol). H_2O_2 induces oxidation and causes a redox reaction. Reaction with reactive oxygen species (ROS) results in the conversion of the non-fluorescent molecule DCF into fluorescent DCF which significantly increases DCF fluorescence intensity, making it an ideal control to validate effects of the DCF probe (**refer to figure 3.9**).

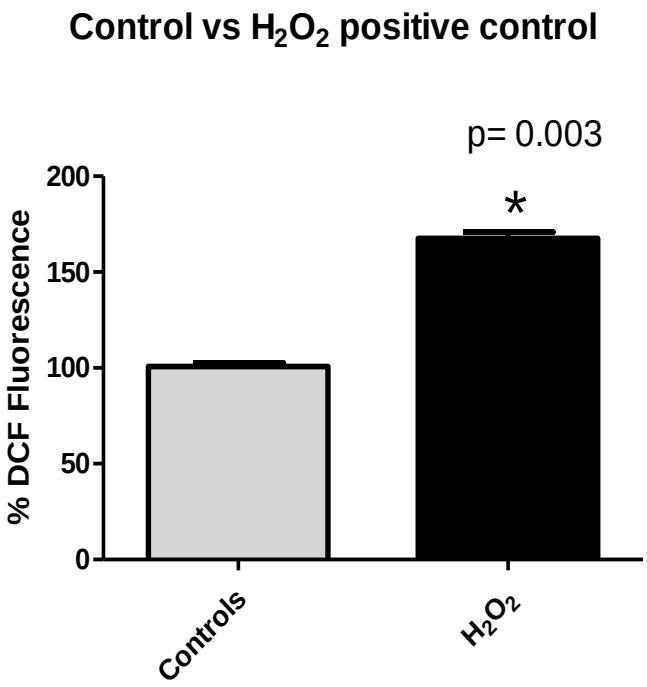

Figure 3.8: H_2O_2 significantly increased mean DCF fluorescence intensity and was included as a positive control in all DCF experiments, n = 8 / group.

3.5.3 Cell viability measurements

3.5.3.1 Propidium Iodide

Propidium iodide is an intercalating agent and a fluorescent molecule that can be used to stain cells. This fluorescent stain is specific for nucleic acids. When cell membrane integrity is lost due to necrosis, it permits propidium iodide to enter the cell due to large holes forming in the membrane. These large enough holes permit the entry of large propidium iodide molecules which subsequently stain the nucleus (Riccardi, Nicoletti 2006, Genis 2014, Westcott 2015). A red fluorescent colour is emitted and analysed in flow channel 2 (FL2-H) of the flow cytometer (FACS caliber). Thus cells that display a high propidium iodide uptake and show an increased propidium iodide fluorescence are considered necrotic (**refer to figure 3.10**) (Wilkins, Kutzner et al. 2002). Please **refer to figure 3.11** for a schematic representation of the propidium iodide protocol used.

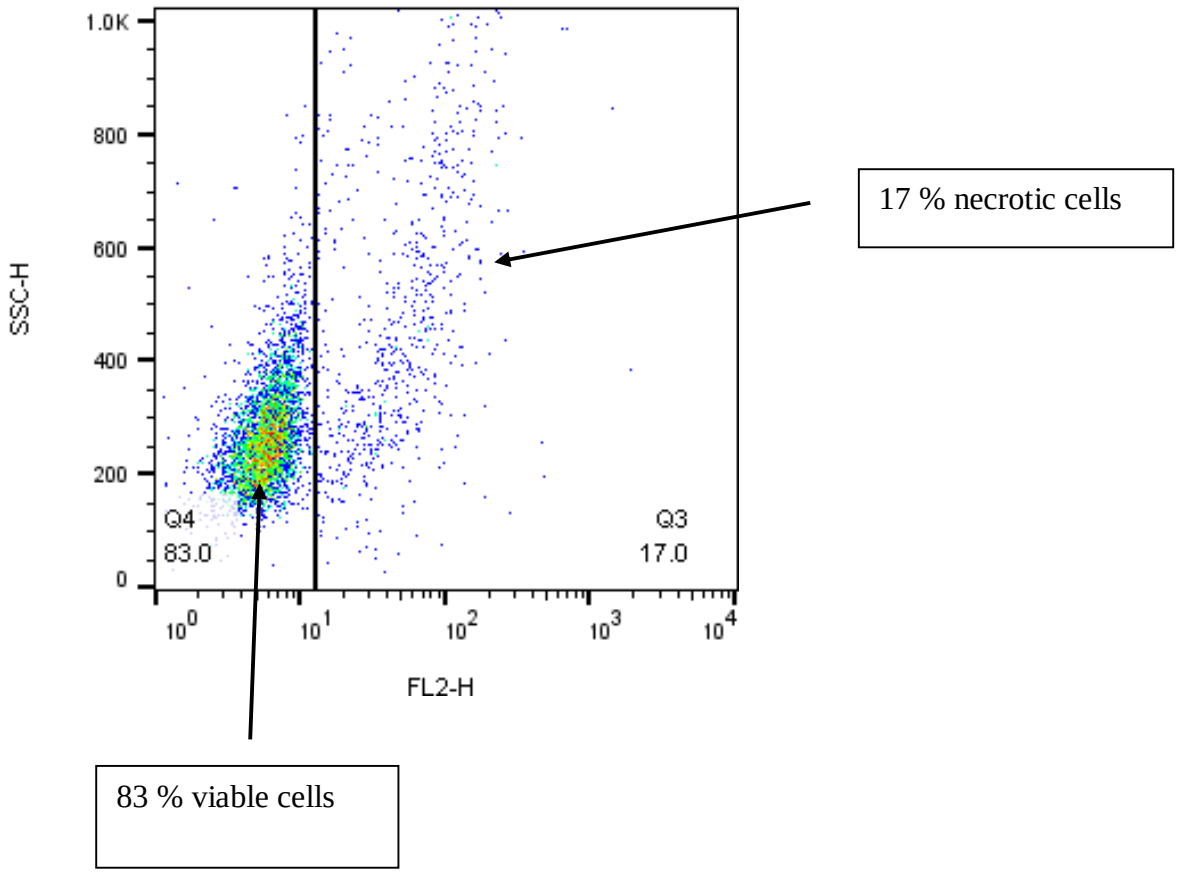

Figure 3.9: A representative scatterplot in which the sub-populations of viable and necrotic cells have been gated. Propidium iodide fluorescence is measured on the x-axis (FL2-H).

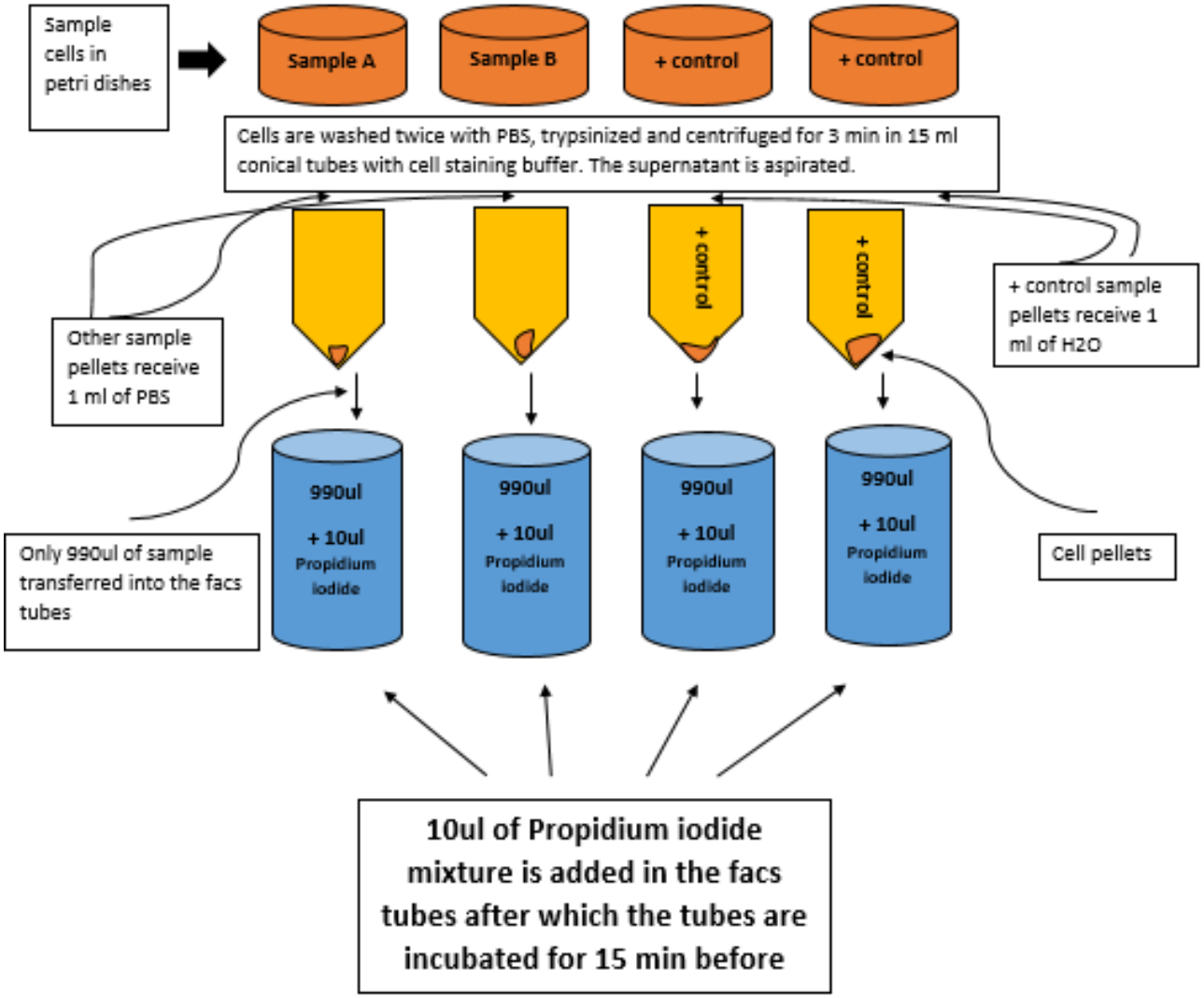

Figure 3.10: Schematic overview of the propidium iodide experimental protocol.

3.5.3.1.1. Positive control: distilled H_2O

Distilled H_2O was used as positive control for the propidium iodide experiments as it increases propidium iodide staining in cells **(Refer to figure 3.12)**. H_2O diffuses across a semi-permeable membrane from an area with low osmolarity to an area of high osmolarity due to osmosis. Therefore, the net result of treating the ECs with distilled H_2O is the movement of H_2O molecules over the cell membrane and into the cell. Cells swell and eventually burst (Westcott 2015). Rupturing of the cell membrane due to necrosis allows the propidium iodide fluorescent probe to enter and stain the nucleus by intercalating with the DNA**.**

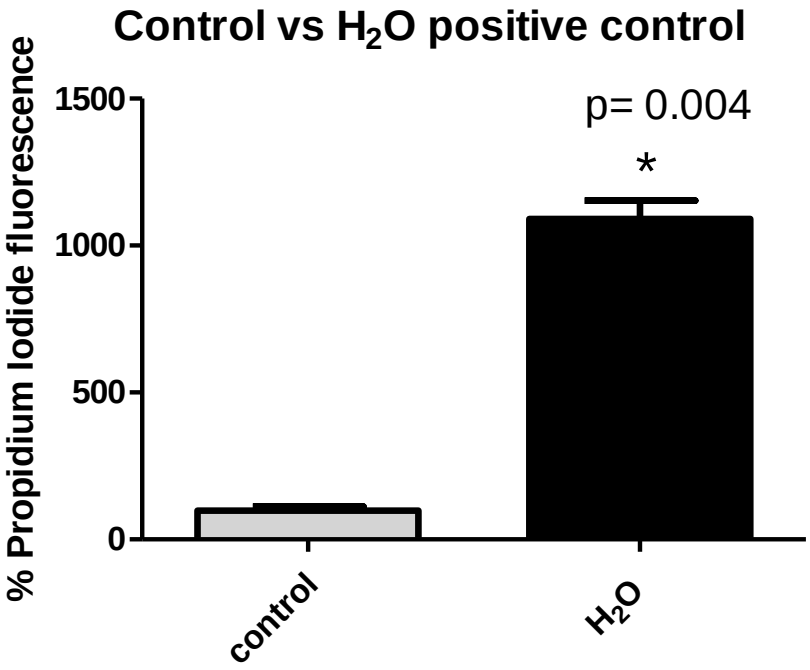

Figure 3.11: Distilled H_2O significantly increased propidium iodide fluorescence and was included in all propidium experiments, n = 7 / group.

3.6 First line and second line ART drug treatment protocol for AECs

As mentioned in the first chapter, the second aim of the *in vitro* study was to investigate the effects of first line and second line ART drugs in AECs in culture on cell viability, NO production and oxidative stress status. The South African first line ART regime comprises of a combination of efavirenz (EFV), emtricitabine (FTC), and tenofovir (TDF), and the second line ART regime comprises of a combination of lopinavir (LPV) and ritonavir (RTV). Therefore, the AECs were treated with a combination of the first line ART drugs and the second line ART drugs for 24hours. The concentrations of first line drugs were based on previous studies by Feng et al (2009) and Grigsby et al (2010), whereas the second line drug's concentration were based on a previous study by Noor et al. Drugs were purchased commercially from SantaCruz Biotechnology (WhiteHead Scientific). They were received in 10mg powder form. For experimental purposes, each of these drugs was prepared in its respective solvents (vehicles) (**refer to table 3.1** for first and second line ART drug concentration and vehicles).

***Table 3.1**: First and second line ART drug concentration and vehicles.*

Drugs	Concentration	Solvents/ vehicles
Efavirenz	5.6nM	The 10mg powder was dissolved in **1ml Methanol**, after which from this stock, it was further dissolved in phosphate buffer saline (**PBS)** buffer to achieve the required concentration
Emtricitabine	1.3uM	The 10mg powder was dissolved in **1ml H_2O,** after which from this stock, it was further dissolved in phosphate buffer saline (**PBS)** buffer to achieve the required concentration.
Tenofovir	500nM	The 10mg powder was dissolved in 1ml dimethyl sulfoxide (**DMSO**), after which from this stock, it was further dissolved in phosphate buffer saline (**PBS)** buffer to achieve the required concentration.
Lopinavir	10uM	The 10mg powder was dissolved in 1ml dimethyl sulfoxide (**DMSO**), after which from this stock, it was further dissolved in phosphate buffer saline (**PBS)** buffer to achieve the required concentration.
Ritonavir	2uM	The 10mg powder was dissolved in 1ml dimethyl sulfoxide (**DMSO)**, after which from this stock, it was further dissolved in phosphate buffer saline (**PBS**) buffer to achieve the required concentration.

Since the drugs were dissolved in their respective vehicles, to eliminate the effects of the vehicles in the experiments, the first line and second line ART drugs were compared to a control group consisting of a combination the vehicles of the drugs. Therefore, the first line combination of ART drugs (efavirenz with emtricitabine and tenofovir) was compared with a control group consisting of combination of vehicles (methanol, H_2O and DMSO in PBS). In the same way, the second line combination of ART drugs (lopinavir and ritonavir) was compared with a combination of vehicles (DMSO in PBS). **Refer to Appendix D**, for detailed ART drug and vehicle protocols and calculations.

Chapter 4 : Results (clinical study)

This chapter describes the results obtained from the clinical (main) study.

4.1 Descriptive and demographic profile of the total study population.

The total population consisted of 152 participants. 83 % (n= 124) of the total population was of the mixed race origin **(refer to figure 4.1 A)**. In addition, 67% (n=102) of the total population was female **(refer to figure 4.1 B)**. Furthermore, the total population consisted of 24% (n= 36) HIV negative, 22 % (n= 33) HIV positive- ART naïve, 32% (n= 50) HIV positive- first line ART, and 22% (n= 33) HIV positive- second line ART **(refer to figure 4.1 C).**

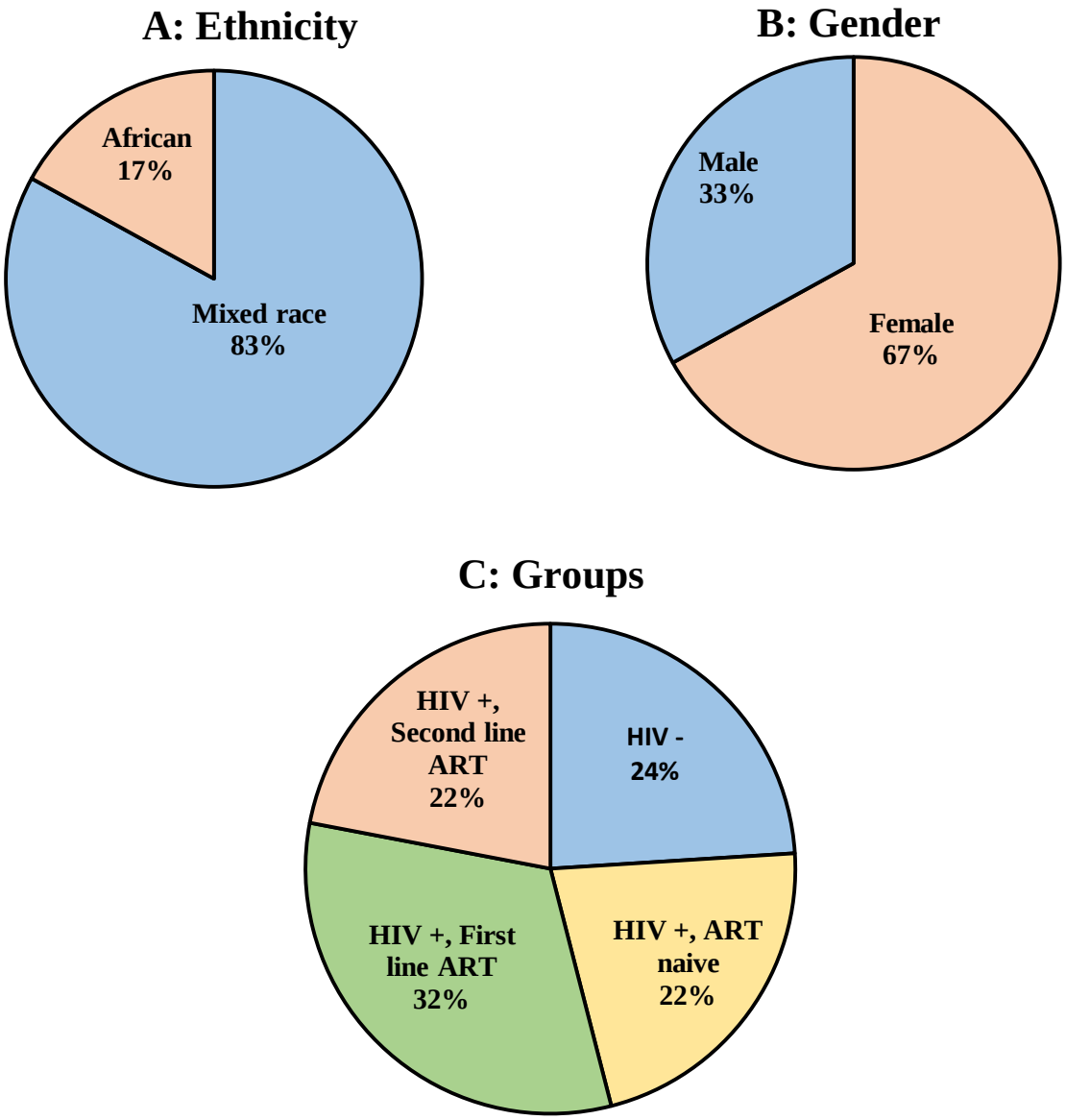

Figure 4.1: Distribution of the study participants, A, ethnicity distribution; B, gender distribution; C, HIV status distribution.

4.2 Descriptive profile of the HIV positive and negative groups

4.2.1 Demographic, life style and anthropometric characteristics

The median BMI of participants in the HIV negative group was significantly higher than that of the HIV positive, ART naïve group (p = 0.01) and the first line ART group (p = 0.01) **(Refer to Table 4.1)**. Furthermore, the median waist circumference was significantly higher in the HIV negative group when compared to the HIV positive- ART naïve, HIV positive- first line ART as well as HIV positive- second line ART (p <0.01, p < 0.01, p= 0.040 respectively). There were no significant differences in terms of age, gender, ethnicity and the use of cardiovascular medication amongst the four study population groups.

***Table 4.1**: Demographic, lifestyle and anthropometric characteristics of the HIV negative, HIV positive ART naive, HIV positive first line ART, and HIV positive second line ART participants.*

	HIV-Negative (n=36)	**HIV-Positive ART Naïve (n=33)**	**HIV-Positive on first line ART (n=50)**	**HIV- positive on second line ART (33)**
Age (years)	40.722 ± 8.74	36.212 ± 10.08	37.740 ± 8.22	41.394 ± 9.38
Gender: F, n (%)	29 (80.6%)	20 (60.6%)	28 (56.6%)	25 (75.8%)
Ethnicity, Mixed Race, n (%)	30 (83.3%)	29 (87.9%)	43 (86.0%)	22 (66.7%)
Smoking, n (%)	5 (13.9%)	13 (40.6%)	18 (36.7%)	8 (24.2%)
CVS Medication, n (%)	5 (13.9%)	5 (15.2%)	10 (20 %)	4 (12.1%)
BMI (kg/m^2)	29.2 (23.8- 35.2)$^{a; b}$	20.7 (19.0- 24.1)^{a}	21.6 (19.8- 25.4)^{b}	24.7 (19.3- 29.9)
WC (cm)	101 (92.0- 112)$^{a, c, d}$	84 (77- 90)^{a}	87 (82-92) $^{b, c}$	92 (83- 99)$^{b, d}$

n indicates number of participants; ART, antiretroviral therapy; BMI, body mass index; WC, waist circumference; CVS, cardiovascular. Normally distributed data are expressed as mean ± standard error of mean, and non-parametric data is expressed as median with 25th and 75th percentiles. N, % of n and p values were obtained with ANOVA for parametric data or Kruskal Wallis for non-parametric data.. **Means with same superscript letter: differ significantly (p <0.05).**

4.2.2 Cardiovascular and biochemical characteristics

Cardiovascular characteristics such as the SBP and DBP were found to not be significantly different amongst the four groups (**refer to table 4.2**). Analysis of specific biochemical blood parameters revealed a significantly higher median total cholesterol level in the HIV negative group in comparison to the HIV positive- first line and second line ART groups ($p = 0.046$, $p = 0.023$ respectively). Interestingly, HIV positive first line group displayed a modest, but significantly higher median total cholesterol level than the HIV positive ART naïve group ($p = 0.041$). In addition, a significantly higher mean LDL-cholesterol level was observed in the HIV negative group in comparison to all three HIV positive groups, ART naïve, first line ART as well as second line ART ($p = 0.010$; $p = 0.001$, $p < 0.01$ respectively). The median HBA1C percentage was observed to be significantly lower in the HIV positive- second line group compared to the HIV positive ART-naïve group ($p = 0.031$). All the other biochemical parameters measured were found to not be significantly different amongst the groups.

Table 4.2: *Cardiovascular and biochemical characteristics of the HIV negative, HIV positive ART naive, HIV positive first line ART, and HIV positive second line ART participants.*

	HIV-Negative (n=36)	**HIV-Positive ART Naïve (n=33)**	**HIV-Positive on first line ART (n=50)**	**HIV- positive on second line ART (33)**
SBP (mm Hg)	128.86 ± 18.38	123.12 ± 18.12	128.64 ± 28.67	122.76 ± 15.38
DBP (mm Hg)	88 (78.5- 94.5)	83 (75.0- 93.0)	87 (77.0-97.0)	84 (78.0- 88.0)
Total Cholesterol (mmol/L)	5.2 (4.1- 5.5)[a, c]	4.2 (3.6- 4.8)[b]	4.3 (3.8- 4.7)[b, c]	4.2 (3.4- 4.8)[a]
HDL-C (mmol/L)	1.3 (1.0- 1.4)	1.2 (1.0-1.5)	1.4 (1.4- 1.0)	1.3 (1.2- 1.5)
LDL-C (mmol/L)	3.09 ± 0.84[a, b, c]	2.50 ± 0.75[a]	2.45 ± 0.65[b]	2.23 ± 0.72[c]
Triglycerides (mmol/L)	1.1 (0.8- 1.5)	0.9 (0.8- 1.1)	0.9 (0.6- 1.4)	1.2 (0.9- 1.6)
Fasting Glucose (mmol/L)	5.0 (4.7- 5.5)	4.7 (4.5- 4.9)	4.8 (4.5- 5.2)	4.7 (4.3- 5.2)
HbA1C (%)	5.6 (5.5- 5.8)	5.6 (4.3- 5.9)[a]	5.4 (5.2-5.8)	5.3 (5.2- 5.5)[a]
CRP (mg/L)	6.0 (3.0-10.0)	3.0 (1.0- 10.0)	5.0 (2.0- 10.6)	3.2 (2.0- 8.0)
CD4 count (cells / uL)	NA	551.0 (424- 764)	358.0 (236.5- 589.5)	348.0 (177.0- 551.0)

n indicates number of participants; ART, antiretroviral therapy; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; HbA1c, glycated haemoglobin; CRP, C-reactive protein. Normally distributed data are expressed as mean with ± standard error of mean, non-parametric data is expressed as median with 25th and 75th percentiles. N, % of n and p values were obtained with ANOVA for parametric data or Kruskal Wallis for non-parametric data. **Means with same superscript letter: differ significantly ($p < 0.05$).**

4.2.3 Vascular function

No significant differences were observed in terms of baseline brachial artery diameter, maximum brachial artery diameter and FMD% amongst the four groups **(refer to table 4.3)**.

Table 4.3: *FMD and related characteristics of the HIV negative, HIV positive ART naive, HIV positive first line ART, and HIV positive second line ART participants.*

	HIV-Negative (n=36)	**HIV-Positive ART Naïve (n=33)**	**HIV-Positive on first line ART (n=50)**	**HIV-positive on second line ART (33)**
Baseline Brachial artery diameter (mm)	3.65 ± 0.68	3.54 ± 0.65	3.54 ± 0.65	3.41 ± 0.73
Maximum Brachial artery diameter (mm)	3.80 ± 0.81	3.77 ± 0.62	3.83 ± 0.75	3.66 ± 0.75
FMD (%)	5.86 ± 4.91	6.71 ± 4.43	7.65 ± 5.38	7.74 ± 4.39

n indicates number of participants; ART, antiretroviral therapy; FMD, Flow mediated dilatation. Normally distributed data are expressed as mean with ± standard error of mean.

4.3 Associations of vascular variables with various demographic and cardiovascular risk variables in the total population, HIV negative population, and HIV positive population

Multiple regression analysis with baseline artery diameter, maximum artery diameter and FMD% as dependent variables **(refer to table 4.4)** showed that the female gender was inversely associated with baseline and maximum artery diameter in the total population ($p < 0.01$), HIV negative population ($p = 0.005$) as well as the total HIV positive population ($p < 0.01$). Whereas, interestingly, smoking history was borderline positively associated with baseline artery diameter in the total population ($p = 0.053$). CRP was inversely associated with the baseline artery diameter in the total population ($p = 0.042$). Fasting glucose levels were inversely associated with baseline artery diameter in the total as well the HIV positive population ($p = 0.018$, $p = 0.038$ respectively). In addition, fasting glucose levels were inversely associated with maximum artery diameter in the HIV positive population ($p = 0.038$). Furthermore, the use of cardiovascular medication was inversely associated with baseline and maximum artery diameter in the HIV positive population ($p = 0.035$, $p = 0.062$ respectively).

Notably, baseline artery diameter was inversely associated with FMD% in the total population as well as the HIV positive population ($p = 0.007$, $p = 0.029$ respectively). Interestingly, no associations were observed between HIV positive status and the dependent variables. Furthermore, no significant associations were found between any of the other independent variables and the three dependent variables **(refer to table 4.4).**

Table 4.4: *Independent associations of baseline artery diameter, maximum artery diameter, and FMD, with various cardiovascular risk factors in the total study population, HIV negative population and HIV positive population.*

Baseline Artery Diameter R^2; Adjusted R^2	**Total Group** 0.4074; 0.353		**HIV negative Group** 0.6409; 0.4613		**HIV positive Group** 0.3996; 0.3281	
	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p
HIV-infection	-0,153 (-0,42; 0,11)	0.255	-	-	-	-
Female Gender	-0,940 (-1,18; -0,70)	**< 0.01**	-1,156 (-1.92; -0.40)	**0.005**	-0,865 (-1,14; -0,59)	**< 0.01**
Age (years)	0,001 (-0,01; 0,01)	0.800	0,018 (-0,57; 0,83)	0.142	-0,001 (-0,01; 0,01)	0.932
CVS Medication, yes	-0,202 (-0,53; 0,13)	0.230	0,337 (-0,01; 0,03)	0.342	-0,453 (-0,87; -0,03)	**0.035**
Smoking history, yes	0,236 (0,00; 0,47)	**0.053**	0,128 (-0,01; 0,01)	0.708	0,215 (-0,05; 0,48)	0.113
Waist circumference (cm)	0,008 (0,00; 0,02)	0.061	0,010 (-0,38; 0,30)	0.183	0,006 (-0,01; 0,02)	0.292
Mean SBP (mmHg)	0,001 (-0,01; 0,01)	0.868	0,001 (-0,87; 0,93)	0.946	-0,004 (-0,01; 0,00)	0.380
LDL cholesterol (mmol/L)	0,097 (-0,04; 0,24)	0.178	-0,041 (-0,04; 0,01)	0.803	0,133 (-0,04; 0,31)	0.131
HDL cholesterol (mmol/L)	0,051 (-0,21; 0,31)	0.703	0,033 (-0,17; 0,03)	0.940	0,091 (-0,20; 0,38)	0.537
CRP (mg/L)	-0,008 (-0,01; 0,00)	**0.042**	-0,012 (-0,38; 1,06)	0.318	-0,007 (-0,01; 0,00)	0.103
Fasting Glucose (mmol/L)	-0,094 (-0,17; -0,02)	**0.018**	-0,069 (-0,57; 0,83)	0.165	-0,140 (-0,27; -0,01)	**0.038**

Table 4.4: *continued*

Maximum Artery Diameter R^2; Adjusted R^2	**Total Group** 0.3457; 0.2826		**HIV negative Group** 0.4660; 0.1990		**HIV positive Group** 0.3790; 0.3050	
	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p
HIV-infection	-0,045 (-0,35; 0,27)	0.775	-	-	-	-
Female Gender	-0,944 (-1,22; -0,67)	**< 0.01**	-1,709 (-2,85; -0,57)	**0.005**	-0,883 (-1,18; -0,58)	**< 0.01**
Age (years)	0,003 (-0,01; 0,02)	0.702	0,021 (-0,02; 0,06)	0.271	-0,001 (-0,02; 0,01)	0.879
CVS Medication, yes	-0,276 (-0,66; 0,11)	0.161	0,142 (-0,95; 1,23)	0.789	-0,434 (-0,89; 0,02)	0.062
Smoking history, yes	0,185 (-0,09; 0,46)	0.191	0,104 (-0,95; 1,16)	0.839	0,154 (-0,14; 0,44)	0.295
Waist circumference (cm)	0,006 (0,00; 0,02)	0.223	0,009 (-0,01; 0,32)	0.455	0,006 (-0,01; 0,02)	0.293
Mean SBP (mmHg)	-0,001 (-0,01; 0,01)	0.859	-0,011 (-0,03; 0,01)	0.251	-0,001 (-0,01; 0,01)	0.889
LDL cholesterol (mmol/L)	0,119 (-0,05; 0,28)	0.160	0,172 (-0,34; 0,69)	0.495	0,134 (-0,05; 0,32)	0.162
HDL cholesterol (mmol/L)	0,068 (-0,24; 0,38)	0.664	0,575 (-0,78; 1,93)	0.387	0,072 (-0,25; 0,39)	0.653
CRP (mg/L)	-0,007 (-0,02; 0,00)	0.125	-0,003 (-0,04; 0,03)	0.854	-0,006 (-0,01; 0,00)	0.170
Fasting Glucose (mmol/L)	-0,069 (-0,16; 0,02)	0.132	-0,037 (-0,19; 0,11)	0.612	-0,152 (-0,30; -0,01)	**0.038**

Table 4.4: *continued*

FMD % R^2; Adjusted R^2	**Total Group** 0.1488; 0.0550		**HIV negative Group** 0.3268; -0.1089		**HIV positive Group** 0.1367; 0.0194	
	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p
HIV-infection	0,721 (-1,79; 3,23)	0.571	-	-		
Female Gender	-0,140 (-2,89; 2,61)	0.920	0,491 (-12,22; 13.20)	0.936	0,022 (-3,01; 3,06)	0.989
Age (years)	-0,038 (-0,14; 0,07)	0.484	-0,072 (-0,38; 0,23)	0.627	-0,060 (-0,19; 0,06)	0.340
CVS Medication, yes	-0,444 (-3,50; 2,61)	0.774	-1,410 (-9,71; 6,89)	0.724	0,868 (-3,01; 4,75)	0.657
Smoking history, yes	-1,052 (-3,27; 1,17)	0.349	2,140 (-5,85; 10,13)	0.580	-1,659 (-4,10; 0,79)	0.181
Waist circumference (cm)	0,016 (-0,06; 0,09)	0.679	-0,035 (-0,22; 0,15)	0.698	0,062 (-0,04; 0,16)	0.229
Mean SBP (mmHg)	-0,010 (-0,07; 0,05)	0.739	-0,042 (-0,19; 0,10)	0.544	0,017 (-0,06; 0,09)	0.667
LDL cholesterol (mmol/L)	0,239 (-1,08; 1,56)	0.720	-0,199 (-4,16; 3,76)	0.917	0,389 (-1,20; 1,98)	0.626
HDL cholesterol (mmol/L)	0,194 (-2,22; 2,61)	0.873	2,337 (-8,26; 12,93)	0.648	0,294 (-2,36; 2,95)	0.826
CRP (mg/L)	0,001 (-0,07; 0,07)	0.966	-0,034 (-0,32; 0,25)	0.804	0,016 (-0,06; 0,09)	0.671
Fasting Glucose (mmol/L)	-0,528 (-1,26; 0,20)	0.155	-0,644 (-1,89; 0,61)	0.292	-0,056 (-1,28; 1,17)	0.927
Baseline artery diameter (mm)	-2.439 (-4.19; -0.68)	**0.007**	-1,884 (-7,69; 3,93)	0.503	-2,266 (-4,29; -0,24)	**0.029**

FMD%, flow mediated dilatation; CI, confidence interval; CVS, cardiovascular; SBP, brachial systolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein. Associations were determined by forward stepwise multiple regression analyses. $P < 0.05$ is regarded as statistically significant. Independent variables in the model: age; gender; HIV status (for total group); waist circumference; CVs medication; smoking history; SBP; LDL-cholesterol; HDL- cholesterol; CRP; fasting glucose and baseline artery diameter (for FMD% variable).

4.4 Associations of vascular variables with various demographic and cardiovascular risk variables in the HIV positive ART naïve, first line ART and second line ART populations respectively.

Multiple regression analysis with baseline artery diameter, maximum artery diameter and FMD% as dependent variables **(refer to table 4.5)** showed that the female gender was inversely associated with baseline and maximum artery diameter in the HIV positive- ART naïve group ($p = 0.002$, $p = 0.006$ respectively), first line ART group ($p= 0.001$, $p = 0.005$ respectively), and second line ART group ($p = 0.027$, $p = 0.019$ respectively). Fasting glucose levels were inversely associated with baseline artery diameter in the ART naïve group ($p = 0.047$). Interestingly, waist circumference ($p = 0.044$) was positively associated, and CRP ($p = 0.065$) borderline negatively associated with baseline artery diameter in the first line ART group. HDL-cholesterol was found to be positively associated with baseline and maximum artery diameter in the second line ART group ($p = 0.044$, $p = 0.023$ respectively).

Smoking history was observed to be borderline negatively associated with FMD% in the first line ART group ($p = 0.052$). Furthermore, CRP and baseline artery diameter were negatively associated with FMD% in the second line ART group ($p = 0.031$, $p= 0.014$ respectively). No associations were observed with FMD% in the ART naïve group. Furthermore, no significant associations were found between any of the other independent variables and the three dependent variables**.**

Table 4.5: *Independent associations of baseline artery diameter, maximum artery diameter, and FMD%* with various cardiovascular risk factors in the HIV positive ART naive population, HIV positive first line ART population and HIV positive second line ART population.

Baseline Artery Diameter R^2; Adjusted R^2	**ART naive** 0.5976; 0.3293		**First Line ART** 0.5437; 0.3915		**Second Line ART** 0.5134; 0.2271	
	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p
Female Gender	-0,992 (-1,56; -0,42)	**0.002**	-0,823 (-1,26; -0,39)	**0.001**	-0,960 (-1,80; -0,12)	**0.027**
Age (years)	0,008 (-0,02; 0,04)	0.605	0,004 (-0,02; 0,03)	0.691	-0,023 (-0,07; 0,02)	0.286
CVS Medication, yes	-0,266 (-1,61; 1,08)	0.680	-0,517 (-1,23; 0,19)	0.148	-0,521 (-1,40; 0,36)	0.229
Smoking history, yes	0,222 (-0,32; 0,76)	0.397	0,279 (-0,18; 0,74)	0.227	-0,061 (-0,70; 0,58)	0.843
Waist circumference (cm)	0,007 (-0,02; 0,04)	0.578	0,021 (0,00; 0,04)	**0.044**	0,010 (-0,02; 0,04)	0.407
Mean SBP (mmHg)	-0,006 (-0,03; 0,02)	0.621	-0,002 (-0,01; 0,01)	0.725	-0,008 (-0,03; 0,02)	0.498
LDL cholesterol (mmol/L)	0,242 (-0,15; 0,63)	0.207	0,185 (-0,11; 0,48)	0.218	-0,046 (-0,46; 0,37)	0.817
HDL cholesterol (mmol/L)	-0,163 (-0,67; 0,35)	0.506	0,253 (-0,29; 0,84)	0.381	1,133 (0,04; 2,23)	**0.044**
CRP (mg/L)	-0,010 (-0,05; 0,03)	0.584	-0,009 (-0,02; 0,00)	**0.065**	0,004 (-0,03; 0,03)	0.769
Fasting Glucose (mmol/L)	-0,213 (-0,42; 0,00)	**0.047**	-0,166 (-0,42; 0,09)	0.198	-0,107 (-0,58; 0,36)	0.639

Table 4.5: *continued*

Maximum Artery Diameter R^2; Adjusted R^2	**ART naive** 0.5128; 0.1880		**First Line ART** 0.5169; 0.3558		**Second Line ART** 0.5739; 0.3232	
	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p
Female Gender	-0,900 (-1,51; -0,29)	**0.006**	-0,787 (-1,31; -0,26)	**0.005**	-0,989 (-1,79; -0,19)	**0.019**
Age (years)	0,004 (-0,03; 0,04)	0.806	0,007 (-0,02; 0,03)	0.610	-0,021 (-0,06; 0,02)	0.322
CVS Medication, yes	-0,127 (-1,55; 1,30)	0.853	-0,634 (-1,49; 0,22)	0.142	-0,502 (-1,35; 0,34)	0.227
Smoking history, yes	0,268 (-0,31; 0,84)	0.337	0,045 (-0,51; 0,60)	0.872	-0,158 (-0,77; 0,46)	0.595
Waist circumference (cm)	0,006 (-0,02; 0,04)	0.645	0,021 (0,00; 0,05)	0.087	0,011 (-0,01; 0,04)	0.361
Mean SBP (mmHg)	-0,007 (-0,03; 0,02)	0.613	0,004 (-0,01; 0,02)	0.605	-0,010 (-0,03; 0,01)	0.371
LDL cholesterol (mmol/L)	0,207 (-0,21; 0,62)	0.303	0,180 (-0,18; 0,54)	0.317	0,008 (-0,39; 0,41)	0.968
HDL cholesterol (mmol/L)	-0,134 (-0,68; 0,41)	0.605	0,225 (-0,48; 0,93)	0.517	1,242 (0,19; 2,29)	**0.023**
CRP (mg/L)	-0,012 (-0,05; 0,03)	0.550	-0,006 (-0,02; 0,01)	0.276	-0,003 (-0,03; 0,03)	0.828
Fasting Glucose (mmol/L)	-0,203 (-0,43; 0,02)	0.071	-0,240 (-0,55; 0,07)	0.126	-0,117 (-0,57; 0,34)	0.593

Table 4.5: *continued*

FMD % R^2; Adjusted R^2	**ART naive** 0.5027; 0.1119		**First Line ART** 0.2505; -0.0549		**Second Line ART** 0.6275; 0.3714	
	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p
Female Gender	3,647 (-2,58; 9,87)	0.230	1,450 (-4,93; 7,83)	0.645	-2,764 (-8,17; 2,64)	0.294
Age (years)	-0,098 (-0,34; 0,15)	0.410	-0,123 (-0,39; 0,14)	0.348	-0,007 (-0,26; 0,25)	0.956
CVS Medication, yes	5,038 (-5,62; 15,70)	0.328	-0,916 (-9,48; 7,65)	0.828	-0,897 (-6,02; 4,23)	0.715
Smoking history, yes	0,967 (-3,42; 5,35)	0.643	-5,683 (-11,41; 0.04)	**0.052**	-3,331 (-6,89; 0,23)	0.065
Waist circumference (cm)	-0,029 (-0,25; 0,19)	0.784	0,156 (-0,11; 0,42)	0.240	0,040 (-0,11; 0,19)	0.571
Mean SBP (mmHg)	0,007 (-0,20; 0,21)	0.940	0,074 (-0,07; 0,22)	0.303	-0,039 (-0,18; 0,10)	0.555
LDL cholesterol (mmol/L)	-0,815 (-4,06; 2,43)	0.599	0,366 (-3,29; 4,02)	0.839	1,917 (-0,39; 4,23)	0.098
HDL cholesterol (mmol/L)	0,590 (-3,49; 4,67)	0.761	0,194 (-6,69; 7,08)	0.954	5,452 (-1,45; 12,35)	0.113
CRP (mg/L)	-0,021 (-0,33; 0,29)	0.886	0,077 (-0,04; 0,19)	0.179	-0,190 (-0,36; -0,02)	**0.031**
Fasting Glucose (mmol/L)	0,338 (-1,55; 2,23)	0.707	-1,357 (-4,57; 1,85)	0.393	-0,198 (-2,84; 2,44)	0.876
Baseline artery diameter (mm)	-0,886 (-5,22; 3,45)	0.668	-2,268 (-7,14; 2,60)	0.348	-3,717 (-6,56; -0,87)	**0.014**

FMD%, flow mediated dilatation; CI, confidence interval; CVS, cardiovascular; SBP, brachial systolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein. Associations were determined by forward stepwise multiple regression analyses. P < 0.05 is regarded as statistically significant. Independent variables in the model: age; gender; waist circumference; CVs medication; smoking history; SBP; LDL-cholesterol; HDL- cholesterol; CRP; fasting glucose and baseline artery diameter (for FMD% dependent variable).

4.5 Associations of vascular variables with CD4 count in the total HIV positive population and ART groups

CD4 count (p = 0.027) was negatively associated with FMD% in the second line ART group. There were no other associations observed with CD4 count in any of the other HIV positive groups **(refer to table 4.6**).

Table 4.6: *Independent associations of baseline artery diameter, maximum artery diameter, and FMD% with CD4 count in the total HIV positive population, HIV positive ART naive population, HIV positive first line ART population and HIV positive second line ART population.*

Baseline Artery Diameter R^2; Adjusted R^2	**Total HIV population** **0.3517; 0.2983**		**ART naive** 0.3832; 0.2430		**First Line ART** 0.4709; 0.3882		**Second Line ART** 0.3115; 0.1475	
	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p
First line ART, yes	-0,038 (-0,33; 0,25)	0.792	-	-	-	-	-	-
Second line ART, yes	-0,035 (-0,37; 0,30)	0.833	-	-	-	-	-	-
CD4	0,001 (-0,01; 0,03)	0.719	0,000 (-0,001; 0,001)	0.754	0,000 (0,000; 0,001)	0.458	0,000 (-0,001; 0,001)	0.639
Female Gender	-0,758 (-1,02; -0,50)	**< 0.01**	-0,871 (-1,41; -0,34)	**0.003**	-0,629 (-1,00; -0,25)	**0.002**	-0,992 (-1,75; -0,24)	**0.012**
Age (years)	0,001 (-0,01; 0,01)	0.919	0,002 (-0,02; 0,03)	0.901	0,012 (-0,01; 0,03)	0.242	-0,016 (-0,05; 0,02)	0.397
CVS Medication, yes	-0,395 (-0,73; -0,06)	**0.020**	-0,184 (-0,96; 0,59)	0.628	-0,571 (-1,00; -0,14)	**0.011**	-0,490 (-1,39; 0,41)	0.269
Smoking history, yes	0,086 (-0,17; 0,34)	0.508	0,116 (-0,39; 0,62)	0.637	0,131 (-0,24; 0,51)	0.482	-0,140 (-0,80; 0,52)	0.663

Table 4.6: *continued*

Maximum Artery Diameter R^2; Adjusted R^2	**Total HIV population** **0.3505; 0.2970**		**ART naive** 0.3125; 0.1562		**First Line ART** 0.4551; 0.3700		**Second Line ART** 0.3850; 0.2386	
	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p
First line ART, yes	0,055 (-0,26; 0,37)	0.724	-	-	-	-	-	-
Second line ART, yes	0,006 (-0,35; 0,36)	0.975	-	-	-	-	-	-
CD4	0,000 (0,00; 0,001)	0.739	0,000 (-0,001; 0,001)	0.918	0,000 (0,000; 0,001)	0.464	0,000 (-0,001; 0,002)	0.399
Female Gender	-0,806 (-1,09; -0,52)	**<0.01**	-0,762 (-1,31; -0,21)	**0.009**	-0,733 (-1,19; -0,28)	**0.003**	-1,109 (-1,85; -0,37)	**0.005**
Age (years)	0,001 (-0,01; 0,02)	0.932	-0,003 (-0,03; 0,02)	0.828	0,015 (-0,01; 0,04)	0.227	-0,015 (-0,05; 0,02)	0.405
CVS Medication, yes	-0,408 (-0,77; -0,05)	**0.026**	-0,063 (-0,86; 0,73)	0.872	-0,654 (-1,18; -0,13)	**0.016**	-0,469 (-1,35; 0,41)	0.280
Smoking history, yes	0,038 (-0,24; 0,31)	0.788	0,145 (-0,37; 0,66)	0.566	0,053 (-0,40; 0,51)	0.813	-0,242 (-0,89; 0,40)	0.444

Table 4.6: *continued*

FMD% R^2; Adjusted R^2	**Total HIV population** 0.1767; 0.0964		**ART naive** 0.4095; 0.2408		**First Line ART** 0.2375; 0.0797		**Second Line ART** 0.4265; 0.2545	
	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p	Regression coefficient (95% CI)	p
First line ART, yes	1,924 (-0,43; 4,28)	0.107	-	-	-	-	-	-
Second line ART, yes	1,747 (-0,95; 4,44)	0.201	-	-	-	-	-	-
CD4	0,002 (0,00; 0,01)	0.131	0,002 (0,00; 0,01)	0.456	0,004 (0,00; 0,01)	0.189	0,007 (0,00; 0,01)	**0.027**
Female Gender	-0,910 (-3,46; 1,64)	0.480	3,193 (-1,25; 7,64)	0.150	-2,343 (-7,32; 2,64)	0.344	-4,609 (-9,18; -0,04)	**0.048**
Age (years)	-0,045 (-0,16; 0,07)	0.426	-0,099 (-0,27; 0,07)	0.237	-0,107 (-0,34; 0,13)	0.360	0,001 (-0,20; 0,20)	0.995
CVS Medication, yes	1,444 (-1,39; 4,27)	0.313	3,799 (-1,47; 9,07)	0.149	0,843 (-4,47; 6,16)	0.748	-0,311 (-5,13; 4,50)	0.894
Smoking history, yes	-1,663 (-3,78; 0,45)	0.121	0,456 (-2,96; 3,87)	0.784	-2,770 (-6,97; 1,43)	0.188	-2,762 (-6,21; 0,68)	0.110
Baseline brachial artery diameter (mm)	-2,479 (-4,33; -0,63)	**0.009**	-1,501 (-4,48; 1,48)	0.307	-3,179 (-7,74; 1,38)	0.165	-3,083 (-5,45; -0,72)	**0.013**

FMD%, flow mediated dilatation; CI, confidence interval; CVS, cardiovascular; SBP, brachial systolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein. Associations were determined by forward stepwise multiple regression analyses. P< 0.05 is regarded as statistically significant. Independent variables in the model: age; gender; first line ART (for total HIV population); second line ART (for total HIV population); CD4; CVs medication; smoking history; and baseline artery diameter (for FMD% dependent variable).

Chapter 5 : Results (*in vitro* sub-study)

This section describes and reports data collected from the *in vitro* sub-study.

5.1 Proteomic analysis of the HIV-conditioned model

Proteomic analysis by the CPGR showed detectable expression levels of HIV-1 glycoprotein Gp160 in the FBS-free HIV-conditioned medium, and no traces of any HIV-1 related proteins were detected in the control medium (**refer to appendix C** for the analytical report from CPGR, proteomics manager, Dr Zac Macdonald)

5.2 The effects of the HIV-conditioned growth medium in an *in vitro* model on AECs

As the initial aim of the *in vitro* study was to develop an HIV-conditioned *in vitro* model of AECs in which the ART drugs could be tested, it was deemed vital to investigate whether and / or how the HIV-conditioned growth medium would affect the AECs as a first step, especially since this protocol was not previously established in our laboratory. AECs treated with HIV-conditioned medium (CM) for 24 hours were compared with AECs treated with normal (non-conditioned) AEC medium (NM) for 24 hours and NO production, cell viability and ROS production was measured. The n value represents the number of individual experiments that were conducted for the endpoints. Each experiment used between 2 and 6 samples for which the mean value was calculated.

5.2.1 The effect of CM on NO production in AECs

AECs exposed to CM (24 hours), showed no change (104.32± 14.58 % vs. 100± 20.35 %NM) in mean DAF-2/DA fluorescence (measuring NO) (**refer to figure 5.1**), compared to untreated controls (AECs in NM).

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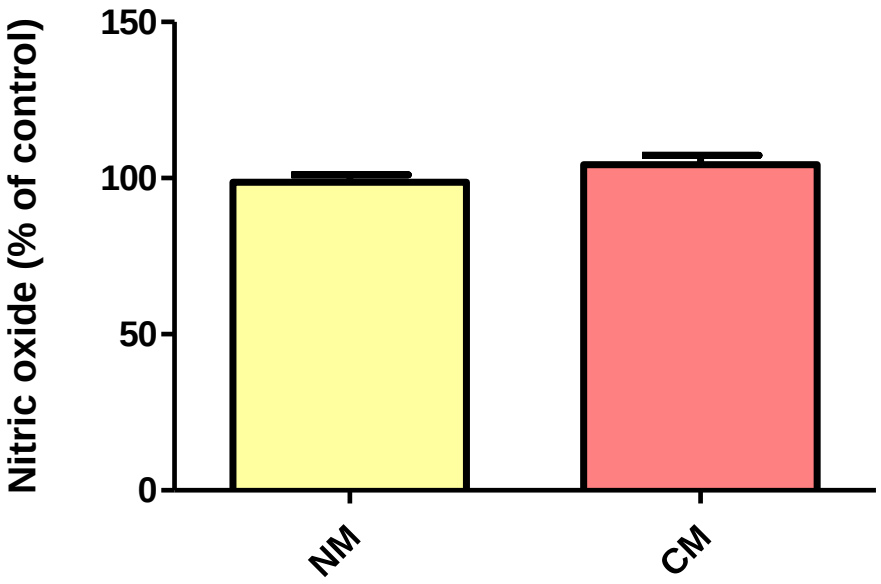


Figure 5.1: Effects of CM vs. NM on NO production as measured by DAF-2/DA fluorescence. NO expressed as % DAF-2/DA stained cells (calculated as a percentage of NM; NM fluorescence adjusted to 100 %), n= 7 / group.

5.2.2 The effect of CM on cell viability in AECs

AECs exposed to CM (24 hours), showed no change (101.59± 27.34 % vs. 100 ± 41.19% NM) in mean PI fluorescence (measuring necrosis) (**refer to figure 5.2**), compared to untreated controls (AECs in NM).

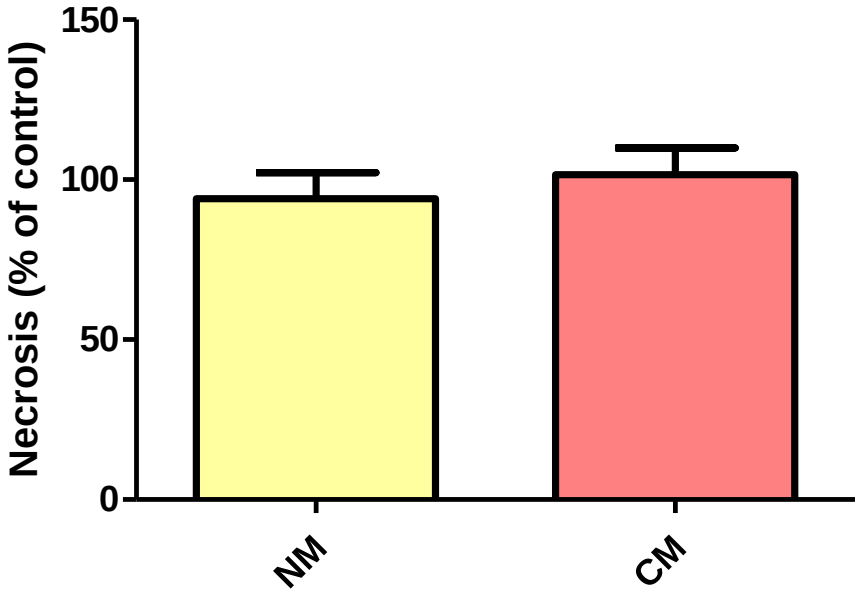


Figure 5.2: Effects of CM vs. NM on necrosis as measured by PI fluorescence. Necrosis expressed as % PI stained cells (calculated as a percentage of NM; NM adjusted to 100 %), n = 3 / group.

5.2.3 The effect of CM on ROS production in AECs

AECs exposed to CM (24 hours), showed no change (93.81 ± 2.55% vs. 100 ± 2.55% NM) in mean DCF fluorescence (measuring ROS) (**refer to figure 5.3**), compared to untreated controls (AECs in NM).

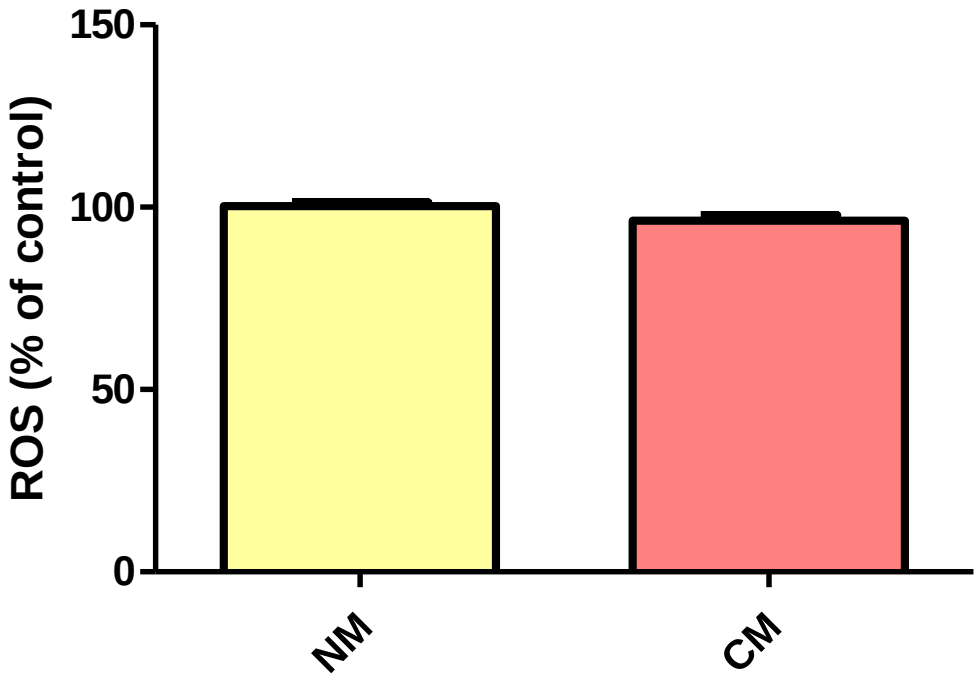


Figure 5.3: Effects of CM vs. NM on ROS production as measured by DCF fluorescence. ROS expressed as % DCF stained cells (calculated as a percentage of NM; NM adjusted to 100 %), n= 4 / group.

5.3 Effects of first line and second line ART on AECs *in vitro*

In view of the fact that the CM had no effect on any of the measured parameters (see previous sections) and in view of time constraints, it was decided to test for the effects of ART drugs on AECs incubated in normal (non-conditioned growth medium) for the purposes of this thesis. The ART containing groups were compared with the vehicle control groups which comprised of a combination of the drug's vehicles. Dose-response studies were designed to determine which dose of the ART combination elicits an effect on the AECs. Normal drug concentration (based on the literature; see *in vitro* Methods Chapter 3, section 3.6) vs. normal vehicle control, half of the normal drug concentration (half drug) vs. half vehicle control, and double the normal drug concentration (double drug) vs. double vehicle control. All of the experimental conditions were included in each separate experiment. The n value represents the number of individual experiments that were conducted for the endpoints, with each experimental group containing 4- 12 samples.

5.3.1 First line ART- dosage response

5.3.1.1 The effect of a first line ART combination drug cocktail (dose-response) on NO production in AECs

AECs exposed to half drug concentration (24 hours), showed no change (104.72 ± 14.03 % vs. 100± 12.60 % half vehicle control) in mean DAF-2/DA fluorescence (measuring NO) (**refer to figure 5.4**). AECs exposed to normal drug concentration (24 hours), showed a significant decrease (88.36 ± 6.66 % vs. 100± 6.75 % normal vehicle control: $p < 0.02$) in mean DAF-2/DA fluorescence (measuring NO) (**refer to figure 5.4**). AECs exposed to double drug concentration (24 hours), showed no change (101.33 ± 11.87 % vs. 100± 7.29 % double vehicle control) in mean DAF-2/DA fluorescence (measuring NO) (**refer to figure 5.4**).

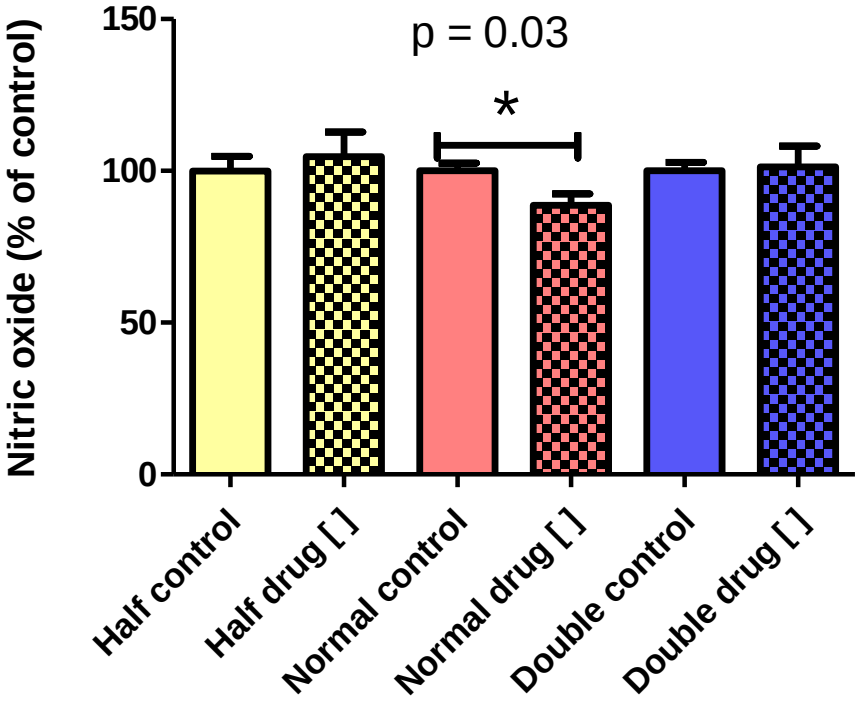


Figure 5.4: Effects of half drug concentration vs. half control, normal drug concentration vs. normal control and double drug concentration vs. double control on NO as measured by DAF-2/DA fluorescence. NO expressed as % DAF-2/DA stained cells (calculated as a percentage of control; control adjusted to 100 %), n= 8 / group. [ ]: Concentration.

5.3.1.2 The effect of a first line ART combination drug cocktail (dose-response) on the cell viability of AECs

AECs exposed to half drug concentration (24 hours), showed no change (97.67 ± 17.07 % vs. 100 ± 14.98 % half vehicle control) in mean PI fluorescence (measuring necrosis) (**refer to figure 5.5**). AECs exposed to normal drug concentration (24 hours), showed no change (95.50 ± 47.20 % vs. 100 ± 46.81 % normal vehicle control) in mean PI fluorescence (measuring necrosis) (**refer to figure 5.5**). AECs exposed to double drug concentration (24 hours), showed no change (106.42 ± 15.35 % vs. 100 ± 35.42 % double vehicle vs. control) in mean PI fluorescence (measuring necrosis) (**refer to figure 5.5**).

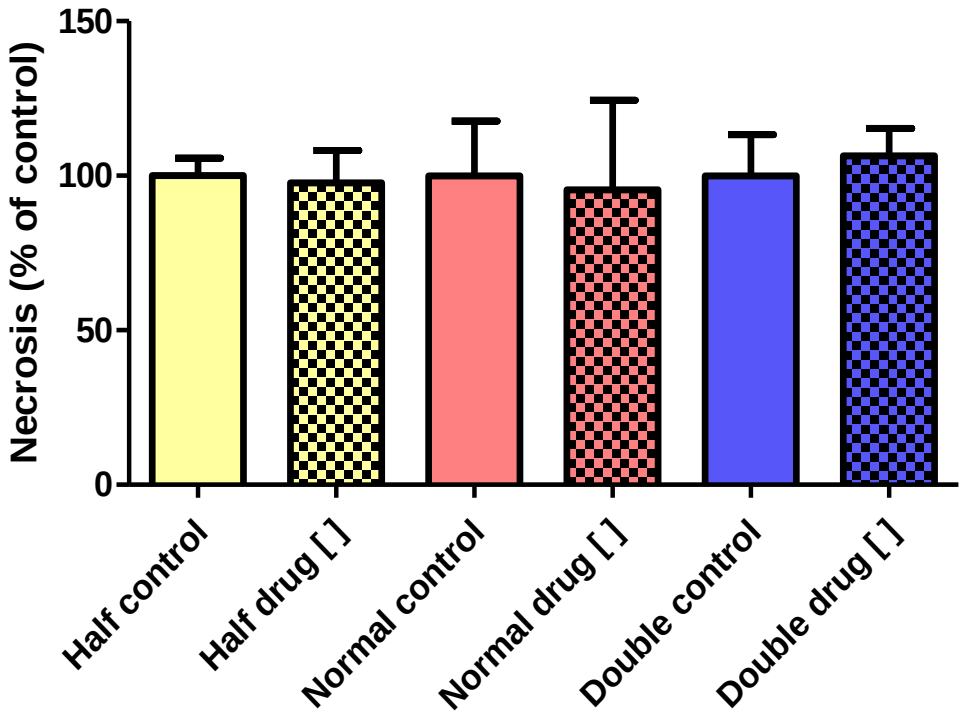


Figure 5.5: Effects of half drug concentration vs. half control, normal drug concentration vs. normal control and double drug concentration vs. double control on necrosis as measured by PI fluorescence. Necrosis expressed as % PI stained cells (calculated as a percentage of control; control adjusted to 100 %), n= 4 / group. [ ]: Concentration.

5.3.1.3 The effect of first line ART combination drug cocktail (dose-response) on ROS production in AECs

AECs exposed to half drug concentration (24 hours), showed a significant increase (127.12 ± 2.59 % vs. 100 ± 1.96 % half vehicle control: $p < 0.01$) in mean DCF fluorescence (measuring ROS) (**refer to figure 5.6**). AECs exposed to normal drug concentration (24 hours), showed no change (95.02 ± 2.50 % vs. 100 ± 5.79 % normal vehicle control: $p = 0.01$) in mean DCF fluorescence (measuring ROS) (**refer to figure 5.6**). AECs exposed to double drug concentration (24 hours), showed a significant decrease (86.23 ± 1.47 % vs. 100 ± 3.43% double vehicle control: $p < 0.05$) in mean DCF fluorescence (measuring ROS) (**refer to figure 5.6**).

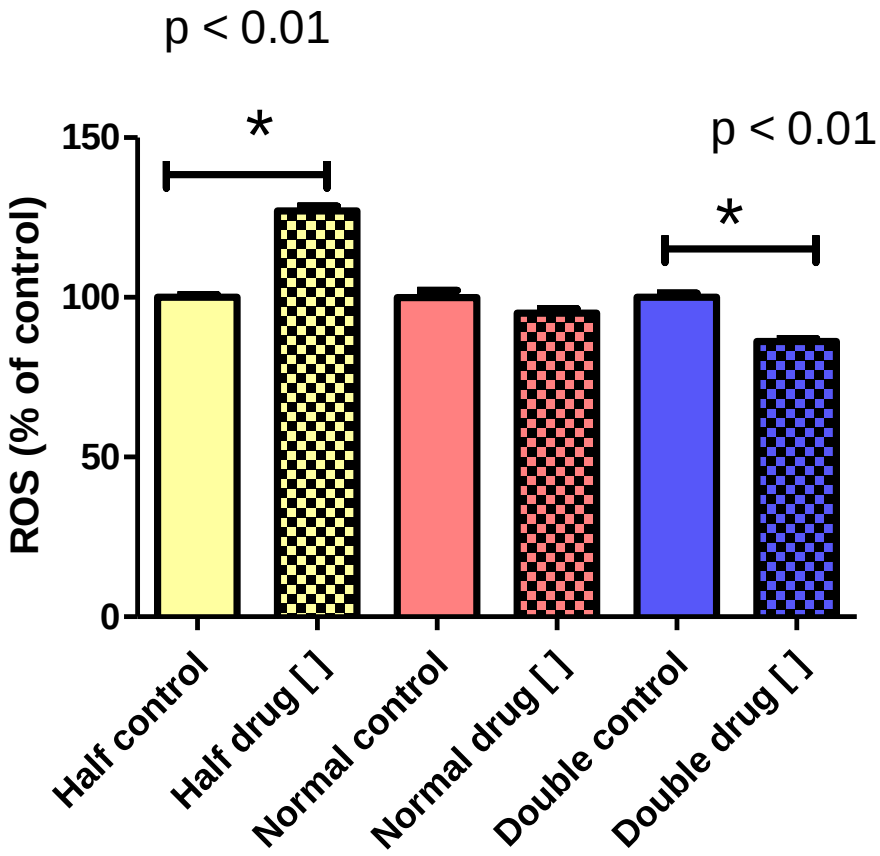


Figure 5.6: Effects of half drug concentration vs. half control, normal drug concentration vs. normal control and double drug concentration vs. double control on ROS production as measured by DCF fluorescence. ROS expressed as % DCF stained cells (calculated as a percentage of control; control adjusted to 100 %), n= 5 / group. [ ]: Concentration.

5.3.2 Second line ART dose-response

5.3.2.1 The effect of a second line ART combination drug cocktail (dose-response) on NO production in AECs

AECs exposed to half drug concentration (24 hours), showed no change (107.62± 7.77% vs. 100 ± 7.25 % half vehicle control) in mean DAF-2/DA fluorescence (measuring NO) (**refer to figure 5.7**). AECs exposed to normal drug concentration (24 hours), showed no change (98.55 ± 11.60 % vs. 100 ± 8.22 % normal vehicle control) in mean DAF-2/DA fluorescence (measuring NO) (**refer to figure 5.7**). AECs exposed to double drug concentration (24 hours), showed no change (96.32 ± 13.68 % vs. 100 ± 15.60 % double vehicle control) in mean DAF-2/DA fluorescence (measuring NO) (**refer to figure 5.7**).

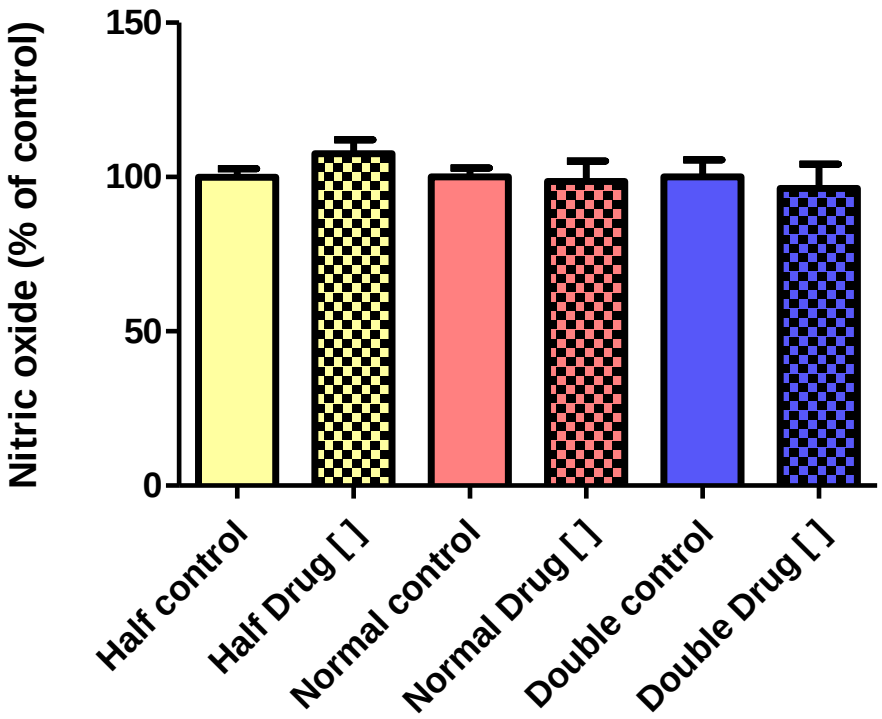


Figure 5.7: Effects of half drug concentration vs. half control, normal drug concentration vs. normal control and double drug concentration vs. double control on NO as measured by DAF-2/DA fluorescence. NO expressed as % DAF-2/DA stained cells (calculated as a percentage of control; control adjusted to 100 %), n= 5 / group. [ ]: Concentration.

5.3.2.2 The effect of second line ART combination drug cocktail (dose-response) on the cell viability of AECs

AECs exposed to half drug concentration (24 hours), showed no change (133.34 ± 22.77 % vs. 100 ± 39.96 % half vehicle control) in mean PI fluorescence (measuring necrosis) (**refer to figure 5.8**). AECs exposed to normal drug concentration (24 hours), showed no change (113.08 ± 22.14 % vs. 100 ± 48.85 % normal vehicle control) in mean PI fluorescence (measuring necrosis) (**refer to figure 5.8**). AECs exposed to double drug concentration (24 hours), showed no change (97.58 ± 17.90 % vs. 100 ± 25.77 % double vehicle control) in mean PI fluorescence (measuring necrosis) (**refer to figure 5.8**).

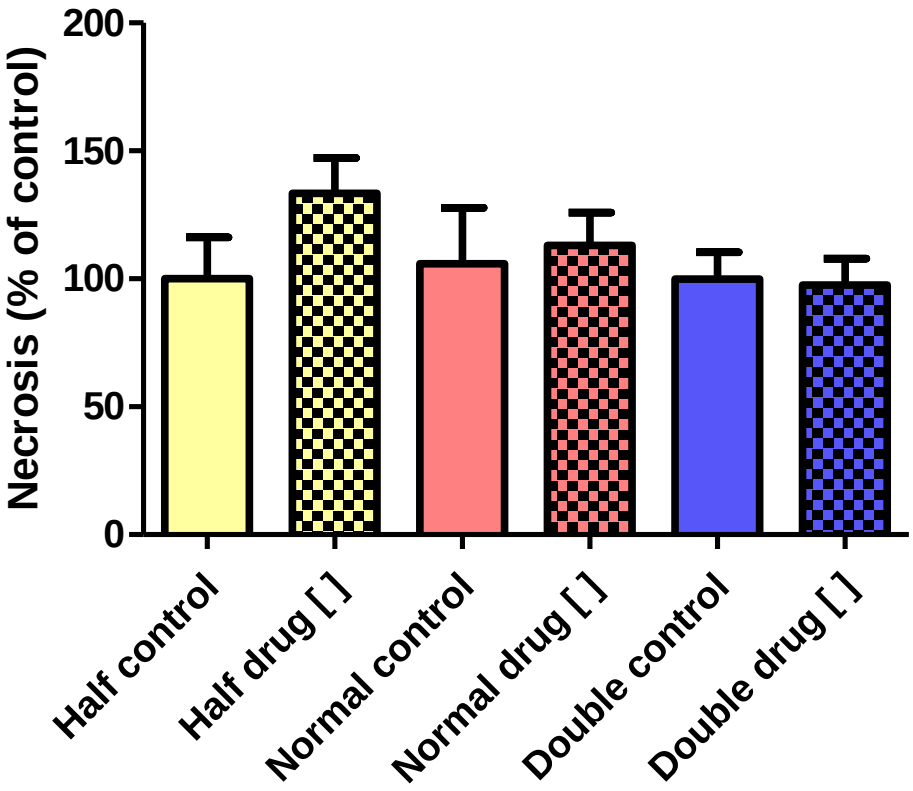


Figure 5.8: Effects of half drug concentration vs. half control, normal drug concentration vs. normal control and double drug concentration vs. double control on necrosis as measured by PI fluorescence. Necrosis expressed as % PI stained cells (calculated as a percentage of control; control adjusted to 100 %), n= 5 / group. [ ]: Concentration.

5.3.2.3 The effect of second line ART combination drug cocktail (dose-response) on ROS production in AECs

AECs exposed to half drug concentration (24 hours), showed a significant increase (115.07 ± 13.86 % vs. 100 ± 16 % half vehicle control) in mean DCF fluorescence (measuring ROS) (**refer to figure 5.9**). AECs exposed to normal drug concentration (24 hours), showed no change (96.82± 2.56 % vs. 100 ± 8.08 % normal vehicle control: p = 0.0046) in mean DCF fluorescence (measuring ROS) (**refer to figure 5.9**). AECs exposed to double drug concentration (24 hours), showed a significant increase (116.51 ± 7.71 % vs. 100 ± 4.92 % double vehicle control: p= 0.008) in mean DCF fluorescence (measuring ROS) (**refer to figure 5.9**).

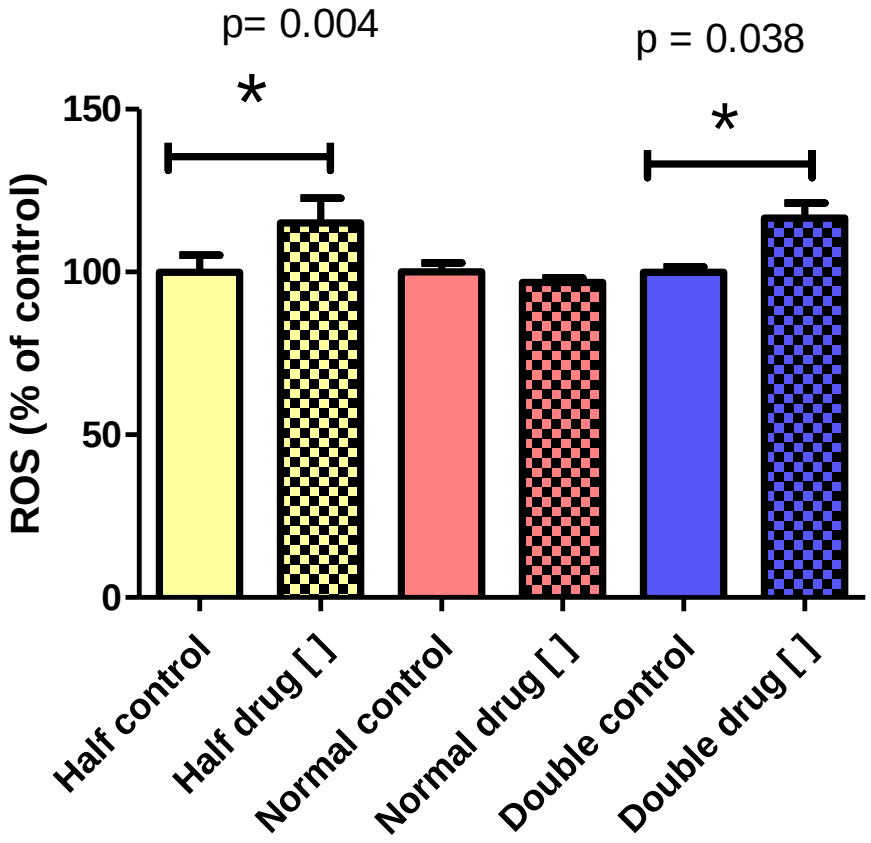


Figure 5.9: Effects of half drug concentration vs. half control, normal drug concentration vs. normal control and double drug concentration vs. double control on ROS production as measured by DCF fluorescence. ROS expressed as % DCF stained cells (calculated as a percentage of control; control adjusted to 100 %), n= 4 / group. [ ]: Concentration.

Chapter 6 : Discussion & Conclusion (clinical study)

This chapter argues the findings from the main cross-sectional study and summarises key findings followed by a conclusion.

6.1 Demographic, life style and anthropometric findings of the study population

In the present study, mean age and the number of females present in each group was not significantly different, ensuring that the four groups were similar in terms of age- and gender. The median BMI and waist circumference levels in all the HIV positive groups were significantly lower than in the HIV negative group. Although recent reports have emerged demonstrating an increased incidence of cardiovascular risk factors (such as increased waist circumference and BMI) in HIV-infected individuals, HIV infection does profoundly impact the nutritional status of these individuals. HIV-infected individuals are riddled with poor appetite, impaired nutrient–absorption, increased basal metabolic rate and opportunistic infections (Lemmer, Badri et al. 2011). Under-nutrition in HIV-infected individuals is manifested by weight loss, loss of lean body mass and micronutrient deficiencies (Macallan, Miller et al. 1999). Interestingly, one study has found a lower BMI in HIV positive individuals to be associated with cardiomyopathy (Lemmer, Badri et al. 2011).

In light of arguing the current finding, the literature is somewhat inconclusive. A recently published study found that, particularly ART exposed HIV-infected individuals in a sub-Saharan African setting showed significantly higher BMI and waist circumference values in comparison to the HIV ART naïve individuals (Nduka, Uthman et al. 2016). In contrast, in the current study, no significant difference is found between the different HIV positive groups. Furthermore, increased abdominal fat deposition is known to occur in more than half of HIV-infected individuals, with ART naïve individuals demonstrating clear increases in their visceral adipose tissue and trunk fat once commencing with ART (Stanley & Grinspoon 2012, Shlay, Bartsch et al. 2007). Furthermore, a study by Shlay et al. specifically demonstrated an increased BMI in HIV individuals' after commencement on PI, NNRTIs as well as PIs in combination with NNRTIs (Shlay, Bartsch et al. 2007). Moreover, many studies have also found no

significant difference in BMI and waist circumference between HIIV-infected and non HIV-infected individuals.

Ultimately our finding does correspond to some previous findings from Southern Africa where HIV infection is associated with a lower BMI and a lower prevalence of obesity and overweight (Malaza, Mossong et al. 2012, Bärnighausen, Welz et al. 2008). As discussed, the literature has observed HIV-infected individuals to be associated with higher BMI and waist circumference, our finding could be attributed to late presentation and initiation of ART. Thus ART participants could be in a more advanced stage of HIV disease which could have resulted in severe weight loss as HIV stage is largely attributed to weight loss in HIV-infected individuals (Brennan-Benson 2009, Malaza, Mossong et al. 2012). However, it is imperative to note that the current study did not record the duration of HIV infection and ART of the participants, this in fact is a limitation and thus the finding cannot be conclusively attributed to late presentation and initiation of ART.

Furthermore, it is interesting to note that according to the WHO recommended BMI cut-off values, the median BMI in the HIV negative group would be considered 'overweight', whereas the median BMI in the HIV positive groups would be considered to fall within the normal range. This is in itself could be considered a finding which is in line with the current SA weight and obesity trends. The general adult population of South Africa is under an increased burden of obesity, in fact South Africa has the highest prevalence of obesity in sub-Saharan Africa (Tugendhaft, Manyema et al. 2015).

6.2 Cardiovascular and biochemical findings of the study population

HIV positive first line and second line ART groups demonstrated significantly lower median total cholesterol and LDL-C levels in comparison to the HIV negative group. Furthermore, LDL-C levels were additionally observed to be significantly lower in the ART naïve compared to the HIV negative group. Our findings seem to contradict the literature, as ART, particularly protease inhibitors (which the present study's second line ART group were using) has shown to elevate the lipid profiles of HIV-infected individuals (**refer to table 6.1** for the overall effects of ARTs on lipids and glucose levels). Furthermore, a recent study investigated the type of PIs and their impact on the lipid profile and it was clear that greater elevations in total cholesterol

was observed in the group that was on lopinavir and ritonavir (second line ARTs in SA) but no differences in the LDL and HDL levels. HIV-infected individuals treated with efavirenz (first line ART in SA) showed similar increases in total cholesterol compared with the individuals taking lopinavir and ritonavir. In addition, HIV infected individuals treated with abacavir/lamivudine, zidovudine/lamiuvudine, or stavudine/lamivudine showed significantly higher elevations in all lipid parameters, in comparison to individuals using tenofovir/emtricitabine. Thus it has been demonstrated in literature that there is definitely a wide range of lipid elevations, which depends on the ART drug class and type used (Hill, Sawyer et al. 2015). PIs such as ritonavir, indinavir, and amprenavir are known to upregulate CD36, a scavenger receptor, mediating cholesterol uptake in macrophages (Dressman, Kincer et al. 2003).

Our finding of modest but significant higher median total cholesterol levels in the HIV positive first line ART group in comparison to ART naïve group is somewhat in line with literature. Although NNRTIs induce a less harmful effect on lipids than PIs, it has been found that efavirenz has a harmful effect on lipids when compared to other NNRTIs (Feeney, Mallon 2011). Furthermore, ART naïve individuals have been observed to have a lower total, LDL-C and HDL-C levels, as well as elevated serum triglycerides (Grunfeld 2008, Feeney, Mallon 2011). HIV-infection was associated with altered lipid metabolism, even before the advent of ART. In the early phase of acute HIV-infection, individuals demonstrate several diverse clinical signs of immunosuppression such as fever, intestinal infections, weight loss and depletion of protein reserves (da Cunha, Maselli et al. 2015). The possibility of HIV infection, by itself, causing changes in lipid metabolism has been postulated, because it is evident that plasma viremia may promote a decrease in the plasma concentrations of total cholesterol, HDL and LDL and, in later stages of infection, an elevation in the concentration of TG (Sellmeyer, Grunfeld 1996, Pedersen, Lindhardt et al. 1989, da Cunha, Maselli et al. 2015). Specifically, the reduction of HDL likely occurs as a result of an activation of the immune system in early HIV infection, which promotes an increase in lipid peroxidation, inflammatory cytokine production, and alterations in the reverse cholesterol transport. This process promotes an imbalance in their antioxidant system, a decrease in the production of anti-inflammatory cytokines and an elevation of proinflammatory cytokines, which increases the chance of developing atherosclerotic diseases (da Cunha, Maselli et al. 2015, Sellmeyer, Grunfeld 1996, Pedersen, Lindhardt et al. 1989).

The present study also observed that the median HBA1C% levels were significantly lower in the HIV positive second line group compared to the HIV positive ART-naïve group. Studies have demonstrated that individuals with HIV infection have lower baseline HbA1c values than the HIV-negative controls (Han, Crane et al. 2012). Furthermore, the type of ART has been related to changes in HbA1c levels (**Refer to table 6.1**). People receiving a PI-based regimen, but not those on a non-PI containing regimen, have shown to have significantly lower HbA1c levels compared to the controls (Han, Crane et al. 2012). In contrast, relatively new evidence is emerging in the literature showing that certain protease inhibitors, such as indinavir, lopinavir, and ritonavir, can reversibly induce insulin resistance, probably by inhibition of glucose translocation through glucose transporter type 4 (GLUT4) (Grunfeld 2008, Brown, Tassiopoulos et al. 2010).

Table 6.1: *Overall effects of main ARTs on lipid profiles (da Cunha, Maselli et al. 2015)*

Antiretroviral class	Drug	Effects on lipids	Effects on glucose
NRTIs	Emtricitabine (FTC)	↑ Dyslipidaemia	No effect
	Lamivudine (3TC)	↑ Dyslipidaemia	No effect
	Tenofovir (TDF)	↑ Dyslipidaemia	No effect
	Zidovudine (AZT)	↑↑ Dyslipidaemia	Insulin resistance
NNRTIs	Efavirenz (EFV)	↑↑ HDL, ↑ Dyslipidaemia	No effect
	Etravirine (ETR)	Neutral effects	No effect
	Nevirapine (NVP)	↑↑ HDL, ↑LDL	No effect
PIs	Lopinavir/ritonavir	↑↑↑ Dyslipidaemia	Insulin resistance
	Amprenavir/ritonavir	↑↑↑ Dyslipidaemia	Insulin resistance
	Atazanavir/ritonavir	↑ Dyslipidaemia	Insulin resistance
	Darunavir/ritonavir ↑	↑ Dyslipidemia	Insulin resistance

↑: increase; ↑↑: 2 X increase; ↑↑↑: 3 X increase.

6.3 Vascular function

The present study did not find any significant differences in the measured vascular function variables amongst the four groups of the total cohort. Our findings correspond with a few studies where FMD% was not significantly different between the HIV-infected and non-infected individuals (Blanco, Garcia et al. 2006, Nolan, Watts et al. 2003, Mondy, de las Fuentes et al. 2008).

Conversely, our findings contradict those of other studies using FMD to assess vascular function that HIV infected individuals present with endothelial dysfunction in comparison to individuals without HIV infection (Stein, Currier et al. 2014). A case-control study of 83 HIV infected-children, found that FMD% was significantly lower in the HIV positive group when compared to the HIV negative group. When comparing the different treatment exposure groups, it was found that FMD % was even worse in children using PIs (which the second line ART group of the present study are using) (Charakida, Donald et al. 2005). In fact, the present study demonstrated the highest mean FMD% for the group on second line ART, albeit not significant. Furthermore, a study of adult HIV-infected individuals by Stein et al. reported on impaired (lower FMD% relative to study's control group's FMD%) endothelial function in HIV-infected individuals using PIs containing regimen while the HIV infected ART naïve individuals presented with normal FMD% ("normal FMD%" was considered more than or equal to the mean FMD% in the study's control group). This study further concluded that PIs are associated with pro-atherogenic lipoprotein changes and with endothelial dysfunction (Stein, Currier et al. 2014, Stein, Klein et al. 2001). Another study evaluating endothelial function in an adult population, also found significantly impaired FMD in HIV-infected individuals in comparison to healthy controls (Solages, Vita et al. 2006). In addition, a most recent study of 2016, based in Ethiopia also found significant differences between their HIV-infected populations. They observed that FMD was impaired in younger adults treated with efavirenz and lopinavir in combination with ritonavir in comparison to ART naïve younger adults. Furthermore, FMD was impaired in efavirenz-treated younger adults group compared to nevaripine-treated young and older adults, and in lopinavir with ritonavir treated group compared to nevaripine-treated older adults (Gleason Jr., Caulk et al. 2016).

On the other hand, there are some studies that have demonstrated improved endothelial function upon commencement of ART (Torriani et al 2008.). This study observed an improvement in the HIV-infected individuals' FMD% after commencement of a PI sparing regimen of NRTIs plus efavirenz, NNRTI-sparing regimen of NRTIs plus lopinavir/ritonavir, or a NRTI-sparing regimen of efavirenz plus lopinavir/ritonavir. The NRTIs were lamivudine, stavudine, zidovudine, or tenofovir. (Torriani, Komarow et al. 2008). In addition, a study by Stein et al 2014, demonstrated that FMD% was impaired in the ART naïve group prior to ART commencement. The participants were assigned to use one of three antiretroviral regimens: efavirenz + 2 NRTI (PI sparing regimen); lopinavir/ritonavir + 2 NRTI (NNRTI sparing regimen); and lopinavir/ritonavir + efavirenz (NRTI sparing regimen). Endothelial function

determined by flow-mediated vasodilatation was measured at baseline, week 4 and at week 24. After 4 weeks of treatment, FMD had significantly improved by 1.1% (p=0.003) and the improvement was of similar magnitude in each arm. After 24 weeks of treatment, FMD had increased significantly by 1.9% (p<0.001) and it was of similar magnitude for each arm. This suggests that the use of the three different antiretroviral regimens rapidly improved endothelial function in HIV positive ART naïve individuals and that the benefits were similar regardless of antiretroviral regimen. Furthermore, the benefits were noticeable as early as after 4 weeks and persisted at 24 weeks of treatment. This study concluded that antiretroviral treatment in HIV positive ART naïve individuals improved vascular reactivity and it may have decreased short term cardiovascular risks (Andrade, Cotter 2006).

In light of contradicting findings in literature with regards to vascular function in HIV-infected populations, the present study's findings could be attributed to the specific ART drugs that the participants are using as well as the difference in population characteristics and a small sample size. It is noteworthy that none of the studies in the literature have assessed endothelial function in groups taking SA approved first line and second line ART.

6.4 Associations of vascular variables with various demographic and cardiovascular risk variables in the total population, HIV negative population, and HIV positive population

One of the major findings of the present study is that HIV-infection was not associated with poorer vascular endothelial function measurements. This includes all the vascular variables, namely baseline artery diameter, maximum artery diameter, and FMD%. In adults, case-control studies investigating the effects of HIV infection on FMD% have had inconsistent results (Solages, Vita et al. 2006, Mondy, de las Fuentes et al. 2008, Blanco, Garcia et al. 2006, Nolan, Watts et al. 2003). Each of these studies have been relatively small with the largest including only 83 HIV-infected individuals and the population characteristics as well as imaging techniques have varied between studies. However, as mentioned earlier, the extent of infection and inflammation as well as the use and type of ART might play a role.

Our findings show that female gender was inversely associated with baseline brachial artery diameter in all the groups. This is in line with literature as it has been established that females generally have a smaller baseline brachial artery diameter than males (Pham, Kim et al. 2016, Kapuku, Treiber et al. 2004). The confounding impact of baseline artery diameter on the FMD% is the underlying reason for forcing it to remain in the regression model where FMD is the dependent variable, hence ensuring that it is adjusted for and that any associations with FMD% are observed independently of the baseline artery diameter.

Since baseline artery diameter is used in the calculation of FMD%, it follows that it is an indirect predictor of endothelial function (FMD%). Therefore, associations with baseline artery diameter will also be briefly discussed. In our study, smoking was positively associated with baseline artery diameter in the total population. This is difficult to explain as the literature largely points to the opposite thus the finding can be attributed to being a statistical finding. Although baseline artery diameter is not a reflection of endothelial function, it has been established that cigarette smoking can induce vascular dysfunction.

Furthermore, the present study did not find any significant associations with various cardiovascular risk factors and FMD in the total HIV-infected group. A large body of scientific evidence, does however point to a range of risk factors to be associated with impaired vascular function in HIV-infected individuals. These risk factors include lipoprotein levels, systolic blood pressure, markers of insulin-glucose metabolism, and presence of metabolic syndrome, among others (Stein, Currier et al. 2014). Our findings could be attributed to a small sample size, difference in population characteristics (this is the first study of its kind in a population of mainly mixed-race adults in the Western Cape with no previous data to which our findings can be compared and validated), and different distributions of traditional cardiovascular risk factors. Additionally, it is important to note that in the regression analysis models, the current study's total HIV-infected group consisted of ART naïve individuals as well as those that are using first line and second line ART, which may have affected the observed associations.

6.5 Associations of vascular variables with various demographic and cardiovascular risk variables in the HIV positive ART naïve, first line ART and second line ART populations respectively.

An important finding of the present study is that CRP-levels were negatively associated with FMD% in the second line ART group. CRP is an inflammatory marker synthesized in hepatocytes in response to stimulation from IL-6. It has been demonstrated that CRP levels increase during acute inflammatory conditions. C-reactive protein can be considered a biomarker of the process of endothelial dysfunction and, at supraphysiological concentrations, as a predictor of vascular disease. It also plays an important role in down regulation of eNOS and in protein transcription of endothelial cells, which causes destabilization of eNOS-RNA. This process results in reduced production of NO.

In literature, the relationship between CRP and FMD% is inconclusive. A study by Mondy et al. found no significant correlation between high-sensitivity CRP and FMD% in HIV positive individuals on long term HAART (Mondy, de las Fuentes et al. 2008). Furthermore, a study investigating endothelial function in HIV-infected individuals on various regimes, including lopinavir and ritonavir (SA approved second line drugs), also could not find an association with CRP and endothelial function (Torriani, Komarow et al. 2008). Additionally, three other studies found no association between CRP and endothelial function (Nolan, Watts et al. 2003, van Wijk, de Koning et al. 2006, Charakida, Donald et al. 2005). It is noteworthy that each study had less than 100 HIV-infected subjects with varying demographic, CVD risk factors, and HIV treatment profiles. Additionally, some of the studies measured high-sensitivity CRP, whereas the current study measured normal CRP.

In light of arguing the current finding, there is no concrete evidence from previous studies that PIs are associated with increased CRP levels in HIV individuals which could then be associated with a lower FMD. It is well known that in HIV-negative patients, inflammation plays an important role in endothelial dysfunction and atherosclerosis. Similarly, higher levels of CRP have been found in HIV-infected patients compared to controls and this has been shown to predict cardiovascular mortality and morbidity even after accounting for viral load and CD4 count (Hsue et al., 2004). In fact, one study has shown that CRP levels did not normalize after 96 weeks of treatment (Shikuma et al., 2011). Furthermore, CRP is not the only marker of

inflammation in HIV infection; HIV appears to be associated with a generalized inflammatory activation of the vascular wall. Proinflammatory markers and adhesion molecules that are implicated in the pathogenesis of cardiovascular disease in non-HIV individuals are similarly studied in the context of HIV. An example of this is, TNF-α, which is also expressed in large quantities by macrophages in HIV-infected individuals (Subbarao, Lowe et al. 2011).

The present study demonstrates the potential of CRP as a biomarker of reduced endothelial function in the second line group. This is a highly interesting finding which has not been demonstrated in the past and warrants further investigation.

Smoking showed a borderline negative association (p= 0.052) with FMD% in the first line ART group. Endothelial dysfunction has been correlated with a wide range of cardiovascular risk factors, including smoking. Cigarette smoking does play a role in atherogenesis and endothelial dysfunction (Messner & Bernhard 2014). Therefore, HIV-infected individuals that smoke, may present with poor vascular endothelial function, possibly even to a greater extent compared to the general smoking population. Furthermore, since smoking is linked to endothelial dysfunction, it was included in the model to 'adjust' for it and find associations with other variables independent of smoking.

6.6. Associations of vascular variables with CD4 count in the total HIV positive population and ART groups

The present study found that CD4 count is positively associated with endothelial function in the second line ART group. CD4 cells play an important and central role in the immune system, coordinating the arms of the adaptive immune system to shape an effective response while simultaneously regulating non-essential or deleterious activities. Their importance is demonstrated most strikingly during AIDs, when there is significant depletion of CD4 cells by the HI virus. The depletion of CD4 cells ultimately results in a host of immune dysfunctions and susceptibility to opportunistic pathogens (Soghoian 2015). In HIV, protease inhibitors based combination therapy has shown to increase CD4 count in children and adolescents (Soh, Oleske et al. 2003).

With regards to CD4 count being positively associated with endothelial function, the present study's finding is somewhat in line with literature. It has been found that 'nadir' CD4 count of less than 350 cells/µl is associated with lower FMD%. In fact, even after adjusting for various

cardiovascular risk factors, the association remained in that study (Ho, Scherzer et al. 2012). Furthermore, another study has found a negative correlation between CD4 count and FMD% (Nolan, Watts et al. 2003). The CD4 count in a way represents the HIV disease stage of the individuals. A lower CD4 count points to a worse stage of HIV disease progression. HIV infection and the stage of it has been linked with endothelial dysfunction (Subbarao, Lowe et al. 2011). Therefore, an increased CD4 count represents healthier HIV individuals and thus can be correlated to a better endothelial function.

Interestingly, this association is only found in the study's second line ART group. HIV-infected individuals that are on second line treatment in South Africa, generally are in a more progressed phase of the infection. In view of that, these participants may be particularly sensitive to any changes in their CD4 counts, which could significantly impact on their vascular and endothelial health. This further could suggest that the immunological status of these individuals impacts their vascular function. Furthermore, previous studies have not investigated the association of CD4 count with vascular endothelial function in HIV positive individuals using the particular SA approved drugs. The particular PI drugs that the participants were using may also play a role in this association.

6.7 Concluding remarks

In the present study, the groups were similar in terms of age, gender and ethnic composition. In this cohort, the mean values of markers of cardiovascular risk were generally lower in HIV positive groups in comparison to the HIV negative group, which suggests a generally more favourable cardiovascular risk profile. This includes BMI, waist circumference and lipid parameters such as total cholesterol and LDL cholesterol. In the present study, endothelial function was not found to be significantly different amongst the four groups. When evaluating independent associations, CD4 count, CRP levels, as well as female gender were found to be independent predictors of endothelial function in HIV positive, second line ART group. Furthermore, cigarette smoking history was found to be a negative independent predictor of endothelial function in the first line ART group.

6.8 Advantages, Limitations and Future Directions

This study has several advantages. It is the first of its kind to assess endothelial function in HIV infected adult individuals in the Western Cape Province of South Africa. Furthermore, it is the first study to assess endothelial function and its association with cardiovascular risk factors in HIV-infected populations receiving the South African approved first line and second line ART.

The clinical study has some limitations. The study was of a cross sectional nature and the number of participants were relatively low, however still higher than several other studies such as of Nolan & Watts (2003), Blanco et al. (2006), Lebech et al. (2007), Torriani et al. (2008), and Mondy et al. (2008), assessing endothelial function in HIV-infected populations. Although the current study found a few independent predictors of endothelial function, some researchers may argue that the findings could be attributed to the statistical regression model having too many independent variables and thus making the model less stable. This could be corrected by running a forward stepwise multiple regression model with a full list of dependent variables. This would allow the model to consider all confounding variables in earlier steps, but at a final stage only the most important variables are considered. Furthermore, the duration of HIV-infection and ART were not measured. Thus more studies are warranted, especially those of longitudinal nature in South Africa. With regards to regression analysis, ART status was not included as an independent variable in the model for the total HIV positive group. This could have impacted the study's finding. Although intra-group regression analyses were conducted in the ART treatment groups, future studies are recommended to include ART in their regression models when measuring independent associations in the HIV population. Additionally, this study did not perform serum analysis for biomarkers of endothelial dysfunction due to funding restrictions. These biomarkers could include VCAM-1, ICAM-1, E-selectin, P-selectin, VEGF, TNF-α. These have previously been demonstrated to be predictors of ED in HIV populations by Graham et al. (2013). Furthermore, with current trends in clinical and health related research, male participant recruitment was not easy and even after planning for equal number of participants in each gender group, although not significant, the present study did have more females than males. In light with our finding of female gender being associated with a smaller baseline artery diameter and FMD%, it would be interesting to compare and assess endothelial function in HIV participants based on their gender. Therefore, future studies are warranted keeping these limitations in mind.

Chapter 7 : Discussion and Conclusion (*in vitro* sub-study)

This chapter argues the findings of the *in vitro* sub-study.

7.1 Findings from Proteomic analysis of the HIV-conditioned model

The present sub study identified detectable expression levels of HIV-1 glycoprotein gp160 in the FBS-free HIV-conditioned medium. This is somewhat in line with current literature. The HL2/3 hela cells are transfected with a clone of the HXB2 strain that lack the reverse transcriptase coding sequence. This results in a high level production of the HIV-1 Gag, Env (particularly gp120), Tat, Rev and Nef proteins without shedding of infectious viral particles (Ciminale & Felber et al. 1990, Klug, Ashkenazi et al. 2014, Africa 2014).

Although, only gp160 protein was identified in the present study, its role in HIV virus replication is crucial. In the HIV replication cycle, Env is translated to a heavily glycosylated 160 kD precursor (gp160) that initially self-assembles as a membrane-bound homotrimer in the lipid bilayer of the trans Golgi network. Host proteases (known as furin) cleave the protein into a 41 kDa transmembrane subunit (gp41) that remains non-covalently associated with a120 kDa surface subunit (gp120) (Moulard & Decroly 2000). This leads to production of the mature trimeric viral spike protein that is ultimately incorporated into the viral envelope during budding from the cell (**refer to figure 7.1**) The viral spike protein has two main parts: the surface protein (SU) and the transmembrane protein (TM). The tropism of the virus is determined by the SU protein domain because it is responsible for the receptor-binding function of the virus. The SU domain therefore determines the specificity of the virus for a single receptor molecule. Env spikes surround the viral surface and mediate events required for cell entry, including CD4/co-receptor binding and membrane fusion steps (Melikyan 2014, Wilen, Tilton et al. 2012). While proteolytic maturation of gp160 is not strictly required for CD4 binding, the immature gp160 trimer spike does not support fusion (Nakatani-Webster, Hu et al. 2015) (**Refer to figure 7.2**).

Interestingly, another study demonstrated a role for HIV-1 glycoproteins, gp160 and gp120 in endothelial cell injury (Ullrich, Groopman et al. 2000). It has been previously established that gp120 can damage the endothelium by its interaction with CXCR4, a receptor for the α-

chemokine stromal cell–derived factor-1 (SDF-1). In addition, this particular study observed that gp160 also was able to induce endothelial apoptosis, especially by activating caspases and mildly enhancing bax (a pro- apoptotic molecule). This novel finding points to a role for these viral envelope proteins in the transition of HIV infected cells from circulation to tissues (Ullrich, Groopman et al. 2000). Ultimately, the authors concluded that gp160 and gp120 are able to disrupt endothelial integrity via their interaction with CXCR4, enabling virus transition from the circulation and further playing a role in endothelial injury syndromes linked with HIV and AIDs (Ullrich, Groopman et al. 2000). This role of gp160 in cultured endothelial cells, largely adds value to the current study's identification of this protein in the conditioned medium. The identification of this protein further validates the current HIV-conditioned *in vitro* model.

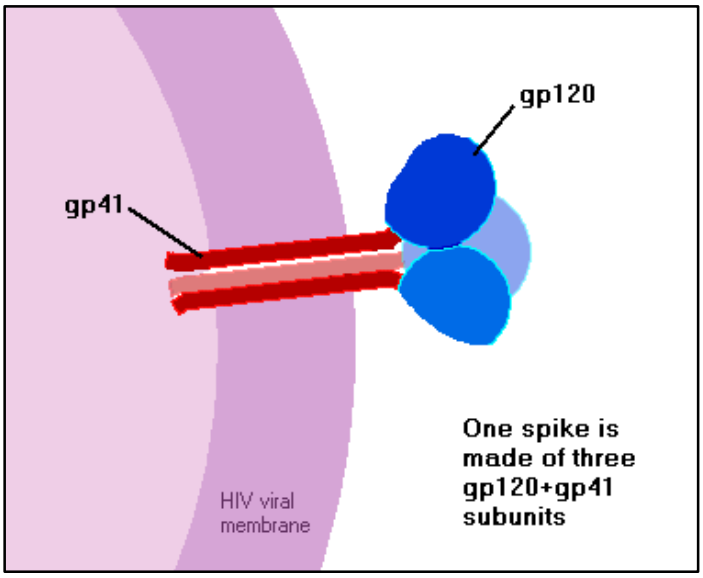


Figure 7.1: HIV viral spike protein.

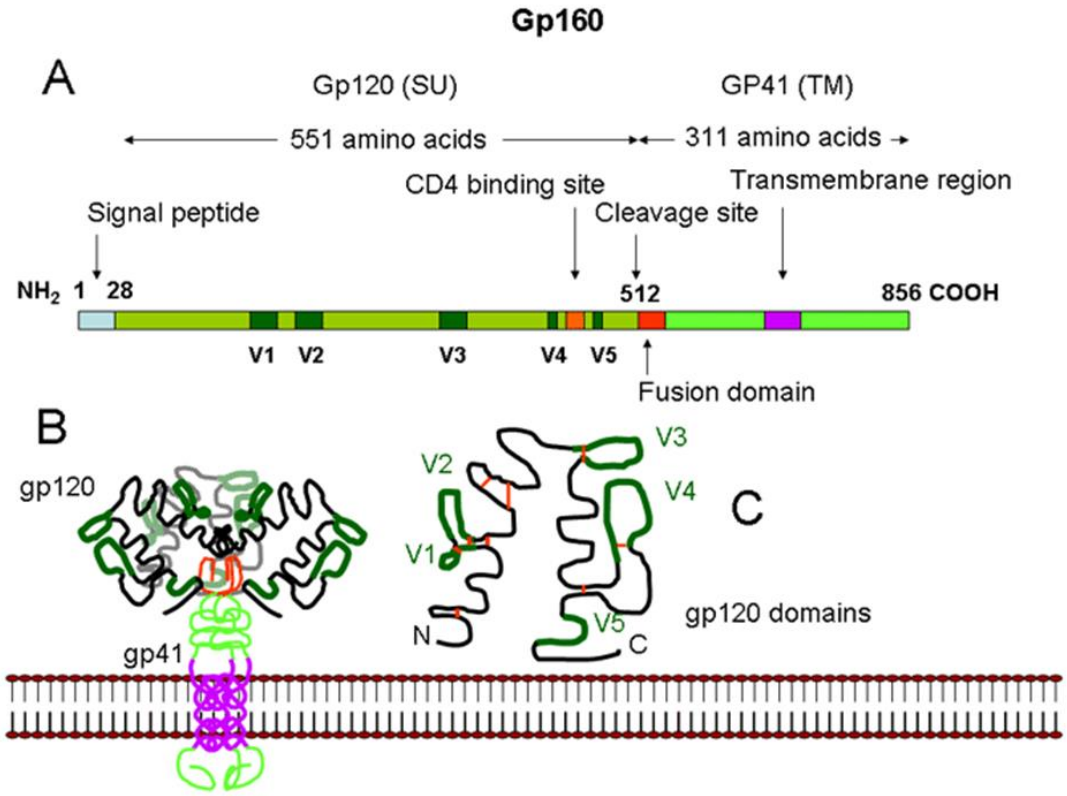


Figure 7.2: The structure of gp160.

A. The linear domain structure of gp160. Gp160 is cleaved into gp120 (the surface protein) and gp41 (the transmembrane fusion protein). B. A trimer of gp120/gp41 is associated with the viral membrane. C. Gp120 has a number of hypervariable domains (V1-V5). The red bars show disulphide bridges (Nakatani-Webster, Hu et al. 2015).

7.2 The effects of a HIV-conditioned *in vitro* model on NO production, cell viability and ROS production of AECs

No significant results were observed with regards to the HIV-conditioned *in vitro* model on NO production, cell viability and ROS production in the AECs. According to the literature, *in vitro* studies correlate endothelial dysfunction to decreased NO production, increase in NO -quenching reactive oxygen species (ROS), endothelial chemokine/adhesion protein expression, and endothelial cell death (apoptosis or necrosis) due to local inflammation in the cells (Wang, Yi et al. 2015). As discussed in previous chapters, untreated HIV-infection has been associated with impaired endothelial function (Torriani, Komarow et al. 2008). This state has been best experimentally shown in literature using whole virus or HIV gp120 in *in vitro* studies. Studies with HIV envelope as recombinant gp120 protein from CXCR4 binding strains have shown that it induces endothelial apoptosis by CXCR4-dependent caspase activation (Fiala, Murphy et al. 2004, Fiala, Polik et al. 2004). Furthermore, a study found that NO production, was reduced by HIV gp120 in tumour necrosis factor α (TNF-α) activated endothelial cells (Jiang, Fu et al. 2010, Wang, Yi et al. 2015b). Gp120 is known to stimulate macrophages to nitric oxide production which can lead to direct endothelial damage (Chi, Henry et al. 2000). Most importantly, in contrast to our findings, another study reported that gp160 (identified protein in the study's HIV-conditioned *in vitro* model) and gp120 are able to induce apoptosis in a time and concentration manner in HUVECs. Furthermore, this study pointed to a role of gp160 in endothelial injury (Ullrich, Groopman et al. 2000). However, it is vital to note that all the mentioned studies used different HIV-infection *in vitro* models and the experiments were done on various endothelial cells (mostly human derived endothelial cells), while the current study utilized adult rat aortic endothelial cells.

In light of arguing the current finding, it is imperative to note that the study's HIV conditioned model did not express detectable levels of gp120 as per proteomics analysis, whereas others have found a role of gp120 in *in vitro* studies. Although Ullrich et al. (2000) did find a harmful role for gp160 by inducing apoptosis, our study did not measure cell apoptosis. Therefore, it is possible that our conditioned medium may well have resulted in adverse effects that were not detected by our chosen end-points of NO production, necrosis and ROS production. Furthermore, it is important to note that the HIV-1 related protein was not quantified; thus the lack of cellular effects could be attributed to insufficient concentrations of the gp160 present in the medium. Finally, the current study incubated the AECs in the HIV-1 conditioned medium for one-time period only, namely 24 hours; a longer incubation period could possibly have been more effective in eliciting injury.

7.3 The effect of first line ART (dose-response) on NO production, cell viability and ROS production in AECs

The current South African approved first line fixed dose combination (FDC) tablet (trade name: Atripla®, or Odimune®) comprises of Tenofovir (NRTI), Emtricitbine (NRTI) and, Efavirenz (NNRTI). Therefore, in order to simulate the FDC tablet, the three drugs were used in combination in the cell culture experiments. The first line ART drug's normal concentration was based on previous studies that demonstrated anti HIV activity with synergistic effects of the three drugs in combination in cell culture (Feng, Ly et al. 2009, Grigsby, Pham et al. 2010). The present study found that, at the normal concentration, the first line ART drugs in combination, significantly reduced NO production in AECs (albeit modestly) in comparison to the control. One study showed that ART agents generally reduce NO serum levels and increase ROS which can accelerate foam cell formation (Skowyra, Zdziechowicz et al. 2012). Endothelial cells are known to be highly sensitive to ROS, and it is believed that the interaction of ROS with endothelial cell-generated nitric oxide (NO) may be one of the reasons for the sensitivity and increased cardiovascular dysfunction observed in patients receiving HIV treatment-related drugs (Skowyra, Zdziechowicz et al. 2012).

The present study did not find any significant difference in cell viability of AECs treated with first line ART agents, as measured by propidium iodide staining. One particular agent in the first line regime, efavirenz has been shown to increase cell necrosis and ROS production in human umbilical vein endothelial cells (HUVECs) at concentrations 10ug/ml and 15ug/ml (Weiß, Kost et al. 2016). This study demonstrated that relevant concentrations of efavirenz led to the generation of ROS in human endothelial cells, associated with the induction of heat-shock proteins, ER stress, and autophagy. The current study's normal concentration for Efavirenz was 5.6nM (0.0018ug/ml), far smaller concentration in comparison to study by Weib et al. Moreover, the stress inducing effects of efavirenz on HUVEC were demonstrated to be aggravated by a drug combination with nelfinavir. Furthermore, efavirenz was also reported to cause oxidative stress in human hepatic cells (Apostolova, Gomez-Sucerquia et al. 2010). The current study contradicted the literature in groups that received normal and double drug concentrations in terms of ROS production, as the present study found significant decreases in ROS levels in first line ART treated AECs. However, the present study did find a significant increase in ROS production with half drug combination. In the literature it is fairly unclear as to how first line ART agents increase ROS levels. Furthermore, the mechanism by which efavirenz, the drug which is largely associated with increase ROS, is also unknown (Weiß, Kost et al. 2016).

The current study's contradicting results with literature could be attributed to the drug concentrations. As mentioned, many previous studies have used a different drug concentration in comparison to the current study. Furthermore, it is imperative to note that most of the previous studies have used human derived endothelial cells, meanwhile the current study has used adult rat aortic endothelial cells. Furthermore, many previous studies showed drug effects using different incubation periods (4- 36 hours), whereas our study only made use of a single incubation period of 24 hours.

7.4 The effect of second line ART (dose-response) on NO production, cell viability and ROS production in AECs

The second line ART normal drug concentration was based on previous *in vitro* studies (Noor, Flint et al. 2006). The present study did not demonstrate any significant difference in AECs NO production in any of the three drug groups. In literature, ritonavir (at 15.5umol/L) has been found to decrease eNOS mRNA and protein levels in cultured human coronary endothelial cells (Fu, Chai et al. 2005, Subbarao, Lowe et al. 2011). Furthermore, ritonavir is found to downregulate eNOS in human pulmonary artery endothelial cells (Wang, Chai et al. 2009). The present study did find a significant increase in cell necrosis in the AECs treated with half drug concentration. Ritonavir at concentrations near clinical plasma levels was shown to cause endothelial mitochondrial DNA damage and cell death mainly through necrosis pathways (Zhong, Lu et al. 2002). However, the said study used concentrations of 7.5, 15 and 30umol/L, whereas our study observed a significant increase in cell necrosis, in AECs treated in 1 uM, (1umol/L) (half of normal drug concentration) (Zhong, Lu et al. 2002).

The present study observed a significant increase in ROS production in the double drug group and a significant decrease in ROS production in the normal drug group, although the latter was by a small margin. In literature, the drugs from the protease inhibitor class are implicated to increase production of ROS in experiments (Kline & Sutliff 2008). They have been found to also increase endothelial cell permeability and leukocyte adhesion in cell culture models (Mondal, Pradhan et al. 2004, Kline & Sutliff 2008, Lowe et al. 2011). However, most of these experiments have investigated the effects of the drug zidovudine on cell ROS production (Kline & Sutliff 2008). Furthermore, it is important to note that all the second line ART studies in literature vary in terms of their ART drug, concentration as well as the type of cells used. Thus making it difficult to make accurate deduction on the effects of ART on endothelial function *in vitro*.

7.5 Concluding Remarks

The present sub-study attempted to establish a HIV-conditioned *in vitro* model and was successful in identifying the HIV-1 envelope protein, gp160, in the conditioned growth medium. However, the effect of this HIV-conditioned *in vitro* model did not elicit any significant effects on the AECs NO production, cell viability and ROS production. With regards to the effect of the first line and second line ART on AECs, mixed results were obtained (**refer to table 7.1** for summary of results). It is imperative to note that the current study combined the first line drugs in a one 'cocktail' and the second line drugs in one 'cocktail', in order to stimulate the SA approved FDC and second line combination regime. Most studies in the literature investigated the effects of these drugs separately, therefore there is a possibility that these drugs may elicit a different effect when investigated separately. However, it is evident from the present study's experiments with first line ART regime, that the double drug concentration did not induce any endothelial damage as it decreased ROS levels, while the half drug concentration increased ROS, and normal drug concentration reduced cellular NO production. For the experiments with the second line ART regime, it appears that the normal drug concentration did not induce endothelial damage as it had no effect on cellular ROS levels, meanwhile the double drug concentration increased ROS levels. Therefore, double drug concentration for first line ART and normal drug concentration for second line ART, could be considered a relevant dose for future investigations into the effects of ART drugs in an HIV-1 infected model.

Table 7.1: *Summary of results from First and Second line ART dose response*

ART regime	Drug concentration	NO production	Necrosis	ROS production
First line ART	Half drug concentration	No effect	No effect	**Increase**
	Normal drug concentration	**Decrease**	No effect	No effect
	Double drug concentration	No effect	No effect	**Decrease**
Second line ART	Half drug concentration	No effect	No effect	**Increase**
	Normal drug concentration	No effect	No effect	No effect
	Double drug concentration	No effect	No effect	**Increase**

7.5 Advantages, Limitations and Future Directions

To the best of our knowledge, there are no previously published studies that investigated the effects of the South African approved ART regimes on endothelial cells with NO production, cellular necrosis and ROS production as end-points.

A limitation in the present study, was that HIV-conditioned in vitro model elicited no effect on the measured parameters of NO production, cell viability and ROS production. More than a year was spent establishing the protocol to develop this model. Several stumbling blocks were faced and unsuccessful experiments performed. The development of this model would be ideal to 'mimic' HIV-infection in *in vitro* for the investigations of ARTs. Furthermore, it would be a cost-effective and safe to work with as it doesn't consist of a live HI virus. Finally, due to time constrains and in view of the unsuccessful HIV-conditioned in vitro model, the effects of the first line and second line ART agents were not investigated on 'HIV exposed' endothelial cells. Instead the effects of these ART regimes was investigated on AECs in their normal AEC medium. Although, the effects of various ART doses were demonstrated on AECs, the results were mixed. Another major limitation of the sub study is that the sample size for each parameter was relatively small and the concentration of the drugs used relatively low compared to other studies in the literature. More studies are warranted with bigger sample size and an established HIV-infected *in vitro* model.

Chapter 8: Overall conclusion

The current study aimed to investigate the effects of the South African Government approved first line and second line ART drugs on HIV-exposed endothelial function. The clinical study highlighted CRP, CD4 as well as female gender to be independent predictors of vascular endothelial function in HIV positive, second line ART individuals. Furthermore, a modest association with vascular endothelial function was noted with smoking history in HIV positive, first line ART individuals. Overall, a more favourable cardiovascular risk profile was noted in the HIV-positive individuals of the present cohort. Additionally, the successful identification of a HIV envelope protein gp160 in the HIV-conditioned medium, to an extent validated the sub-study's HIV-conditioned *in vitro* model. The identification of a safe combination dose of the specific first line and second line ART drug combinations used in South Africa appear to be findings that have never been demonstrated in the past in an *in vitro* model of endothelial cells.

Furthermore, the study's interdisciplinary nature of both clinical and *in vitro* aspects, enabled a broader (macro view) on the topic. It permitted a cellular as well as clinical overview of endothelial function in HIV infection treated with ART. Although it was not possible to fully align the findings of the two models, the two aspects further exposed the study to new exciting challenges. However, future studies are warranted to shed a clearer light on the effect of HIV-infection and SA approved ART regimes on endothelial function, both from a clinical as well as from a basic scientific point of view.

8.1 Research outputs associated with the study

Published Abstracts:

- Mashele, N, **Charania, S**, Essop, F, Webster, I, Westcott, C, Goswami, N, De Boever, P, Nawrot, T. and Strijdom, H, 2016. The effects of HIV-infection and anti-retroviral treatment on endothelial function in a South African cohort. *Atherosclerosis*, *252*, pp.e162-e163.
- Strijdom H, De Boever P, Nawrot T, Walzl G, Webster I, Westcott C, Mashele N, **Charania S**, Stelzl E, Kessler HH, Goswami N. HIV and cardiovascular risk in a study population of mixed ancestry living in Cape Town, South Africa: preliminary data from the EndoAfrica study. 11th International Symposium on Molecular Diagnostics, Graz, Austria, May 2016. Clin Chem Lab Med 2016; 54(5): eA10.

International conference output:

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

Other outputs:

- **Charania S**, Mashele N, Genis A, Essop F.M, Webster I, Westcott C, Strijdom H. The effects of HIV and antiretroviral therapy on cardiovascular risk and vascular endothelial function- A cross sectional study of a population in Worcester. 44th conference of the Physiology Society of Southern Africa, hosted by university of Cape Town, 28- 31 August 2016.
- **Charania S**, Mashele N, Genis A, Essop F.M, Westcott C, Webster I, Strijdom H. Cardiovascular risk and vascular endothelial function in a Worcester study population of people living with HIV. Biomedical day, Faculty of Medicine and Health Sciences, Stellenbosch University, 23 November 2016.
- Westcott C, Mashele N, **Charania S**, Essop F, Webster I, Goswami N, De Boever P, Nawrot T, Strijdom H. Cardiovascular risk and vascular endothelial function in people living with and without HIV: A cross-sectional study in Worcester. SASCAR conference, Cape Town, September 2016.

Appendix A: Informed consent document

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT:

WINELANDS HAART to HEART STUDY

REFERENCE NUMBER: N12/12/086

PRINCIPAL INVESTIGATOR: Prof MF Essop

ADDRESS:

Department of Physiological Sciences

Mike de Vries Building

Room 2005a

Stellenbosch University

CONTACT NUMBER: 021 8083146

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is entirely voluntary and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the Health Research Ethics Committee at Stellenbosch University and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

This study will only be done in the Stellenbosch district and surrounding areas. We are hoping to include 3300 people. We are trying to get more information on the amount of fat, muscle and fluid in the body, as well as how the heart and metabolism of the body functions when people with HIV are on anti-retroviral (ARV) medication. By getting this information we would be able to see how the body uses and stores sugar and fat and that the ARV treatment does not cause any problems with the heart.

Additionally, we will also do a sonar imaging of your arm. The sonar test imaging and testing will involve inflating a blood pressure cuff around your arm for a few minutes – this may cause mild discomfort, but will not cause any damage.

If you agree to volunteer to take part in the study, a blood sample will be taken by a nurse. It will then be taken to a laboratory, called Pathcare in Stellenbosch, where the blood sample will be analysed for glucose and the different types of proteins that carry fat in the blood. Other tests that will be done by the laboratory will tell us more about the infection. The remainder of your blood can be used to obtain genetic material (RNA), however, this will be explained to you in a separate consent form, which you will need to sign if you agree that we can use your blood sample for genetic testing.

We will also take a blood pressure measurement, look and listen to your heart with a special machine and we will determine the amount of fat, muscle and fluid you have in your body with a special machine. We will also ask you to complete a questionnaire regarding your lifestyle, including diet and physical activity.

There is also a machine (SONOST 3000) in which you will be asked to take off your shoes so that your foot can be placed in this machine that will not hurt or harm you in anyway. This machine will tell us how your bone health looks like.

Why have you been invited to participate?

You have been invited to voluntary participate because you are HIV positive and you are receiving ARV therapy or not.

By agreeing to participate in this study, you will be helping us to determine if the current ARV treatment (those on treatment only) affects your metabolism, body composition, the function of your heart and your blood vessels.

What will your responsibilities be?

We will ask if we can do a physical examination on you as one of the selected patients. A blood sample will then be taken from your arm for laboratory tests. We will ask you to complete the Health and lifestyle questionnaire that contains questions regarding your family history of heart disease, diet, physical activity and what medication you are taking. One of the researchers will help you to complete this questionnaire. A registered scientist, Dr Theo Nell, will also perform body measurements on you. These measurements include your waist circumference, hip circumference, and arm circumference, the back of your arm's skin fold, height and weight. You will be asked to wear light or minimal clothing when these measurements are taken. However, the research team will ensure that these measurements are taken in a completely private area. You will also be asked to lie on the examination bed where Dr. Nell will use a special machine that will tell us how much fat, fluid and muscle is in your body. This machine will not harm you in anyway. The machine works by placing stickers on your hands and feet which will tell us how much fat, muscle and fluid is in your body.

You will then be asked to sit in front of the bone machine that will be used to see how healthy your bones are. A small amount of gel will be placed on your ankles and then the machine will test your bone health by using two rubbers that are touching your ankles.

Before you come for the test you must not eat as well as drink any fluids such as alcohol or caffeine, exercise or take medication that make you want to urinate. You would need to empty your bladder and remove all metallic objects from your body.

We will additionally ask to perform a sonar test on you. This test involves inflating a blood pressure cuff around your arm for a few minutes – this may cause mild discomfort, but will not cause any damage. This test will involve sonar imaging of your arm and may require you to wear clothing that is not too tight around the arms. You will be asked not to eat or drink anything the morning prior to your clinic visit.

Will you benefit from taking part in this research?

There are no direct benefits to you as the participant of this research project. By voluntarily taking part in this research, you will be helping the researchers to find out more about HIV positive patients on ARV treatment and how this can affect your body.

If you choose to know the results of your blood tests we will make these available to you. However, you would have to discuss this information with your personal doctor, in order to assess your medical status with ARV treatment (metabolic and cardiac status).

If we find any problems, we will inform your physician and also refer you for medical assessment.

Are there any risks involved in your taking part in this research?

There are no more than minimal medical or physiological risks associated with this study. All the researchers that are part of this study have been properly trained.

You may feel some pain associated with having blood drawn from a vein in my arm, and may experience some discomfort, bruising and/or slight bleeding at the site where the needle will go in the vein.

The body shape test will require you to take some of your clothes (not naked) and shoes off, but there is no pain involved during this procedure. All measurements will be done in private and confidentiality is very important.

The machine that will be used to determine the fat in the body uses a very small electrical current that you will not feel and is not dangerous to your health.

The bone machine will also not hurt you in any way. It feels like two soft rubber balls touching the ankles.

You may feel discomfort during the inflation stage of the sonar test; however, this will not harm you in anyway.

If you do not agree to take part, what alternatives do you have?

It is your decision to voluntary participate or not, and nothing will be done from the researchers' part or medical staff at the clinic/hospital to in any way to persuade you to take part. If you decide not to participate, this decision will not influence your current medical treatment.

Who will have access to your medical records?

Only the principal researcher (Prof Faadiel Essop and the following masters students: Dr. Faten Abaid, Sana Charania, and Post-doctoral fellow: Dr. Nyiko Mashele) will have access to your data and records. All information will be treated with respect and utmost confidentiality. Under no circumstances will your name or any form of identification be used in any publication, poster, lecture or thesis that results from this study. Professor Faadiel Essop, Dr. Faten Abaid, Sana Charania and Dr. Nyiko Mashele will be the only authorised personnel who will have access to your results from this study as well as the lifestyle questionnaire and anthropometric measurements. No personal contact information will be collected in the study. If you agree to participate, you will receive a subject number and we will use that subject number to link all your data. The subject number will not be linked to your name / contact details (which will not be collected). This ensures that all your information is anonymous.

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

There are no risks involved that could lead to injury. In the event of a test that shows abnormal the researcher will write a referral to the attending doctor at the clinic/hospital.

Will you be paid to take part in this study and are there any costs involved?

No, you will not be paid to take part in the study. There will be no costs involved for you, if you do take part. Travelling costs in order to get to the Department of Physiological Sciences will be covered by this study, and you will not need to pay for taxi fare if so needed.

Would you like to know the results of your blood tests?

Please indicate by marking the correct box with an X

YES ☐

NO ☐

Is there anything else that you should know or do?

You can contact Professor MF Essop on 021 808 3146 if you have any further queries or encounter any problems.

You can contact the Health Research Ethics Committee at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor. You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I (name) ……..……. agree to take part in a research study entitled (WINELANDS HAART to HEART STUDY).

I declare that:

I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

I have had a chance to ask questions and all my questions have been adequately answered.

I understand that taking part in this study is voluntary and I have not been pressurised to take part.

I may choose to leave the study at any time and will not be penalised or prejudiced in any way.

I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....….....…….. on (*date*) ……...…….. 2016.

Signature of participant Signature of witness

Declaration by investigator

I *(name)* ……..…… declare that:

I explained the information in this document to (participant name):

……..

I encouraged him/her to ask questions and took adequate time to answer them.

I am satisfied that he/she adequately understands all aspects of the research, as discussed above

I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) .....….....…….. on (*date*) ……...……... 2016.

Signature of investigator Signature of witness

Declaration by interpreter

I *(name)* ……..…… declare that:

I assisted the investigator (participant *name*) ……. to explain the information in this document to (*name of participant*) ……..…….. using the language medium of Afrikaans/Xhosa/Zulu/English.

We encouraged him/her to ask questions and took adequate time to answer them.

I conveyed a factually correct version of what was related to me.

I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....….....…….. on (*date*) ……...……..

Signature of interpreter Signature of witness

Appendix B: Questionnaire example

Study ID	_____
Date subject signed consent	_____ ((dd-mm-yyyy))
Date and time start of interview	_____
Date of birth	_____
Age when signed consent	_____
Gender	Male Female
Ethnicity	Black White Coloured Indian Other (please specify) Refused
Ethnicity other	_____

Have you had anything to eat or drink from last night?

Do you smoke? If so, how often?

Did you smoke this morning?

Are you currently menstruating?

Past

Have you had a heart attack in the past? ○ Yes ○ No

Have you previously had TB? ○ Yes ○ No

If yes, when did you previously have TB? _____

(If patient cannot recall date, enter year (yyyy))

Have you had a stroke in the past? ○ Yes ○ No

Present

Do you have high blood pressure (hypertension)? ○ Yes ○ No ○ Unknown

If yes, what year were you first diagnosed? _____

Do you have a heart disease? ○ Yes ○ No ○ Unknown

If yes, what year were you diagnosed? _____

Do you have high cholesterol? ○ Yes ○ No ○ Unknown

If yes, what year were you diagnosed? _____

Do you have any other long lasting health problems? (For example: kidney stones, arthritis, asthma, bilharzia, malaria) ○ Yes ○ No

If yes, please specify what the health problem is and what year you were diagnosed? _____

Do you currently have pulmonary Tuberculosis (TB)? ○ Yes ○ No

If yes, are you on treatment? ○ Yes ○ No

When did you start treatment? _____

(If the patient cannot recall date, enter year (yyyy))

Diabetes

Do you have Diabetes ○ Yes ○ No

What type of Diabetes do you have?

○ Type I Diabetes (also known as Juvenile Onset or Insulin Dependent Diabetes)
○ Type II Diabetes (also known as Non-insulin Dependent Diabetes)
○ Don't know

How long ago were you told you have diabetes?

- ○ < 12 months
- ○ 1-5 years
- ○ 6-15 years
- ○ 15+ years

Which of the following do you use to manage your diabetes?

- ☐ Diet
- ☐ Pills
- ☐ Injection
- ☐ Nothing
- ☐ Other

((tick all that apply))

If other, please specify. _____

Family History of Cardiovascular diseases

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

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

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

Family Planning

Do you currently have a baby younger than 3 months?	○ Yes	○ No	
Are you currently pregnant?	○ Yes	○ No	○ Not applicable
Are you currently breast feeding?	○ Yes	○ No	
Are you on family planning?	○ Yes	○ No	

Drug History

Do you take any medications? ○ Yes ○ No

Which medications do you take?

- ☐ Beta blockers
- ☐ Statins
- ☐ Aspirin
- ☐ Calcium channel blockers
- ☐ ACE inhibitors
- ☐ Other
- ☐ Anti-inflammatory

If other, specify _____

What dosage of aspirin do you take? _____ (mg)

Alcohol

Have you consumed an alcoholic drink within the past 12 months? ○ Yes ○ No

How often do you typically drink?

○ Daily
○ 8 or more days a month
○ Less than 8 days a month

At what age did you start drinking regularly (at least once a week)? _____ ((answer in years))

What do you drink?

	Yes	No
Beer	○	○
Spirits (brandy, vodka, cane etc.)	○	○
Red Wine	○	○
White Wine	○	○
Other	○	○

When you drink beer, how many standard units do you typically have on a single occassion? (see reference card). _____

When you drink spirits, how many standard units do you typically have on a single occassion? (see reference card). _____

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

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

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

Standard unit reference card

1 standard drink=

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

1 single measure of spirits (30ml)

1 medium size glass of wine (120ml)

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

Exercise

Are you physically active? ○ Yes ○ No

If yes, how many times a week?
- ○ Once
- ○ Twice
- ○ Three times
- ○ More than three times

How would you describe the type of exercise you do?
- ○ Mild
- ○ Moderate
- ○ Intensive

Education

What level of education have you completed?
- ☐ None
- ☐ Primary school
- ☐ High school
- ☐ ABET (Adult Basic Education Training)
- ☐ College/University/Other tertiary institution

(Tick all that apply)

Employment

Which of the following applies to your current employment situation?
- ○ Unemployed
- ○ Employed (full time)
- ○ Employed (part time)
- ○ Self-employed

As someone who is unemployed, which of the following applies to you?
- ○ Looking for work
- ○ Discourage job seeker - not looking for work
- ○ Student
- ○ Homemaker
- ○ Illness/disability prevent me to work
- ○ Too old to work
- ○ Other

If other, specify? _____

Income

Do you or someone in your household receive a Government Social Grant?

○ Yes ○ No

What is the total of your household income per month?

○ less than R1,000
○ R1,000 - R4,999
○ R5,000 - R9,999
○ R10,000 - R20,000
○ more than R20,000

Appendix C: Proteomics Analytical report

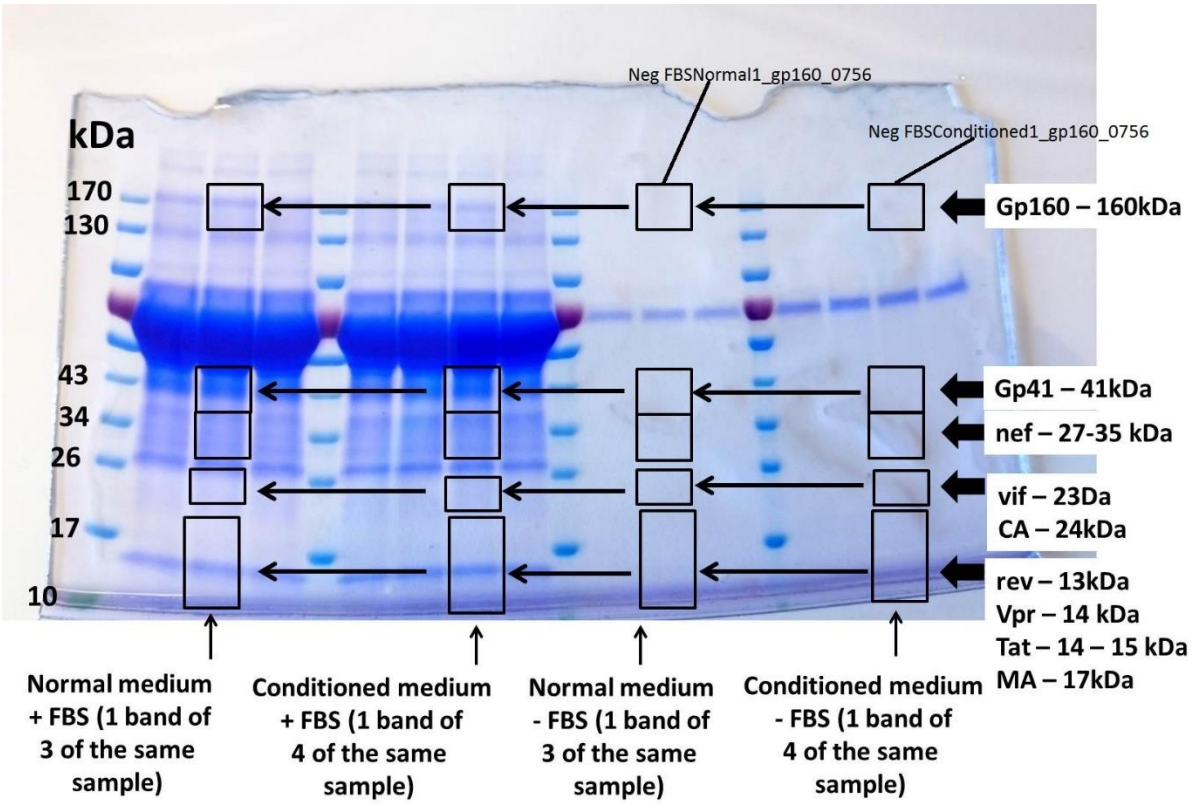
By Dr Zac Mcdonald (Proteomics Manager

1. Background and samples submitted

The aim of the project was to identify HIV derived proteins in conditioned cell culture media. Initially two in-solution samples were provided. No HIV proteins could be identified in the initial samples. To deal with the high concentrations of foetal bovine serum (FBS) following samples (14 in-gel samples) were gel fractionated and FBS was excluded in half of the samples. 24 bands were excised and digested.

2. Analysis synopsis

HIV gp160 was identified in conditioned media without FBS added. HIV gp160 was found not to be present in the corresponding normal media sample.



3. Experimental procedures

3.1. In gel digestion

All reagents are analytical grade or equivalent. Bands were excised from the provided gel and were destained in a 96-well perforated plate (Glygen PER.HOL.10) with 100mM ammo-nium bicarbonate (Ambic, Sigma 40867F) , 50% acetonitrile (ACN, Anatech BJ015CS) until clear. Samples were dehydrated and desiccated before reduction with 2mM triscarboxyeth-yl phosphine (TCEP; Fluka 646547) in 25mM Ambic for 15 minutes at room temperature with agitation. Excess TCEP was removed and the gel pieces again dehydrated. Cysteine res-idues were carbamidomethylated with 20mM iodoacetamide (Sigma I6125) in 25mM Ambic for 30 minutes at room temperature in the dark. After carbamidomethylation the gel pieces were dehydrated and washed with 25mM Ambic followed by another dehydration step. Proteins were digested by rehydrating the gel pieces in trypsin (Promega PRV5111) solution (20ng/uL) and incubating at 37°C overnight. Peptides were extracted from the gel pieces once with 50µl 0.1% trifluoroacetic acid (TFA, Sigma T6508). The samples were dried down and 200µl water added and concentrated to less than 20µl to remove residual Ambic.

The samples were dried and re-dissolved in 0.1% formic acid (Sigma 56302), 2.5% ACN for LCMS analysis.

3.2. LCMS

LC−MS/MS analysis was conducted with a Q-Exactive quadrupole-Orbitrap mass spectrome-ter (Thermo Fisher Scientific, USA) coupled with a Dionex Ultimate 3000 nano-HPLC system. The extracted peptides were dissolved in sample loading buffer (97.5% water, 2.5% Acetoni-trile, 0.1% FA) and loaded on a C18 trap column (300 µm×5mm×5 µm). Chromatographic separation was performed with a C18 column (75 µm×250 mm×3.5 µm). The solvent system employed was solvent A, Water; 0.1% Formic Acid and solvent B, Acetonitrile; 0.1% FA. The linear gradient for peptide separation was generated at 250nL/min as follows- Time change: 50min, gradient change: 6-25 % Solvent B. The mass spectrometer was operated in positive ion mode with a capillary temperature of 320°C. The applied electrospray voltage was 1.95 kV. Details of data acquisition are in following table. An inclusion list was created to target possible gp160 derived peptides for MS/MS analysis (based on preliminary analysis). In the absence of these peptide targets other precursors were chosen for fragmentation.

Full Scan	
Resolution	70,000 (@ *m/z* 200)
AGC target value	3e6
Scan range	320-1750 m/z
Maximal injection time (ms)	200
Data-dependent MS/MS	
Inclusion	On
Resolution	17,500 (@ *m/z* 200)
AGC target value	1e5
Maximal injection time (ms)	200
Loop Count	10
Isolation window width (Da)	3
NCE (%)	26
Data-dependent Settings	
Underfill ratio (%)	1
Charge exclusion	Charge states 1,6-8,>8
Peptide match	preferred
Exclusion isotopes	on
Dynamic exclusion (s)	5

INCLUSION LIST

Mass [m/z]	Formula [M]	Species	CS [z]	Polarity	Start [min]	End [min]	NCE	Comment
484.23200			2	Positive	36.00	44.00		gp160
530.78500			2	Positive	34.00	40.00		gp160
652.34500			2	Positive	32.00	38.00		gp160
512.80600			2	Positive	26.00	30.00		gp160

3.3. Data analysis

Database interrogation was performed with the Byonic algorithm (Proteinmetrics, San Carlos,CA; version PMI-Byonic-Com:v2.5.6) using a combination of all Bos Taurus and HIV (organism) reviewed and unreviewed sequences in a uniprotKB sourced database (http://www.uniprot.org/). Search parameters are detailed in appendices. Proteins with at least two unique peptides and with a log probability score above 5 were considered high confidence hits.

Appendix D: ART drug calculations

First line ART drugs: Efavirenz (EFV), Emtricitabine (FTC) and, Tenofovir (TDF).

Calculation of dosage - EFV: 5.6nM

For a final concentration of 5.6nM EFV:

Molecular weight (MW) of EFV: 315.67g/mol

1M = 315.67g/L

1mM = 315.67mg/L

1µM = 315.67µg/L

1nM = 0.31567µg/L

5.6nM = 1.767752µg/L

= 0.0017677µg/ml

Prepare a stock x1000 times concentrated:

5.6µM = 1.767752µg/ml

= 176.7752µg/100ml

* The powder is received in 10mg that will be dissolved in 1ml *methanol (*0.1767752mg / 10mg x 1ml = 0.01767752ml = 17.67752µl)

Thus 17.67752µl (18µl) of the above solution (*) should be added to 100ml PBS to get the x1000 concentrated stock of 5.6µM.

$C_1 \times V_1 = C_2 \times V_2$

5.6µM x X (volume that should be added to 1ml of medium) = 5.6nM x 1ml

5.6µM x X = 0.0056µM x 1000µl

X = 5.6 / 5.6 = 1µl

Thus 1µl from the x1000 concentrated stock should be added to 1ml of medium to get a final concentration of 5.6nM.

Calculation of dosage - FTC: 1.3µM

For a final concentration of 1.3µM FTC:

Molecular weight (MW) of FTC: 247.25g/mol

1M = 247.25g/L

1mM = 247.25mg/L

1µM = 247.25µg/L

1.3µM = 321.425µg/L

= 0.321425µg/ml

Prepare a stock x1000 times concentrated:

1.3mM = 321.425µg/ml

* The powder is received in 10mg that will be dissolved in 1ml distilled *water (0.*321425mg / 10mg x 1ml = 0.032143ml = 32.143µl) ~~in 1mL~~

Thus 32.143µl (32ul) of the above solution (*) should be added to 968ul of PBS to get x1000 concentrated stock of 1.3mM.

$C_1 \times V_1 = C_2 \times V_2$

1.3mM x X (volume that should be added to 1ml of medium) = 1.3µM x 1ml

1300µM x X = 1.3µM x 1000µl

X = 1300 / 1300 = 1µl

Thus 1µl from the x1000 concentrated stock should be added to 1ml of medium to get a final concentration of 1.3µM.

Calculation of dosage TDF: 500nM

For a final concentration of 500nM TDF:

Molecular weight (MW) of TDF = 635.51g/mol

1M = 635.51g/L

1mM = 635.51mg/L

1µM = 635.51µg/L

1nM = 0.63551µg/L

500nM = 317.755µg/L

= 0.317755µg/ml

Prepare a stock x1000 times concentrated:

500µM = 317.755µg/ml

* The powder is received in 10mg that will be dissolved in 1ml DMSO (0.317755mg / 10mg x 1ml = 0.0317755ml = 31.776µl)

Thus 31.776µl (32ul) of the above solution (*) should be added to 968ul of PBS to get the x1000 concentrated stock of 500µM.

$C_1 \times V_1 = C_2 \times V_2$

500µM x X (volume that should be added to 1ml of medium) = 500nM x 1ml

500µM x X = 0.5µM x 1000µl

X = 500 / 500 = 1µl

Thus 1µl from the x1000 concentrated stock should be added to 1ml of medium to get a final concentration of 500nM.

Protocol of first line ART drugs in AECs.

All three drugs were added simultaneously to the AEC medium. In other words, 1ml of Medium contained 1ul of each of the drugs (1ul EFV + 1ul FTC+ 1ul TDF).

First line Vehicle control protocol and calculations:

Final concentration of methanol in 1 ml of medium:

17.67752µl of methanol in 100ml PBS

0.1767752µl in 1ml

Diluted x1000 when 1ul of this is added to 1ml of medium = 0.0001767752ul/ml

Final concentration of water in 1ml of medium:

32.143ul of water in 1ml PBS

Diluted x1000 when 1ul of this is added to 1ml of medium = 0.032143ul/ml

Final concentration of DMSO in 1 ml of medium:

31.776ul of DMSO in 1ml

Diluted x1000 when 1ul of this is added to 1 ml of medium = 0.031776ul/ml

- Therefore, a vehicle cocktail was made up of: 18ul of methanol + 32ul of water + 32ul of DMSO + 918ul of PBS.
- From this cocktail, 3ul was added in 1ml AEC medium in the control groups to ensure that the concentration of vehicle per ml is the same in the controls groups as in the experimental groups.

Second line ART drugs: Lopinavir (LPV) and Ritonavir (RTV).

Calculation of dosage - LPV: 10µM

Molecular weight (MW) of LPV = 628.80g/mol

For a concentration of 10µM LPV:

1M = 628.80g/L

1mM = 628.80mg/L

1µM = 628.80µg/L

= 0.62880µg/mL

10µM = 6.2880µg/mL

Prepare Stock x1000 times concentrated:

10mM = 6288.0µg/ml

* The powder is received in 10mg that will be dissolved in 1ml DMSO (6.2880mg / 10mg x 1ml = 0.62880ml = 628.80µl)

Thus 628.80µl (629ul) of the above solution (*) should be added to 371ul of PBS to get the x1000 concentrated stock of 10mM.

$C_1 \times V_1 = C_2 \times V_2$

10mM x X (volume that should be added to 1ml of medium) = 10µM x 1ml

10mM x X = 0.01mM x 1ml

X = 0.01 / 10 = 0.001ml = 1µl

Thus 1µl from the x1000 concentrated stock should be added to 1ml of medium to get a final concentration of 10µM.

Calculation of dosage - RTV: 2µM

Molecular weight (MW) of RTV = 720.94g/mol

For a concentration of 2µM RTV:

Molecular weight (MW) of RTV = 720.94g/mol

1M = 720.94g/L

1mM = 720.94mg/L

1µM = 720.94µg/L

= 0.72094µg/mL

2µM = 1.4419µg/mL

Prepare Stock x1000 times concentrated:

2mM = 1441.88µg/ml

* The powder is received in 10mg that will be dissolved in 1ml DMSO (1.44188mg / 10mg x 1ml = 0.144188ml = 144.188µl)

Thus 144.188µl (144ul) of the above solution (*) should be added to 856ul of PBS to get the x1000 concentrated stock of 2mM.

$C_1 \times V_1 = C_2 \times V_2$

2mM x X (volume that should be added to 1ml of medium) = 2µM x 1ml

2mM x X = 0.002mM x 1ml

X = 0.002 / 2 = 0.001ml = 1µl

Thus 1µl from the x1000 concentrated stock should be added to 1ml of medium to get a final concentration of 2µM.

Protocol of second line ART drugs in AECs.

Both the drugs were added simultaneously to the AEC medium. In other words, 1ml of Medium contained 1ul of each of the drugs (1ul LPV + 1µl RTV).

Final concentration of DMSO in 1ml of medium:

- For LPV:

628.8ul of DMSO in 1ml

Diluted x1000 when 1ul of this is added to 1ml of medium = 0.6288ul/ml

- For RTV:

144.19µl of DMSO in 1ml

Diluted x1000 when 1ul of this added to 1ml of medium = 0.144188ul/ml

- Therefore, a vehicle cocktail was made up of: containing 0.6288ul of DMSO (from LPV) + 0.14188ul of DMSO (from RTV) = 0.773ul of DMSO in 1 ml of PBS.
- From this cocktail, 2ul was added in 1ml AEC medium in the control groups to ensure that the concentration of vehicle per ml is the same in the controls groups as in the experimental groups.

References

Aberg, J.A. 2009. Cardiovascular Complications in HIV Management: Past, Present, and Future. *Journal of acquired immune deficiency syndromes (1999),* 50(1). :54-64.

Africa, L.D. 2014. *HIV-1 associated neuroinflammation: effects of two complimentary medicines illustrated in an in vitro model of the blood-brain barrier*, PhD, Stellenbosch University.

Africa, L.D. and Smith, C., 2015. Sutherlandia frutescens may exacerbate HIV-associated neuroinflammation. *Journal of negative results in biomedicine*, *14*(1), p.14.

Ananworanich, J. 2006. CD4-Guided Scheduled Treatment Interruptions Compared with Continuous Therapy for Patients Infected with HIV-1: Results of the Staccato Randomised Trial. *The Lancet,* 368(9534). :459-465.

Andrade, A.C.O. & Cotter, B.R. 2006. Endothelial Function and Cardiovascular Diseases in HIV Infected Patient. *Brazilian Journal of Infectious Diseases,* 10(2). :139-145.

Andrew, P.J. & Mayer, B. 1999. Enzymatic Function of Nitric Oxide Synthases. *Cardiovascular research,* 43(3). :521-531.

Apostolova, N., Gomez-Sucerquia, L., Moran, A., Alvarez, A., Blas-Garcia, A. & Esplugues, J. 2010. Enhanced Oxidative Stress and Increased Mitochondrial Mass during Efavirenz-induced Apoptosis in Human Hepatic Cells. *British journal of pharmacology,* 160(8). :2069-2084.

Arts, E.J. & Hazuda, D.J. 2012. HIV-1 Antiretroviral Drug Therapy. *Cold Spring Harbor perspectives in medicine,* 2(4). :007161.

Bai, Y. 2013. Covalent Fusion Inhibitors Targeting HIV-1 gp41 Deep Pocket. *Amino acids,* 44(2). :701-713.

Barbaro, G. 2003. HIV Infection, Highly Active Antiretroviral Therapy and the Cardiovascular System. *Cardiovascular research,* 60(1). :87-95.

Bärnighausen, T. 2008. Hiding in the Shadows of the HIV Epidemic: Obesity and Hypertension in a Rural Population with very High HIV Prevalence in South Africa. *Journal of human hypertension,* 22(3). :236-239.

Blanco, J.J. 2006. Endothelial Function in HIV-Infected Patients with Low Or Mild Cardiovascular Risk. *The Journal of antimicrobial chemotherapy,* 58(1). :133-139.

Bonetti, P.O., Lerman, L.O. & Lerman, A. 2003. Endothelial Dysfunction: A Marker of Atherosclerotic Risk. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 23(2). :168-175.

Bourque, S.L., Davidge, S.T. & Adams, M.A. 2011. The Interaction between Endothelin-1 and Nitric Oxide in the Vasculature: New Perspectives. *American journal of physiology.Regulatory, integrative and comparative physiology,* 300(6). :R1288-95.

Brennan-Benson, P. 2009. Metabolic and Cardiovascular Complications of HIV. *Medicine,* 37(7). :348-351.

Brown, T.T. & Glesby, M.J. 2012. Management of the Metabolic Effects of HIV and HIV Drugs. *Nature Reviews Endocrinology,* 8(1). :11-21.

Brown, T.T., Tassiopoulos, K., Bosch, R.J., Shikuma, C. & McComsey, G.A. 2010. Association between Systemic Inflammation and Incident Diabetes in HIV-Infected Patients After Initiation of Antiretroviral Therapy. *Diabetes care,* 33(10). :2244-2249.

Cai, H. & Harrison, D.G. 2000. Endothelial Dysfunction in Cardiovascular Diseases: The Role of Oxidant Stress. *Circulation research,* 87(10). :840-844.

Cappuccio, F.P. & Miller, M.A. 2016. Cardiovascular Disease and Hypertension in Sub-Saharan Africa: Burden, Risk and Interventions. *Internal and emergency medicine,* 11(3). :299-305.

Capra, V., Bäck, M., Angiolillo, D., Cattaneo, M. & Sakariassen, K. 2014. Impact of Vascular Thromboxane Prostanoid Receptor Activation on Hemostasis, Thrombosis, Oxidative Stress, and Inflammation. *Journal of Thrombosis and Haemostasis,* 12(2). :126-137.

Caron, M., Auclair, M., Vigouroux, C., Glorian, M., Forest, C. & Capeau, J. 2001. The HIV Protease Inhibitor Indinavir Impairs Sterol Regulatory Element-Binding Protein-1 Intranuclear Localization, Inhibits Preadipocyte Differentiation, and Induces Insulin Resistance. *Diabetes,* 50(6). :1378-1388.

Cat, A.N.D. & Touyz, R.M. 2011. Cell Signaling of Angiotensin II on Vascular Tone: Novel Mechanisms. *Current hypertension reports,* 13(2). :122-128.

Chan, W., Sviridov, D. & Dart, A.M. 2009. HIV, Atherosclerosis and Inflammation: Implications for Treatment. *Journal of HIV therapy,* 14(3). :61-69.

Charakida, M. 2005. Early Structural and Functional Changes of the Vasculature in HIV-Infected Children: Impact of Disease and Antiretroviral Therapy. *Circulation,* 112(1). :103-109.

Chi, D., Henry, J., Kelley, J., Thorpe, R., Smith, J.K. & Krishnaswamy, G. 2000. The Effects of HIV Infection on Endothelial Function. *Endothelium,* 7(4). :223-242.

Ciminale, V., Felber, B.K., Campbell, M. & Pavlakis, G.N. 1990. A Bioassay for HIV-1 Based on Env-CD4 Interaction. *AIDS Research and Human Retroviruses,* 6(11). :1281-1287.

Clark, S.J. 2015. Cardiometabolic Disease Risk and HIV Status in Rural South Africa: Establishing a Baseline. *BMC public health,* 15(1). :1.

Clavel, F. & Hance, A.J. 2004. HIV Drug Resistance. *New England Journal of Medicine,* 350(10). :1023-1035.

Conaldi, P. 1995. Productive HIV-1 Infection of Human Vascular Endothelial Cells Requires Cell Proliferation and is Stimulated by Combined Treatment with Interleukin-1β Plus Tumor Necrosis Factor-α. *Journal of medical virology,* 47(4). :355-363.

Corbeil, J. 1995. Productive in Vitro Infection of Human Umbilical Vein Endothelial Cells and Three Colon Carcinoma Cell Lines with HIV-1. *Immunology & Cell Biology,* 73(2).

Cox, S.W., Aperla, K., Albert, J. & Wahren, B. 1994. Comparison of the Sensitivities of Primary Isolates of HIV Type 2 and HIV Type 1 to Antiviral Drugs and Drug Combinations. *AIDS Research and Human Retroviruses,* 10(12). :1725-1729.

Crabtree, M.J., Hale, A.B. & Channon, K.M. 2011. Dihydrofolate Reductase Protects Endothelial Nitric Oxide Synthase from Uncoupling in Tetrahydrobiopterin Deficiency. *Free Radical Biology and Medicine,* 50(11). :1639-1646.

Da Cunha, J., Maselli, L.M.F., Stern, A.C.B., Spada, C. & Paulo, S. 2015. Wjv. *World,* 4(2). :56-77.

DAD Study Group. 2007. Class of Antiretroviral Drugs and the Risk of Myocardial Infarction. *N Engl J Med,* 2007(356). :1723-1735.

Dalal, S. 2011. Non-Communicable Diseases in Sub-Saharan Africa: What we Know Now. *International journal of epidemiology,* 40(4). :885-901.

Davies, N. 2013. Fixed-Dose Combination for Adults Accessing Antiretroviral Therapy. *Southern African Journal of HIV Medicine,* 14(1).

Davignon, J. & Ganz, P. 2004. Role of Endothelial Dysfunction in Atherosclerosis. *Circulation,* 109(23 Suppl 1). :III27-32.

De Cock, K.M., Jaffe, H.W. & Curran, J.W. 2012. The Evolving Epidemiology of HIV/AIDS. *AIDS (London, England),* 26(10). :1205-1213.

de Gaetano Donati, K., Cauda, R. & Iacoviello, L. 2010. HIV Infection, Antiretroviral Therapy and Cardiovascular Risk. *Mediterranean journal of hematology and infectious diseases,* 2(3). :2010034.

de Gaetano Donati, K. 2003. Increased Soluble Markers of Endothelial Dysfunction in HIV-Positive Patients Under Highly Active Antiretroviral Therapy. *Aids,* 17(5). :765-768.

Deanfield, J.E., Halcox, J.P. & Rabelink, T.J. 2007. Endothelial Function and Dysfunction: Testing and Clinical Relevance. *Circulation,* 115(10). :1285-1295.

Deeks, S.G., Lewin, S.R. & Havlir, D.V. 2013. The End of AIDS: HIV Infection as a Chronic Disease. *The Lancet,* 382(9903). :1525-1533.

Delpech, V. 2013. The HIV Epidemic: Global and UK Trends. *Medicine,* 41(8). :417-419.

Doughan, A.K., Harrison, D.G. & Dikalov, S.I. 2008. Molecular Mechanisms of Angiotensin II-Mediated Mitochondrial Dysfunction: Linking Mitochondrial Oxidative Damage and Vascular Endothelial Dysfunction. *Circulation research,* 102(4). :488-496.

Dressman, J. 2003. HIV Protease Inhibitors Promote Atherosclerotic Lesion Formation Independent of Dyslipidemia by Increasing CD36-Dependent Cholesteryl Ester Accumulation in Macrophages. *The Journal of clinical investigation,* 111(3). :389-397.

Dube, M.P., Shen, C., Greenwald, M. & Mather, K.J. 2008. No Impairment of Endothelial Function Or Insulin Sensitivity with 4 Weeks of the HIV Protease Inhibitors Atazanavir Or Lopinavir-Ritonavir in Healthy Subjects without HIV Infection: A Placebo-Controlled Trial. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America,* 47(4). :567-574.

Ellinsworth, D.C., Shukla, N., Fleming, I. & Jeremy, J.Y. 2014. Interactions between Thromboxane A(2), Thromboxane/Prostaglandin (TP) Receptors, and Endothelium-Derived Hyperpolarization. *Cardiovascular research,* 102(1). :9-16.

Endemann DH, S.E. 2004. Endothelial Dysfunction. *Journal of the American Society of Nephrology : JASN,* 15(8). :1983-92.

Fanales-Belasio, E., Raimondo, M., Suligoi, B. & Buttò, S. 2010. HIV Virology and Pathogenetic Mechanisms of Infection: A Brief Overview. *Annali dell'Istituto Superiore di Sanita*, 46(1). :5-14.

Fauci, A.S. & Folkers, G.K. 2012. Toward an AIDS-Free Generation. *Jama*, 308(4). :343-344.

Fedele, F., Bruno, N. & Mancone, M. 2011. Cardiovascular Risk Factors and HIV Disease. *AIDS reviews*, 13(2). :119-129.

Feeney, E.R. & Mallon, P.W. 2011. HIV and HAART-Associated Dyslipidemia. *Open Cardiovascular Medicine Journal*, 5:49-63.

Feng, J.Y. 2009. The Triple Combination of Tenofovir, Emtricitabine and Efavirenz shows Synergistic Anti-HIV-1 Activity in Vitro: A Mechanism of Action Study. *Retrovirology*, 6(1). :1.

Fiala, M. 2004. HAART Drugs Induce Mitochondrial Damage and Intercellular Gaps and Gp 120 Causes Apoptosis. *Cardiovascular toxicology*, 4(4). :327-337.

Fiala, M. 2004. HIV-1 Induces Cardiomyopathy by Cardiomyocyte Invasion and gp120, Tat, and Cytokine Apoptotic Signaling. *Cardiovascular toxicology*, 4(2). :97-107.

Fitch, K.V. 2010. Increased Coronary Artery Calcium Score and Noncalcified Plaque among HIV-Infected Men: Relationship to Metabolic Syndrome and Cardiac Risk Parameters. *Journal of acquired immune deficiency syndromes (1999)*, 55(4). :495-499.

Fitridge, R. & Thompson, M. 2011. *Mechanisms of vascular disease*. University of Adelaide Press

Flammer & Luscher ,. 2011. Three Decades of Endothelium Research. *SWISS MEDICAL WEEKLY*, 141:3-8.

Flammer, A.J. & Lüscher, T.F. 2010. Human Endothelial Dysfunction: EDRFs. *Pflügers Archiv-European Journal of Physiology*, 459(6). :1005-1013.

Flammer, A.J. 2012. The Assessment of Endothelial Function: From Research into Clinical Practice. *Circulation,* 126(6). :753-767.

Flexner, C. 1998. HIV-Protease Inhibitors. *New England Journal of Medicine,* 338(18). :1281-1293.

Fourie, C.M.T., Van Rooyen, J.M., Kruger, A. & Schutte, A.E. 2010. Lipid Abnormalities in a Never-Treated HIV-1 Subtype C-Infected African Population. *Lipids,* 45(1). :73-80.

Fourie, C.M.T., Schutte, A.E., Smith, W., Kruger, A. & van Rooyen, J.M. 2015. Endothelial Activation and Cardiometabolic Profiles of Treated and Never-Treated HIV Infected Africans. *Atherosclerosis,* 240(1). :154-160.

Francisci, D. 2009. HIV Type 1 Infection, and Not Short-Term HAART, Induces Endothelial Dysfunction. *AIDS (London, England),* 23(5). :589-596.

Fu, W., Chai, H., Yao, Q. & Chen, C. 2005. Effects of HIV Protease Inhibitor Ritonavir on Vasomotor Function and Endothelial Nitric Oxide Synthase Expression. *JAIDS Journal of Acquired Immune Deficiency Syndromes,* 39(2). :152-158.

Gallant, J.E. 2006. Tenofovir DF, Emtricitabine, and Efavirenz Vs. Zidovudine, Lamivudine, and Efavirenz for HIV. *New England Journal of Medicine,* 354(3). :251-260.

Gallant, J.E. 2004. Efficacy and Safety of Tenofovir DF Vs Stavudine in Combination Therapy in Antiretroviral-Naive Patients: A 3-Year Randomized Trial. *Jama,* 292(2). :191-201.

Genis, A. 2014. *Exposure of cardiac microvascular endothelial cells to harmful stimuli: A study of the cellular responses and mechanisms.* PhD. Stellenbosch University

Gewaltig, M.T. & Kojda, G. 2002. Vasoprotection by Nitric Oxide: Mechanisms and Therapeutic Potential. *Cardiovascular research,* 55(2). :250-260.

Gleason Jr., R.L. 2016. Efavirenz and Ritonavir-Boosted Lopinavir use Exhibited Elevated Markers of Atherosclerosis Across Age Groups in People Living with HIV in Ethiopia. *Journal of Biomechanics*, 49(13). :2584-2592.

Graham, S.M., Mwilu, R. and Liles, W.C., 2013. Clinical utility of biomarkers of endothelial activation and coagulation for prognosis in HIV infection: a systematic review. *Virulence*, *4*(6), pp.564-571.

Green, D.J., Dawson, E.A., Groenewoud, H.M., Jones, H. & Thijssen, D.H. 2014. Is Flow-Mediated Dilation Nitric Oxide Mediated?: A Meta-Analysis. *Hypertension (Dallas, Tex.: 1979)*, 63(2). :376-382.

Green, L.A. 2014. HIV Envelope Protein gp120-Induced Apoptosis in Lung Microvascular Endothelial Cells by Concerted Upregulation of EMAP II and its Receptor, CXCR3. *American journal of physiology.Lung cellular and molecular physiology*, 306(4). :L372-82.

Gresele, P., Falcinelli, E., Sebastiano, M. & Baldelli, F. 2012. Endothelial and Platelet Function Alterations in HIV-Infected Patients. *Thrombosis research*, 129(3). :301-308.

Grigsby, I.F., Pham, L., Mansky, L.M., Gopalakrishnan, R., Carlson, A.E. & Mansky, K.C. 2010. Tenofovir Treatment of Primary Osteoblasts Alters Gene Expression Profiles: Implications for Bone Mineral Density Loss. *Biochemical and biophysical research communications*, 394(1). :48-53.

Grubb, J.R. 2006. Lopinavir-Ritonavir: Effects on Endothelial Cell Function in Healthy Subjects. *The Journal of infectious diseases*, 193(11). :1516-1519.

Grunfeld, C. 2008. Insulin Resistance in HIV Infection: Drugs, Host Responses, Or Restoration to Health? *Topics in HIV medicine : a publication of the International AIDS Society, USA*, 16(2). :89-93.

Hall, J.E. & Gyton, A.C. 2015. *Guyton and Hall textbook of medical physiology*. Elsevier Health Sciences.

Han, J.H. 2012. HIV Infection and Glycemic Response to Newly Initiated Diabetic Medical Therapy. *AIDS (London, England),* 26(16). :2087-2095.

Hans Strijdom, N.C. & Lochner, A. 2009. Nitric Oxide in the Cardiovascular System: A Simple Molecule with Complex Actions. *Cardiovascular journal of Africa,* 20(5). :303-310.

Hebert, V.Y., Crenshaw, B.L., Romanoff, R.L., Ekshyyan, V.P. & Dugas, T.R. 2004a. Effects of HIV Drug Combinations on Endothelin-1 and Vascular Cell Proliferation. *Cardiovascular toxicology,* 4(2). :117-131.

Hebert, V.Y., Crenshaw, B.L., Romanoff, R.L., Ekshyyan, V.P. & Dugas, T.R. 2004b. Effects of HIV Drug Combinations on Endothelin-1 and Vascular Cell Proliferation. *Cardiovascular toxicology,* 4(2). :117-131.

Hill, A., Sawyer, W. & Gazzard, B. 2015. Effects of First-Line use of Nucleoside Analogues, Efavirenz, and Ritonavir-Boosted Protease Inhibitors on Lipid Levels. *HIV clinical trials,*

Ho, J.E. 2012. The Association of CD4+ T-Cell Counts and Cardiovascular Risk in Treated HIV Disease. *AIDS (London, England),* 26(9). :1115-1120.

Hsue, P.Y. 2009. Role of Viral Replication, Antiretroviral Therapy, and Immunodeficiency in HIV-Associated Atherosclerosis. *AIDS (London, England),* 23(9). :1059-1067.

Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E. & Chaudhuri, G. 1987. Endothelium-Derived Relaxing Factor Produced and Released from Artery and Vein is Nitric Oxide. *Proceedings of the National Academy of Sciences of the United States of America,* 84(24). :9265-9269.

Jarrete, A.P., Zanesco, A. & Delbin, M.A. 2016. Assessment of Endothelial Function by Flow-Mediated Dilation in Diabetic Patients: Effects of Physical Exercise. *Motriz: Revista de Educação Física,* 22(1). :3-11.

Jiang, B., Hebert, V.Y., Li, Y., Mathis, J.M., Alexander, J.S. & Dugas, T.R. 2007. HIV Antiretroviral Drug Combination Induces Endothelial Mitochondrial Dysfunction and

Reactive Oxygen Species Production, but Not Apoptosis. *Toxicology and applied pharmacology,* 224(1). :60-71.

Jiang, J., Fu, W., Wang, X., Lin, P.H., Yao, Q. & Chen, C. 2010. HIV gp120 Induces Endothelial Dysfunction in Tumour Necrosis Factor-Alpha-Activated Porcine and Human Endothelial Cells. *Cardiovascular research,* 87(2). :366-374.

Joy, T. 2007. Dietary Fat Intake and Relationship to Serum Lipid Levels in HIV-Infected Patients with Metabolic Abnormalities in the HAART Era. *AIDS (London, England),* 21(12). :1591-1600.

Kannel, W.B., Dawber, T.R., Kagan, A., Revotskie, N. & Stokes, J. 1961. Factors of Risk in the Development of Coronary Heart Disease—six-Year Follow-Up Experience: The Framingham Study. *Annals of Internal Medicine,* 55(1). :33-50.

Kaplan, R.C. 2007. Ten-Year Predicted Coronary Heart Disease Risk in HIV-Infected Men and Women. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America,* 45(8). :1074-1081.

Kapuku, G.K., Treiber, F.A., Hartley, B. & Ludwig, D.A. 2004. Gender Influences Endothelial-Dependent Arterial Dilatation Via Arterial Size in Youth. *The American Journal of the Medical Sciences,* 327(6). :305-309.

Karlsson, M., Kurz, T., Brunk, U.T., Nilsson, S.E. & Frennesson, C.I. 2010. What does the Commonly used DCF Test for Oxidative Stress really show? *The Biochemical journal,* 428(2). :183-190.

Kim, R. & Baxter, J.D. 2008. Protease Inhibitor Resistance Update: Where are we Now? *AIDS Patient Care and STDs,* 22(4). :267-277.

Kirchhoff, F. 2013. HIV Life Cycle: Overview. *Encyclopedia of AIDS,* :1-9.

Kline, E.R. & Sutliff, R.L. 2008. The Roles of HIV-1 Proteins and Antiretroviral Drug Therapy in HIV-1-Associated Endothelial Dysfunction. *Journal of Investigative Medicine*, 56(5). :752-769.

Klug, Y.A., Ashkenazi, A., Viard, M., Porat, Z., Blumenthal, R. & Shai, Y. 2014. Early and Late HIV-1 Membrane Fusion Events are Impaired by Sphinganine Lipidated Peptides that Target the Fusion Site. *The Biochemical journal,* 461(2). :213-222.

Kuller, L.H. 2008. Inflammatory and Coagulation Biomarkers and Mortality in Patients with HIV Infection. *PLoS med,* 5(10). :e203.

Landmesser, U. 2007. Angiotensin II Induces Endothelial Xanthine Oxidase Activation: Role for Endothelial Dysfunction in Patients with Coronary Disease. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 27(4). :943-948.

Lemmer, C., Badri, M., Visser, M. & Mayosi, B. 2011. A Lower Body Mass Index is Associated with Cardiomyopathy in People with HIV Infection: Evidence from a Case Comparison Study. *SAMJ: South African Medical Journal,* 101(2). :119-121.

Limaye, V. & Vadas, M. 2007. The Vascular Endothelium: Structure and Function. *Mechanisms of Vascular DIsease,* :1-10.

Lin, W.H., Zhang, H. & Zhang, Y.T. 2013. Investigation on Cardiovascular Risk Prediction using Physiological Parameters. *Computational and mathematical methods in medicine,* 2013:272691.

Luciw, P.A. 1996. Human Immunodeficiency Viruses and their Replication. *Fields virology,* 2:1881-1952.

Ludmer, P.L., Selwyn, A.P., Shook, T.L., Wayne, R.R., Mudge, G.H., Alexander, R.W. and Ganz, P., 1986. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. New England Journal of Medicine, 315(17), pp.1046-1051.

Luscher, T.F. 1990. Interaction between Endothelin-1 and Endothelium-Derived Relaxing Factor in Human Arteries and Veins. *Circulation research,* 66(4). :1088-1094.

Maartens, Gary, Celum, Connie, Lewin,Sharon R,. 2014. HIV Infection: Epidemiology, Pathogenesis, Treatment, and Prevention. *LANCET The Lancet,* 384(9939). :258-271.

Macallan, D., Miller, T. & Gorbach, S. 1999. Dietary Intake and Weight Loss Patterns in HIV Infection. *Nutritional aspects of HIV infection.,* :18-26.

Maggi, P., Lillo, A., Perilli, F., Maserati, R., Chirianni, A. & PREVALEAT Group. 2004. Colour-Doppler Ultrasonography of Carotid Vessels in Patients Treated with Antiretroviral Therapy: A Comparative Study. *Aids,* 18(7). :1023-1028.

Malaza, A., Mossong, J., Bärnighausen, T. & Newell, M. 2012. Hypertension and Obesity in Adults Living in a High HIV Prevalence Rural Area in South Africa. *PloS one,* 7(10). :e47761.

Mashinya, F., Alberts, M. & Colebunders, R. 2015. Assessment of Cardiovascular Risk Factors in People with HIV Infection Treated with ART in Rural South Africa: A Cross Sectional Study. *AIDS research and therapy,* 12(1). :1.

Mayosi, B.M., Flisher, A.J., Lalloo, U.G., Sitas, F., Tollman, S.M. & Bradshaw, D. 2009. The Burden of Non-Communicable Diseases in South Africa. *The Lancet,* 374(9693). :934-947.

Meintjes, G. 2014. Adult Antiretroviral Therapy Guidelines 2014. *Southern African Journal of HIV Medicine,* 15(4). :121-143.

Melikyan, G.B. 2014. HIV Entry: A Game of Hide-and-Fuse? *Current opinion in virology,* 4:1-7.

Mendis, S. 2014. *Global status report on non-communicable diseases 2014.*

Mendis, S., Puska, P. & Norrving, B. 2011. *Global atlas on cardiovascular disease prevention and control.* World Health Organization

Mensah, G.A. 2013. Descriptive Epidemiology of Cardiovascular Risk Factors and Diabetes in Sub-Saharan Africa. *Progress in cardiovascular diseases,* 56(3). :240-250.

Messner, B. & Bernhard, D. 2014. Smoking and Cardiovascular Disease: Mechanisms of Endothelial Dysfunction and Early Atherogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 34(3). :509-515.

Miceli, M.C. & Parnes, J.R. 1993. Role of CD4 and CD8 in T Cell Activation and Differentiation. *Advances in Immunology*, 53. :59-122.

Mondal, D., Pradhan, L., Ali, M. & Agrawal, K.C. 2004. HAART Drugs Induce Oxidative Stress in Human Endothelial Cells and Increase Endothelial Recruitment of Mononuclear Cells. *Cardiovascular toxicology,* 4(3). :287-302.

Mondy, K.E. 2008. Insulin Resistance Predicts Endothelial Dysfunction and Cardiovascular Risk in HIV-Infected Persons on Long-Term Highly Active Antiretroviral Therapy. *AIDS (London, England)*, 22(7). :849-856.

Monsuez, J. 2009. HIV-Associated Vascular Diseases: Structural and Functional Changes, Clinical Implications. *International journal of cardiology,* 133(3). :293-306.

Moore, R.D. & Chaisson, R.E. 1999. Natural History of HIV Infection in The_era of Combination Antiretroviral Therapy. *Aids,* 13(14). :1933-1942.

Mooser, V. 2003. Atherosclerosis and HIV in the Highly Active Antiretroviral Therapy Era: Towards an Epidemic of Cardiovascular Disease? *Aids,* 17:S65-S69.

Moulard, M. & Decroly, E. 2000. Maturation of HIV Envelope Glycoprotein Precursors by Cellular Endoproteases. *Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes,* 1469(3). :121-132.

Mpe, M.T. 2010. Are we Doing enough to Prevent a Heart Disease Epidemic?: Editorial. *SA Heart,* 7(3). :146-149.

Mudau, M., Genis, A., Lochner, A. & Strijdom, H. 2012. Endothelial Dysfunction: The Early Predictor of Atherosclerosis. *Cardiovascular journal of Africa,* 23(4). :222-231.

Mustafa, A.K. 2011. Hydrogen Sulfide as Endothelium-Derived Hyperpolarizing Factor Sulfhydrates Potassium Channels. *Circulation research,* 109(11). :1259-1268.

Nakatani-Webster, E., Hu, S., Atkins, W.M. & Catalano, C.E. 2015. Assembly and Characterization of gp160-Nanodiscs: A New Platform for Biochemical Characterization of HIV Envelope Spikes. *Journal of virological methods,* 226. :15-24.

Naseem, K.M. 2005. The Role of Nitric Oxide in Cardiovascular Diseases. *Molecular aspects of medicine,* 26(1–2). :33-65.

Nduka, C.U., Uthman, O.A., Kimani, P.K., Malu, A.O. & Stranges, S. 2016. Impact of Body Fat Changes in Mediating the Effects of Antiretroviral Therapy on Blood Pressure in HIV-Infected Persons in a Sub-Saharan African Setting. *Infectious diseases of poverty,* 5(1). :1.

Nolan, D., Watts, G.F., Herrmann, S.E., French, M.A., John, M. & Mallal, S. 2003. Endothelial Function in HIV-Infected Patients Receiving Protease Inhibitor Therapy: Does Immune Competence Affect Cardiovascular Risk? *QJM : monthly journal of the Association of Physicians,* 96(11). :825-832.

Nonnis, S. 2016. Effect of Fetal Bovine Serum in Culture Media on MS Analysis of Mesenchymal Stromal Cells Secretome. *EuPA Open Proteomics,* 10:28-30.

Noor, M.A., Flint, O.P., Maa, J.F. & Parker, R.A. 2006. Effects of Atazanavir/Ritonavir and Lopinavir/Ritonavir on Glucose Uptake and Insulin Sensitivity: Demonstrable Differences in Vitro and Clinically. *AIDS (London, England),* 20(14). :1813-1821.

O'Cofaigh, E. & Lewthwaite, P. 2013. Natural History of HIV and AIDS. *Medicine,* 41(8). :411-416.

Ozkor, M.A. & Quyyumi, A.A. 2011. Endothelium-Derived Hyperpolarizing Factor and Vascular Function. *Cardiology research and practice,* 2011:156146.

Paik, I.J. & Kotler, D.P. 2011. The Prevalence and Pathogenesis of Diabetes Mellitus in Treated HIV-Infection. *Best Practice & Research Clinical Endocrinology & Metabolism,* 25(3). :469-478.

Pedersen, C. 1989. Clinical Course of Primary HIV Infection: Consequences for Subsequent Course of Infection. *BMJ (Clinical research ed.),* 299(6692). :154-157.

Pham, X.D. 2016. Racial and Gender Differences in Arterial Anatomy of the Arm. *The American Surgeon,* 82(10). :973-976.

Phillips, A.N. 2008. Interruption of Antiretroviral Therapy and Risk of Cardiovascular Disease in Persons with HIV-1 Infection: Exploratory Analyses from the SMART Trial. *Antiviral Therapy,* 13(2). :177.

Rajendran, P. 2013. The Vascular Endothelium and Human Diseases. *Int J Biol Sci,* 9(10). :1057-1069.

Ramadhani, H.O. 2014. Association of First-Line and Second-Line Antiretroviral Therapy Adherence. Paper presented at Open forum infectious diseases.

Reyskens, K.M. & Essop, M.F. 2014. HIV Protease Inhibitors and Onset of Cardiovascular Diseases: A Central Role for Oxidative Stress and Dysregulation of the Ubiquitin–proteasome System. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease,* 1842(2). :256-268.

Riccardi, C. & Nicoletti, I. 2006. Analysis of Apoptosis by Propidium Iodide Staining and Flow Cytometry. *Nature protocols,* 1(3). :1458-1461.

Roald, H.E., Barstad, R.M., Engen, A., Kierulf, P., Skjorten, F. & Sakariassen, K.S. 1994. HN-11500--a Novel Thromboxane A2 Receptor Antagonist with Antithrombotic Activity in Humans at Arterial Blood Flow Conditions. *Thrombosis and haemostasis,* 71(1). :103-109.

Rubanyi, G.M. & Vanhoutte, P.M. 1986. Superoxide Anions and Hyperoxia Inactivate Endothelium-Derived Relaxing Factor. *The American Journal of Physiology,* 250(5 Pt 2). :H822-7.

Ruelas, D. & Greene, W. 2013. An Integrated Overview of HIV-1 Latency. *Cell,* 155(3). :519-529.

Sakakura, K., Nakano, M., Otsuka, F., Ladich, E., Kolodgie, F.D. & Virmani, R. 2013. Pathophysiology of Atherosclerosis Plaque Progression. *Heart, Lung and Circulation,* 22(6). :399-411.

Sampson, U.K., Engelgau, M.M., Peprah, E.K. & Mensah, G.A. 2015. Endothelial Dysfunction: A Unifying Hypothesis for the Burden of Cardiovascular Diseases in Sub-Saharan Africa. *Cardiovascular journal of Africa,* 26(2 Suppl 1). :S56-60.

Sandler, N.G. 2011. Plasma Levels of Soluble CD14 Independently Predict Mortality in HIV Infection. *The Journal of infectious diseases,* 203(6). :780-790.

Sellmeyer, D.E. & Grunfeld, C. 1996. Endocrine and Metabolic Disturbances in Human Immunodeficiency Virus Infection and the Acquired Immune Deficiency Syndrome*. *Endocrine reviews,* 17(5). :518-532.

Shen, L. 2008. Dose-Response Curve Slope Sets Class-Specific Limits on Inhibitory Potential of Anti-HIV Drugs. *Nature medicine,* 14(7). :762-766.

Shimokawa, H. 1996. The Importance of the Hyperpolarizing Mechanism Increases as the Vessel Size Decreases in Endothelium-Dependent Relaxations in Rat Mesenteric Circulation. *Journal of cardiovascular pharmacology,* 28(5). :703-711.

Shlay, J.C. 2007. Long-Term Body Composition and Metabolic Changes in Antiretroviral Naive Persons Randomized to Protease Inhibitor-, Nonnucleoside Reverse Transcriptase Inhibitor-, Or Protease Inhibitor Plus Nonnucleoside Reverse Transcriptase Inhibitor-Based Strategy. *Journal of acquired immune deficiency syndromes (1999),* 44(5). :506-517.

Skowyra, A., Zdziechowicz, I., Mikuła, T. & Wiercińska-Drapało, A. 2012. Endothelial dysfunction—An Important Factor in the Progression of Atherosclerosis in HIV-Infected Persons. *HIV & AIDS Review,* 11(3). :57-60.

Smith, D.D., Tan, X., Tawfik, O., Milne, G., Stechschulte, D.J. & Dileepan, K.N. 2010. Increased Aortic Atherosclerotic Plaque Development in Female Apolipoprotein E-Null Mice is Associated with Elevated Thromboxane A2 and Decreased Prostacyclin Production. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society,* 61(3). :309-316.

Soghoian, D.Z. 2015. *HIV-Specific CD4 T Cells and Viral Control,*

Soh, C. 2003. Long-Term Effects of Protease-Inhibitor-Based Combination Therapy on CD4 T-Cell Recovery in HIV-1-Infected Children and Adolescents. *The Lancet,* 362(9401). :2045-2051.

Solages, A. 2006b. Endothelial Function in HIV-Infected Persons. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America,* 42(9). :1325-1332.

Soriano, V. & Mendoza, C.d. 2002. Genetic Mechanisms of Resistance to NRTI and NNRTI. *HIV clinical trials,* 3(3). :237-248.

Stanley, T.L. & Grinspoon, S.K. 2012. Body Composition and Metabolic Changes in HIV-Infected Patients. *The Journal of infectious diseases,* 205 Suppl 3:S383-90.

Stein, J.H., Currier, J.S. & Hsue, P.Y. 2014. Arterial Disease in Patients with Human Immunodeficiency Virus Infection: What has Imaging Taught Us? *JACC: Cardiovascular Imaging,* 7(5). :515-525.

Stein, J.H. 2001. Use of Human Immunodeficiency Virus-1 Protease Inhibitors is Associated with Atherogenic Lipoprotein Changes and Endothelial Dysfunction. *Circulation,* 104(3). :257-262.

Steyn, K. 2005. Risk Factors Associated with Myocardial Infarction in Africa: The INTERHEART Africa Study. *Circulation,* 112(23). :3554-3561.

Subbarao, V., Lowe, D., Aghamohammadzadeh, R. & Wilkinson, R.J. 2011. *Endothelial dysfunction in HIV.* INTECH Open Access Publisher

Sutliff, R.L. 2002. Nucleoside Reverse Transcriptase Inhibitors Impair Endothelium-Dependent Relaxation by Increasing Superoxide. *American journal of physiology.Heart and circulatory physiology,* 283(6). :H2363-70.

Taddei, S., Ghiadoni, L., Virdis, A., Buralli, S. & Salvetti, A. 1999. Vasodilation to Bradykinin is Mediated by an Ouabain-Sensitive Pathway as a Compensatory Mechanism for Impaired Nitric Oxide Availability in Essential Hypertensive Patients. *Circulation,* 100(13). :1400-1405.

Teofili, L. 2010. Endothelial Progenitor Cell Trafficking in Human Immunodeficiency Virus-Infected Persons. *AIDS,* 24(16). :2443-2450.

Torriani, F.J. 2008. Endothelial Function in Human Immunodeficiency Virus-Infected Antiretroviral-Naive Subjects before and After Starting Potent Antiretroviral Therapy: The ACTG (AIDS Clinical Trials Group) Study 5152s. *Journal of the American College of Cardiology,* 52(7). :569-576.

Townsend, N., Nichols, M., Scarborough, P. & Rayner, M. 2015. Cardiovascular Disease in Europe--Epidemiological Update 2015. *European heart journal,* 36(40). :2696-2705.

Tugendhaft, A., Manyema, M., Veerman, L.J., Chola, L., Labadarios, D. & Hofman, K.J. 2015. Cost of Inaction on Sugar-Sweetened Beverage Consumption: Implications for Obesity in South Africa. *Public health nutrition,* :1-9.

Ullrich, C.K., Groopman, J.E. & Ganju, R.K. 2000. HIV-1 gp120- and gp160-Induced Apoptosis in Cultured Endothelial Cells is Mediated by Caspases. *Blood,* 96(4). :1438-1442.

Van den Oever, I.A., Raterman, H.G., Nurmohamed, M.T. & Simsek, S. 2010. Endothelial Dysfunction, Inflammation, and Apoptosis in Diabetes Mellitus. *Mediators of inflammation,* 2010:792393.

Van Wijk, J.P. 2006. Functional and Structural Markers of Atherosclerosis in Human Immunodeficiency Virus-Infected Patients. *Journal of the American College of Cardiology,* 47(6). :1117-1123.

Verma, S. 2002. A Self-Fulfilling Prophecy: C-Reactive Protein Attenuates Nitric Oxide Production and Inhibits Angiogenesis. *Circulation,* 106(8). :913-919.

Wang, X., Chai, H., Lin, P.H., Yao, Q. & Chen, C. 2009. Roles and Mechanisms of Human Immunodeficiency Virus Protease Inhibitor Ritonavir and Other Anti-Human Immunodeficiency Virus Drugs in Endothelial Dysfunction of Porcine Pulmonary Arteries and Human Pulmonary Artery Endothelial Cells. *The American journal of pathology,* 174(3). :771-781.

Wang, D. 2013. Microvascular Endothelial Dysfunction and Enhanced Thromboxane and Endothelial Contractility in Patients with HIV. *Journal of AIDS & clinical research,* 4(12). :267.

Wang, T., Yi, R., Green, L.A., Chelvanambi, S., Seimetz, M. & Clauss, M. 2015a. Increased Cardiovascular Disease Risk in the HIV-Positive Population on ART: Potential Role of HIV-Nef and Tat. *Cardiovascular Pathology,* 24(5). :279-282.

Wang, T., Yi, R., Green, L.A., Chelvanambi, S., Seimetz, M. & Clauss, M. 2015b. Increased Cardiovascular Disease Risk in the HIV-Positive Population on ART: Potential Role of HIV-Nef and Tat. *Cardiovascular Pathology,* 24(5). :279-282.

Wang, X., Chai, H., Yao, Q. & Chen, C. 2007. Molecular Mechanisms of HIV Protease Inhibitor-Induced Endothelial Dysfunction. *Journal of acquired immune deficiency syndromes (1999),* 44(5). :493-499.

Weiß, M., Kost, B., Renner-Müller, I., Wolf, E., Mylonas, I. & Brüning, A. 2016. Efavirenz Causes Oxidative Stress, Endoplasmic Reticulum Stress, and Autophagy in Endothelial Cells. *Cardiovascular toxicology,* 16(1). :90-99.

Weiss, R.A. 1993. Cellular receptors and viral glycoproteins involved in retrovirus entry, *The retroviridae.* Springer.Pages 1-108 in

Weller, I.V.D. & Williams, I.G. 2001. Antiretroviral Drugs. 322:1410.

Westcott, C. 2015. *Investigating the cholesterol-independent (pleiotropic) effects of selected hypolipidaemic agents in functional and dysfunctional endothelial cells,* PhD. Stellenbosch University.

Wilen, C.B., Tilton, J.C. & Doms, R.W. 2012. HIV: Cell Binding and Entry. *Cold Spring Harbor perspectives in medicine,* 2(8). :10.1101/cshperspect.a006866.

Wilkins, R., Kutzner, B., Truong, M., Sanchez-Dardon, J. & McLean, J. 2002. Analysis of Radiation-induced Apoptosis in Human Lymphocytes: Flow Cytometry using Annexin V and Propidium Iodide Versus the Neutral Comet Assay. *Cytometry,* 48(1). :14-19.

Willerson, J.T. & Ridker, P.M. 2004. Inflammation as a Cardiovascular Risk Factor. *Circulation,* 109(21 Suppl 1). :2-10.

World Health Organization. 2010. *World health statistics 2010.*

World Health Organization (WHO). 2015. *Global status report on noncommunicable diseases, 2014: attaining the nine global noncommunicable diseases targets; a shared responsibility.Geneva: World Health Organization; 2014,*

Zhong, D.S. 2002a. HIV Protease Inhibitor Ritonavir Induces Cytotoxicity of Human Endothelial Cells. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 22(10). :1560-1566.

Zhou, X. & He, P. 2011. Improved Measurements of Intracellular Nitric Oxide in Intact Microvessels using 4,5-Diaminofluorescein Diacetate. *American journal of physiology. Heart and circulatory physiology,* 301(1). :H108-14.