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Identification of HIV-1 nef polymorphisms in HIV-positive cardiac patients

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Identification of HIV-1 *nef* Polymorphisms in HIV-Positive Cardiac Patients

Onkemetse Mannafela

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine (Chemical Pathology)

Johannesburg, 05 August 2022.

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Co-supervisor: Prof Nigel Crowther

DECLARATION

I, Mannafela Onkemetse declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine (Chemical Pathology) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

Signature: 

5th day of August 2022

ABSTRACT

Background: Infection with HIV is associated with an increased risk of development of cardiovascular diseases (CVDs), including coronary artery disease (CAD). Potential influential key players involved in the interaction between HIV and CVD include factors such as highly active antiretroviral therapy (HAART) and HIV viral proteins. The HIV-1 Nef viral protein has been associated with increased plasma levels of biomarkers of endothelial dysfunction in HIV-infected subjects, which may be an intermediary process in the development of atherosclerotic plaques. Therefore, given the highly polymorphic nature of the *nef* gene, the aim of this study was to determine if HIV-1 *nef* polymorphisms are associated with endothelial dysfunction and confirmed CAD.

Materials and Methods: Thirty-three (33) HIV-infected subjects with CAD (HIV+/CVD+), of which 31 were on HAART, were recruited alongside 115 HIV-uninfected subjects with CAD (HIV–/CVD+). In addition, 60 HIV-infected subjects on HAART without CAD (HIV+/CVD–) and 60 HIV-uninfected healthy subjects (HIV–/CVD–) were obtained from a previous study. Subjects' demographic information (age, gender, ethnicity), anthropometric data (body mass index; BMI, hip and waist circumference, waist-to-hip ratio), CVD risk factors (diabetes mellitus, CVD and diabetes family history, systolic and diastolic blood pressure, alcohol intake, smoking) and biochemical and immunological factors (triglycerides, total cholesterol, high density lipoprotein- (HDL-C) and low density lipoprotein-cholesterol (LDL-C), and CD4 count were recorded from patients' files. Biomarkers of endothelial dysfunction i.e. intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), von Willebrand factor (vWF) and thrombomodulin (TM), were quantified by a Human Magnetic Luminex Screening Assay from 33, 38, 33 and 30 subjects of the HIV+/CVD+, HIV+/CVD–, HIV–/CVD+ and HIV–/CVD– groups, respectively. HIV-1 *nef* was sequenced from 25 subjects of the HIV+/CVD+ group and *nef* sequences of the 38 subjects in whom

biomarkers of endothelial dysfunction were quantified from the HIV+/CVD– group were obtained from a previous study. The HIV-1 *nef* gene was sequenced by Sanger sequencing and sequences from the HIV+/CVD+ and HIV+/CVD– groups were compared to identify polymorphisms that differed in prevalence between the two groups and associated with plasma levels of ICAM-1, VCAM-1, vWF and thrombomodulin.

Results: The HIV–/CVD+ group had significantly higher plasma levels of ICAM-1 and VCAM-1 versus all the other groups. Significantly higher vWF levels were observed in the HIV+/CVD+ group compared to the HIV+/CVD– and HIV–/CVD–groups while vWF plasma levels of HIV–/CVD+ group were significantly higher in comparison to the HIV+/CVD– group. The HIV+/CVD–group had significantly higher thrombomodulin levels in comparison to the HIV+/CVD+ and HIV–/CVD+ groups, and significantly higher levels of thrombomodulin were observed in the HIV–/CVD–group in comparison to all other study groups. A total of seven HIV-1 *nef* polymorphisms (N51T, E65G, A84G, F86V, R152K, E175D and K185Q) were identified to significantly differ in prevalence between the HIV-infected groups of which N51T, E65G, F86V and K185Q were each associated with higher levels of vWF, while N51T, E175D and K185Q were each associated with lower levels of thrombomodulin, and F86V was associated with a higher level of ICAM-1. Multivariable logistic regression models demonstrated that the significant association of the *nef* polymorphisms with CAD were attenuated after adjusting for levels of vWF, ICAM-1 and thrombomodulin.

Conclusions: This study shows that HIV-1 *nef* polymorphisms are associated with endothelial dysfunction and with CAD which suggests that HIV Nef may play a role in the development of CAD in the HIV-infected population. The study further suggests that endothelial dysfunction may mediate the effect of Nef on CAD development.

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LIST OF ABBREVAITIONS

AIDS – Acquired immunodeficiency syndrome

Akt – protein kinase B

ANCOVA – Analysis of covariance

ANOVA – Analysis of variance

ART – Antiretroviral therapy

ATP – Adenosine triphosphate

Bcl_2 – B-cell lymphoma 2

BH_4 – Tetrahydrobiopterin

BMI – Body mass index

CA – Capsid

CAD – Coronary artery disease

CCR – cell-surface co-receptor

CD4 – Cluster of differentiation 4

CHD – Coronary heart disease

CIMT – Carotid intima-media thickness

CMV – Cytomegalovirus

CTL – Cytotoxic T lymphocyte

CVDs – Cardiovascular diseases

CVs – Coefficient of variations

ddNTPs – Dideoxynucleotide triphosphate

DHFR – Dihydrofolate reductase

DNA – Deoxyribonucleic acid

ECs – Endothelial cells

EDTA – Ethylenediamine tetra-acetic acid

ELISA – Enzyme-linked immunosorbent assay

eNOS – Nitric oxide synthase

EPCR – Endothelial cell protein C receptor

ER – Endoplasmic reticulum

Fas – Fatty acid synthase

FasL – Fas ligand

FIs – Fusion inhibitors

Gag – Group-specific antigen

gp120 – Glycoprotein 120

gp41 – Glycoprotein 41

GTPCH – Guanosine triphosphate cyclohydrolase

H_2O_2 – Hydrogen peroxide

HAART – Highly active antiretroviral therapy

HDL-C – High density lipoprotein-cholesterol

HIV – Human immunodeficiency virus

HIV-1 – Human immunodeficiency virus type-1

HIV-2 - Human immunodeficiency virus type-2

HLA – Human leukocyte antigen

HSV – Herpes simplex virus

ICAM-1 – Intercellular adhesion molecule-l

IL – Interleukin

IN – Integrase

InSTIs – Integrase strand transfer inhibitors

LDL-C – Low density lipoprotein-cholesterol

LEDGF – Lens epithelium-derived growth factor

LOX-1 – Oxidized low-density lipoprotein receptor

MA – Matrix

MHC – Major histocompatibility complex

MI – Myocardial infarction

MVs – Microvesicles

NADP – Nicotinamide adenine dinucleotide phosphate

NC – Nucleocapsid

NCDs – Non-communicable diseases

Nef – Negative regulator factor

NNRTIs – Non-nucleoside reverse transcriptase inhibitors

NO – Nitric oxide

NRTIs – Nucleoside reverse transcriptase inhibitors

O_2 – Oxygen

$ONOO^-$ – Peroxynitrite

PAD – peripheral artery disease

PBMCs – Peripheral blood mononuclear cell

PCR – Polymerase chain reaction

PIs – Protease inhibitors

Pol – Polymerase

PR – Protease

P-TEFb – Positive transcription elongation factor-b

Rev – Regulator of virion gene expression

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RT – Transcriptase

Tac – T-cell antigen coupler

Tat – Transcriptional transactivator

TGs – Triglycerides

TM – Thrombomodulin

TNF-α – Tumour necrosis factor-α

VCAM-1 – Vascular cell adhesion molecule-1

Vif – Viral infectivity factor

VLDL-C – very-low density lipoprotein-cholesterol

Vpr – Viral protein R

Vpu – Viral protein unique

vWF – von-Willebrand factor

WHO – World health organization

CHAPTER ONE – INTRODUCTION

1.1 Context of the study

This MSc study is part of a previous PhD study conducted by Dr Genevieve Mezoh (MSc supervisor), which was supervised by Professor Nigel J. Crowther (MSc co-supervisor) and Professor Penny L Moore. The overall aim of the PhD study by Dr Genevieve Mezoh was to assess endothelial dysfunction in the Black South African HIV-infected population and identify associated HIV-1 *nef* and *tat* polymorphisms. The study by Dr Genevieve Mezoh consisted of collaborators from the University of the Witwatersrand, Dr Alinda G. Vos and Dr Nereshni Lutchman. Additional collaborators from the Cardiology Unit and Vascular Unit of the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), Professor N Tsabedze and Professor ATO Carrim, respectively, were invited for the purpose of recruiting HIV-infected and HIV-uninfected subjects with coronary artery disease (CAD). Therefore, the current MSc study included HIV-infected and HIV-uninfected subjects with and without CAD.

Cardiovascular diseases (CVDs) and HIV are the leading causes of death in South Africa (Shah *et al*., 2018). As the HIV-infected population ages due to effective administration of HAART, a new pattern of increased development of CVD in these individuals is observed which poses a challenge to health authorities and clinicians (Wada *et al*., 2015; Nanni *et al.,* 2015; Kearns *et al.,* 2017). Endothelial dysfunction is associated with coronary artery disease (CAD) and HIV infection (Triant, 2012; Kearns *et al.,* 2017). Mutations in the HIV-1 Nef protein have been associated with plasma markers of endothelial dysfunction in HIV-infected subjects without CAD, which may be one of the mechanisms by which HIV infection increases risk of atherosclerotic disease (Mezoh *et al*. 2020). Therefore, the current study aimed to

investigate whether HIV-1 *nef* variants are associated with CAD and with biomarkers of endothelial dysfunction.

1.2 The structure of the dissertation

This dissertation is divided into six chapters, incorporating the current chapter one. Chapter two is divided into 10 sections which take an in depth look at the literature surrounding CVD and HIV. An overview of CVDs is covered in section 2.1, followed by the pathophysiology in section 2.2. Section 2.3 explores an overview of HIV, followed by a comprehensive literature regarding HIV-1 Nef and envelope proteins in section 2.4 and 2.5, respectively. The development of CVD in the HIV-infected population is explored in section 2.6, while section 2.7 covers thrombosis within this population. The association of *nef* mutations and disease outcome is covered in section 2.8, followed by a concluding problem statement, and aims and objectives, in section 2.9 and 2.10, respectively. Chapter three covers the methodology applied in this study, followed by chapter four comprising of study results. Subsequent to that is chapter five in which results are discussed and conclusions drawn. References used in this dissertation are shown in chapter six.

CHAPTER TWO – LITERATURE REVIEW

2.1 Overview of Cardiovascular Diseases

Cardiovascular diseases (CVDs) describe a group of medical conditions which affect the heart and blood vessels (Shah *et al.*, 2018), with the most serious of these diseases involving atherosclerosis such as coronary artery disease (CAD) (Kearns *et al.*, 2017). Atherosclerosis is characterized by extreme cellular proliferation of inflammatory cells, endothelial cells and vascular smooth muscle cells which result in a chronic and progressive inflammatory disorder presented as occlusive arterial disease, stroke and myocardial infarction (Boehm & Nabel, 2003). Traditional risk factors for CVDs include obesity, smoking, hypertension, diabetes mellitus, abnormal serum lipid levels and physical inactivity (Nguyen *et al.*, 2016). The prevalence of these risk factors is higher in the adult population (Christofaro *et al.*, 2011).

Cardiovascular diseases are the leading causes of mortality and morbidity worldwide, accounting for about 31% of all deaths globally (Shah *et al.*, 2018). Approximately 17.3 million people die from CVDs each year, with stroke and coronary heart disease accounting for up to 39% and 43% of these deaths, respectively (Byrne *et al.*, 2017; Victor *et al.*, 2019). The Global Burden of Diseases study estimated approximately 422.7 million CVD cases from 1990 to 2015 and that 17.92 million deaths were due to CVDs (Roth *et al.*, 2017). In South Africa, the mortality rate due to non-communicable diseases (NCDs) is higher than those of tuberculosis and HIV/AIDS combined, with CVD being the leading category of NCDs (Schulte, 2019).

2.2 Pathophysiology of cardiovascular disease

2.2.1 Development of endothelial dysfunction

Endothelial cells (ECs) are the main constituents of the endothelium, which lines the internal wall of blood vessels, and are significant for the regulation of vascular health (Brevetti *et al.*, 2008). They are affected by changes in blood composition and haemodynamic forces and respond by inducing different paracrine factors within the lumen and blood vessel wall. Vascular homeostasis is maintained by these factors under normal physiological conditions (Verma & Anderson, 2002). Moreover, ECs control the vascular permeability to plasma constituents, leukocyte adhesion and aggregation, and thrombosis (Sitia *et al.*, 2010). Injury to ECs may disrupt their normal physiological function resulting in endothelial dysfunction (Brevetti *et al.*, 2008). The latter has been documented as a crucial contributor to the development of CVDs such as chronic heart failure, coronary artery disease, hypertension, peripheral arterial disease, stroke, and atherosclerosis (Endemann & Schiffrin, 2004; Roquer *et al.*, 2009; Hadi *et al.*, 2005).

Endothelial dysfunction refers to a number of pathological conditions incorporating anti-inflammatory and anti-coagulant properties of the endothelium, dysregulation of vascular remodelling, and impaired modulation of vascular growth (Cai & Harrison, 2000). The pathophysiology of endothelial dysfunction incorporates several mechanisms that are characterised by increased oxidative stress, expression of adhesion molecules and pro-inflammatory cytokines, thrombosis and abnormal modulation of vascular tone (Sitia *et al.*, 2010; Endemann & Schiffrin, 2004).

Endothelial nitric oxide synthase (eNOS) is a crucial enzyme in the balance of vascular pressure by its production of nitric oxide (NO) (Kietadisorn *et al.*, 2012). Nitric oxide is a

molecule that has several intracellular effects that result in endothelial regeneration, vasorelaxation, inhibition of platelet aggregation, vessel tone regulation, leukocyte adhesion and migration, and proliferation and migration of smooth muscle cells (Napoli *et al.*, 2006; Bermudez *et al.*, 2008). Therefore, the bioavailability of eNOS-derived NO is critical for modulation of endothelial function and it may be affected by several processes (Kietadisorn *et al.*, 2012) which include decreased eNOS protein levels, decreased eNOS enzymatic activity, abnormalities of eNOS phosphorylation, abnormalities in eNOS trafficking to the caveolae, and uncoupling of eNOS activity (Huang, 2003).

Elevated reactive oxygen species (ROS) and inflammation, which are linked to decreased NO production and bioavailability, are the main mechanisms underlying the pathophysiology of endothelial dysfunction (Napoli *et al.*, 2006; Bermudez *et al.*, 2008, Premer *et al.*, 2019). The ROS are produced in the vascular wall by several enzymes including eNOS, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase (Figure 2.1) (Münzel *et al.*, 2017). Under normal physiological conditions, ROS act as signalling molecules and play a key role in the modulation of vascular tone, inflammatory responses, cell growth and proliferation, oxygen sensing and apoptosis (Li *et al.*, 2014). However, the balance between ROS production and elimination can be perturbed by several factors. These factors include inactivation of antioxidant enzymes and elevated levels of exogenous and endogenous compounds that are prone to autoxidation and coupled to ROS production (Lushchak, 2014). Superoxide is the key mediator of oxidative stress which reduces the activity of NO by its conversion to peroxynitrite ($ONOO^-$), which in turn promotes uncoupling of eNOS and NO^- generation via tetrahydrobiopterin (BH_4) oxidation. The enzymes GTPCHI and DHFR opposes eNOS uncoupling by replenishing BH_4 levels via *de novo* synthesis and regeneration, respectively (Münzel *et al.*, 2017). All risk factors for CVDs are associated with elevated ROS

production in the vascular wall, a state that ultimately culminates in oxidative stress. The latter results in oxidative modification of phospholipids and lipoproteins, mechanisms that contribute to atherogenesis (Förstermann, 2008). Additionally, oxidation of the cofactor BH_4, may result in eNOS uncoupling and thus potentiation of oxidative stress and eNOS-derived NO, which have been shown to protect the vasculature (Li *et al.*, 2014).

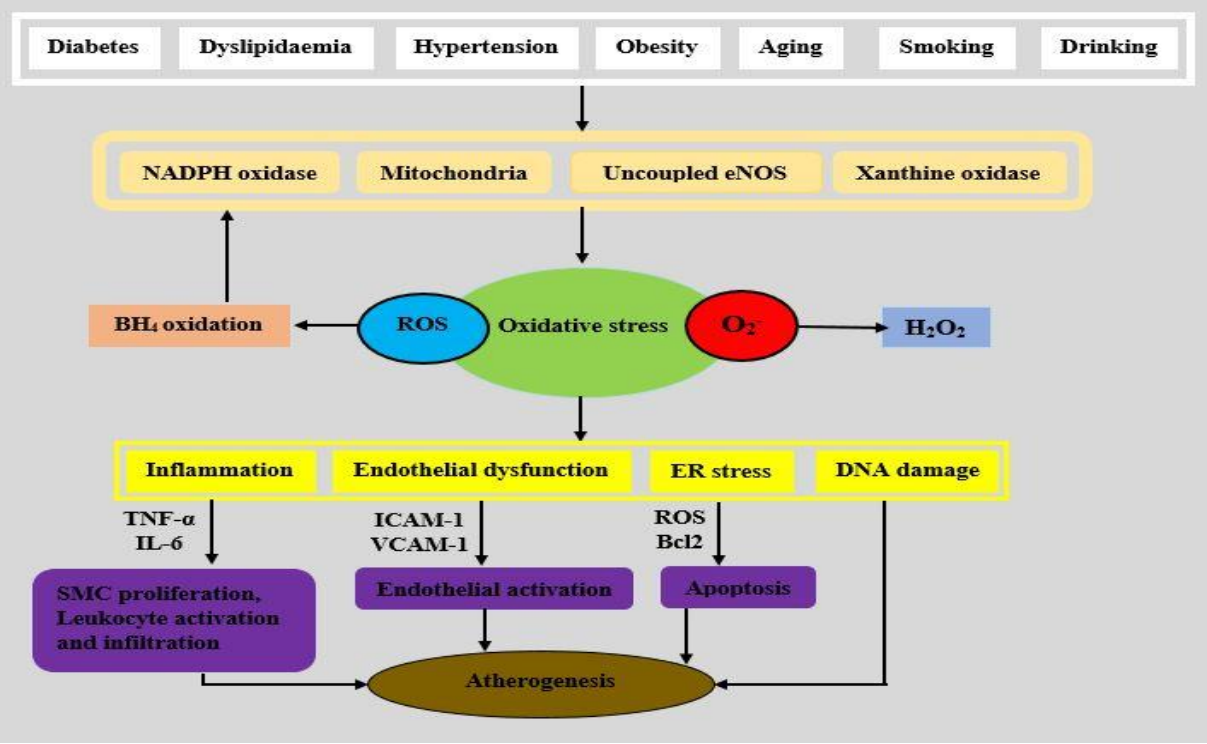


Figure 2.1: Mechanisms through which traditional risk factors of CVDs mediate atherogenesis. Cardiovascular risk factors (shown in white boxes) promote oxidative stress by several mechanisms which stimulates pro-oxidant enzymes such as NADPH oxidase, xanthine oxidase and eNOS, and triggers mitochondrial dysfunction. Oxidative stress results in expression of pro-inflammatory cytokines and cell adhesion molecules, which in turn, triggers leukocyte activation and endothelial activation respectively and thus atherogenesis. The latter may occur due to ER stress and DNA damage as a result of oxidative stress. Key: NADP, nicotinamide adenine dinucleotide phosphate hydrogen; eNOS, endothelial nitric oxide synthase; BH_4, tetrahydrobiopterin; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; ICAM-1, intercellular adhesion molecule-l; VCAM-1, vascular cell adhesion molecule-1; Bcl2, B-cell lymphoma 2; H_2O_2, hydrogen peroxide; O_2^-, superoxide; ER, endoplasmic reticulum; ROS, reactive oxygen species.

2.2.2 Role of inflammation in atherogenesis

Inflammation, characterised by the release of inflammatory cytokines, impairs endothelial function in isolated human veins and animal models (Clapp *et al.*, 2004). Interleukins are a group of cytokines secreted by cells of the immune system of which some have a pro-inflammatory effect while others have anti-inflammatory effects (Zhang & An, 2007). They trigger the recruitment of leukocytes to sites of injury and stimulation of fibroblast proliferation, thus activating the acute phase reaction (Mozos *et al.*, 2017). Interleukin-1 (IL-1) and interleukin-6 (IL-6) contribute towards the modulation of vascular tone by impairing the release of subendothelial NO and endothelin-1 by ECs (Mozos *et al.*, 2017).

TNF-α is one of the most significant cytokines being identified as a major effector of macrophage-mediated host defence and tissue injury, while also playing a key role in innate and adaptive immunity, apoptosis and cell proliferation (Chen *et al.*, 2008). Tissue-fixed macrophages, such as Langerhans and Kupffer cells, are known to be the major sources of TNF-α in pathological processes (Luster *et al.*, 1999). However, other cell types, including ECs, monocytes and T-cells, produce significant amounts of TNF-α upon exposure to a stimuli (Chen *et al.*, 2008).

Chronic inflammation is characterised by interactions between ECs, platelets and leukocytes, that initiate autocrine and paracrine signalling mechanisms (Gawaz *et al.*, 2005). These processes result in leukocyte recruitment into the vascular wall and ultimately development of atherosclerotic lesions and thrombosis (Gawaz *et al.*, 2005). Cellular adhesion molecules, particularly members of the immunoglobin superfamily and selectin family, are intimately involved in the recruitment of leukocytes (Table 2.1) to the sites of inflammation (Hope & Meredith, 2003).

Table 2.1: Distribution and functions of adhesion molecules

	Adhesion molecule	Distribution	Function	References
Selectin family	E-selectin	Endothelium	Leukocyte rolling	Hope & Meredith, 2003
	P-selection	Endothelium, platelets	Platelet/endothelial interaction, leukocyte rolling	Hope & Meredith, 2003
Immunoglobin superfamily	I-CAM-1	Endothelium, leukocytes, fibroblasts, atherosclerotic lesion, smooth muscle cells	Adherence and transmigration of leukocytes	Hope & Meredith, 2003
	ICAM-2	Endothelial cells, lymphocytes, monocytes	Adherence and transmigration of leukocytes	Bö *et al.*, 1996; Krieglstein & Granger, 2001
	ICAM-3	Leukocytes	Adherence of lymphocytes	Bö *et al.,* 1996; Montoya *et al.*, 2002.
	V-CAM-1	Endothelium, atherosclerotic lesion smooth muscle cells	Adherence of leukocytes	Hope & Meredith, 2003

Cell adhesion molecules include endothelial selectin (E-selectin), platelet selectin (P-selectin), intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Mozos *et al.*, 2017). They are expressed on the plasma membrane of cells but also exist in the circulation in a soluble form (Mulhem *et al.*, 2021). These molecules are glycoproteins and are important for tissue integrity, cellular communication and interactions, and extracellular matrix contact (Mozos *et al.*, 2017). Their expression is elevated in obesity, vascular remodelling and endothelial dysfunction. Furthermore, they have been regarded as mediators and biomarkers of

several cardiovascular disorders such as stroke, hypertension and coronary heart disease (Mozos *et al.*, 2017).

von Willebrand Factor (vWF) is an adhesive glycoprotein produced in the megakaryocytes and endothelial cells (Gragnano *et al.*, 2017). Under normal physiological conditions, vWF plays a homeostatic role by mediating adhesion and subsequent aggregation of platelets to injured vascular sub-endothelium thereby promoting coagulation and consolidating blood clots to stop bleeding (Peyvandi *et al.*, 2011; Belyaev *et al.*, 2018). In situations of high fluidic shear stress as seen at sites of arterial stenosis or injury, vWF may be activated inappropriately triggering a dangerous thrombosis scenario (Belyaev *et al.*, 2018). Recent studies have outlined the role of vWF in vascular inflammation and immunothrombosis (Gragnano *et al.*, 2017). Moreover, vWF is implicated in the pathophysiology of atherosclerosis and has been proposed as a prognostic biomarker in metabolic, cardiovascular, and inflammatory diseases, such as myocardial infarction, diabetes mellitus, and stroke (Gragnano *et al.*, 2017)).

2.2.3 Development of the atherosclerotic plaque

Atherosclerosis is a disorder of the arterial wall that is localised at susceptible areas in the major conduit arteries (Insull Jr, 2009). In the general population, atherosclerosis develops over a period of 50 years, beginning from the early teens (Insull Jr, 2009). This process involves the progressive retention of lipids in the arterial intima, that undergo oxidation and modification, which results in chronic inflammation at susceptible sites of the vasculature (Insull Jr, 2009). Fatty streaks are the earliest visible lesion of atherosclerosis which develop during early childhood and adolescence,

and progress into fibrous plaques over the years (Crowther, 2005; Insull Jr, 2009). The latter evolves into vulnerable forms, liable to rupture, resulting in thrombosis or stenosis of the overlying artery (Crowther, 2005). The process of plaque formation is the same across different populations (Insull Jr, 2009). Traditional risk factors for CVDs have been shown to accelerate the rate of atherosclerosis development (Hong *et al.*, 2015).

Atherosclerosis is the precursor of several CVDs (Robinson *et al.*, 2009). Myocardial infarction, stroke and a majority of heart diseases are associated with the evolution of atherosclerosis in the intima of the arterial vessels (Lnsis, 2000). Atherosclerosis is more common in medium and large-sized arteries as a result of the formation of atherosclerotic plaques (Gibellini *et al.*, 2013). These plaques are lesions of the intima composed of calcified and necrotic regions, inflamed smooth muscle cells, accumulated modified lipids, leukocytes and foam cells (Gibellini *et al.*, 2013). Formation of atherosclerotic lesions is believed to be initiated by local inflammation in the vascular wall that is triggered by dyslipidaemia such as high remnant lipoprotein levels and elevated LDL-C levels, as well as endothelial dysfunction and other contributing factors of CVDs such as arterial hypertension, obesity, diabetes mellitus and infections (Katakami, 2017; Wang *et al.*, 2015; Badimon & Vilahur, 2014). The mechanism of atherosclerotic plaque formation is summarised in Figure 2.2. (Wilck, & Ludwig, 2014).

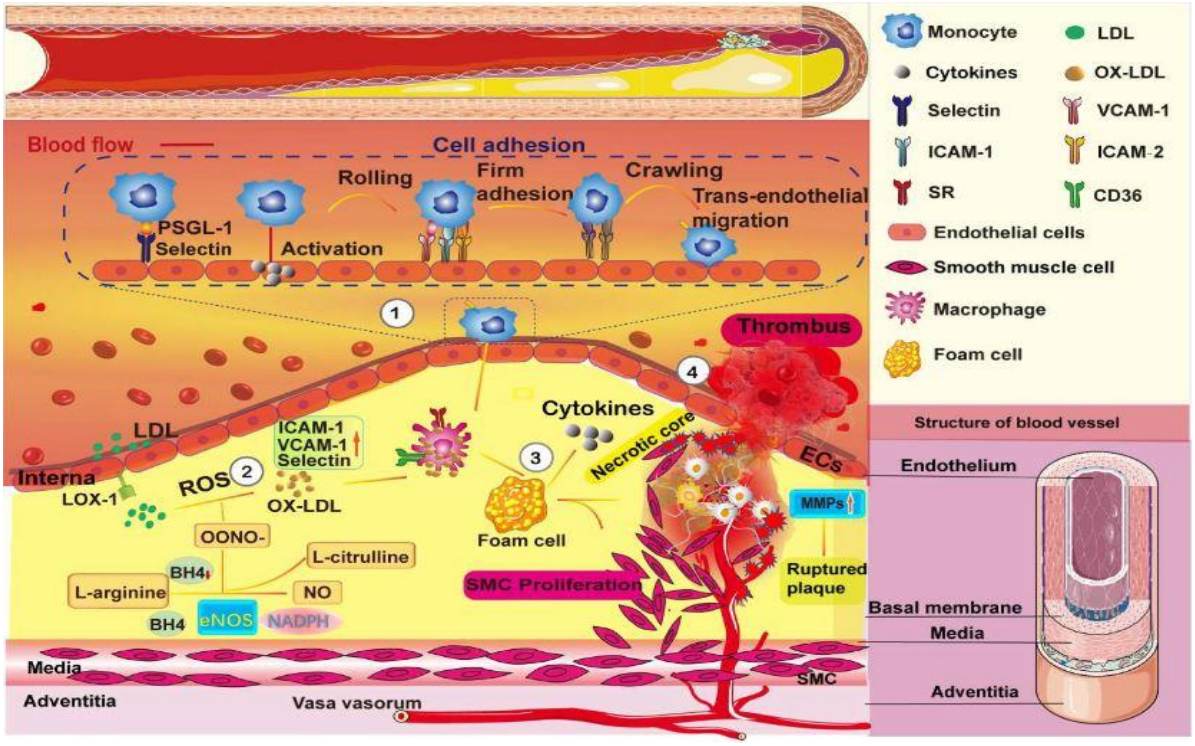


Figure 2.2: Formation of an atherosclerotic plaque. Pro-inflammatory cytokines and cell adhesion molecules mediate recruitment, adhesion and transmigration of monocytes from the lumen into the intima (1). In the intima, monocytes differentiate into macrophage which absorb ox-LDL and form foam cells (2). Foam cells favour production of pro-inflammatory cytokines and undergo an apoptotic process which results in the formation of the necrotic core of a mature atherosclerotic plaque (3). Macrophages increase the inflammatory response which may cause plaque rupture and thus thrombosis (4). Key: ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; LDL, low density lipoprotein; ox-LDL, oxidised low density lipoprotein; BH_4, tetrahydrobiopterin; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; eNOS, endothelial nitric oxide synthase; ROS, reactive oxygen species; LOX-1, Oxidized low-density lipoprotein receptor. Reproduced with permission from Yin ***et al.***, (2021).

2.3 The Human Immunodeficiency Virus

2.3.1 Overview of the Human Immunodeficiency Virus

Human immunodeficiency virus infection has a dramatic impact on individuals, households, and economies worldwide despite the global efforts to control its infection since the epidemic began 40 years ago (Noubissi *et al.*, 2018). As HIV continues to exist as a major public health concern worldwide, it is recorded that approximately 36.9 million people are living with HIV globally (UNAIDS, 2018), with approximately 1.8 million new HIV infections per year (Hatzold *et al.,* 2019). Sub-Saharan Africa is the most profoundly affected area (Ballocca *et al.,* 2017) of which South Africa is home to approximately 7 million individuals living with HIV, with more than 180,000 AIDS-related deaths annually (Bassett *et al.*, 2017).

The human immunodeficiency virus (HIV) is a retrovirus that has a nucleocapsid surrounded by a glycoprotein-rich envelope (Anand *et al.*, 2018). The virus is classified into two major types based on their genetic variability: HIV type-1 (HIV-1) and HIV type-2 (HIV-2). In comparison, HIV-1 is more virulent and more common, accountable for the AIDS pandemic, while HIV-2 is rare and less virulent (Santoro & Perno, 2013).

The HIV-1 subtype is differentiated by its vast genetic variability as a result of its elevated and error-prone replication (Beloukas *et al.*, 2016). The latter is due to the inability of the reverse transcriptase to perform proof-reading (Beloukas *et al.*, 2016). Deletions and insertions are the most common mutations in the viral genome of HIV-1 (Hemelaar, 2012). These mutations lead to an abrupt generation of genetically diverse viral populations within an infected individual (Hemelaar, 2012).

The HIV-1 is divided into three different groups; namely, M, N and O, with most of them being classified under group M. The latter is further categorised into subtypes, A to J, according to their phylogenetic clustering of sequences (Dwivedi & Sengupta, 2012). These HIV-1 subtypes take credit for the current global AIDS pandemic (Maartens *et al.*, 2014). The HIV-1 subtype C predominates in sub-Saharan Africa and India while the other subtypes predominate in the other regions of the world as shown in Figure 2.3 (Maartens *et al.*, 2014).

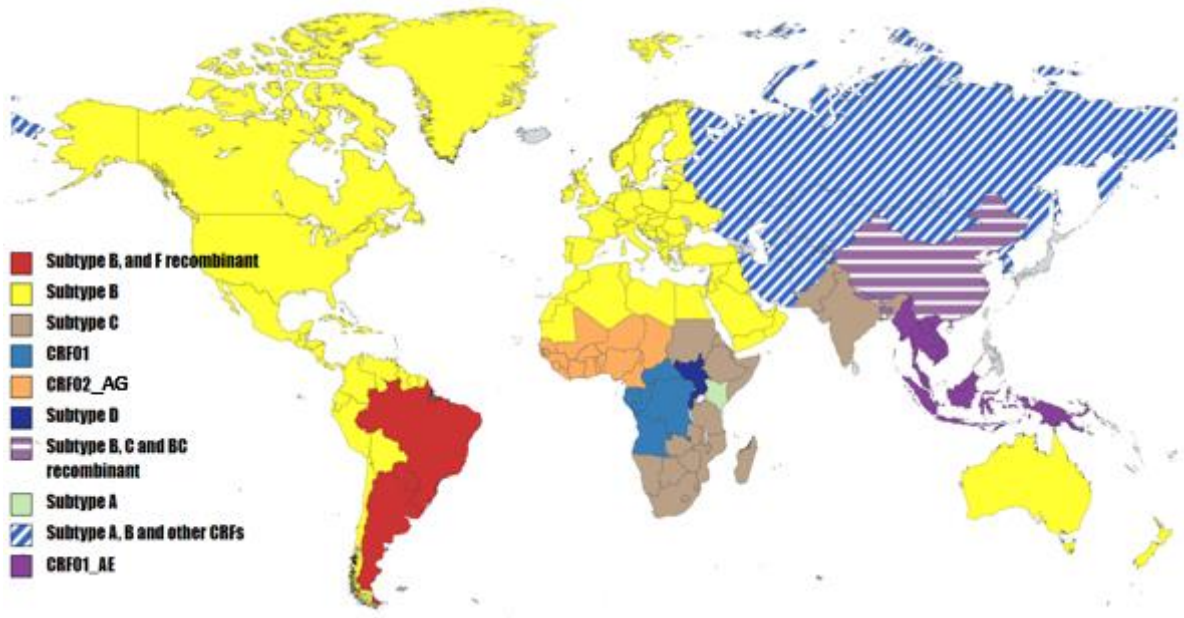


Figure 2.3: Global distribution of major HIV-1 subtypes. Ten different epidemic patterns are indicated by the different colours. The Australian epidemic is subtype B. Africa has the widest variety of HIV subtypes with specific geographical hotspots of subtype A and D and subtype C being very prevalent in the south and east. Subtypes CRF02 AG and CRF01 are primarily found in West Central and West Africa. A mixture of subtype B and CRF01 AE, subtype C, and a mixture of B, C, and BC recombinants have all been found in Asia. In South America there is a significant number of F recombinants plus subtype B, whilst in the rest of the Americas and in Western Europe subtype B is the most common variety. Subtypes of A, B, and other CRFs dominate the epidemic in eastern Europe. Key: CRF01 AE, first circulating recombinant form of HIV-1; CRF02 AG, second circulating recombinant form of HIV-1. Source: World map customized to show distribution of HIV-1 subtypes worldwide. https://mapchart.net/world.html

2.3.2 The HIV replication cycle

The life cycle of HIV-1 is complex and is divided into an early and late replication phase (Kirchhoff, 2013). The attachment of the virion to the cell surface represents the early phase of the cycle, which concludes with the proviral DNA being incorporated into the genome of the host (Kirchhoff, 2013). Subsequently, the late replication phase commences with proviral transcription and ends with the release of active infectious progeny virions as shown in Figure 2.4 (Kirchhoff, 2013; Gonzalo-Gil *et al.*, 2017).

The attachment of virions to the cell surface is required for infection and involves binding of the viral envelope glycoprotein to CD4 receptor which, in turn, mediates the interaction of the virion with CCR4 or CCR5 co-receptors (Wilen *et al.*, 2012). This triggers a cascade of conformational changes that results in fusion of the virion with the host cell membrane and release of viral RNA and viral proteins, such reverse transcriptase and integrase, into the cytoplasm (Engelman & Cherepanov, 2012). The viral RNA is reverse transcribed by reverse transcriptase into viral DNA which is transported into the nucleus and inserted into the host cell genome by the viral integrase enzyme aided by the host chromatin-binding protein lens epithelium-derived growth factor (LEDGF) (Pasternak *et al.*, 2013). Host RNA polymerase II and positive transcription elongation factor-b (P-TEFb) modulate the transcription of integrated viral RNA to produce messenger RNAs of different sizes which are then exported to the nucleus of the host cell (Pasternak *et al.*, 2013).

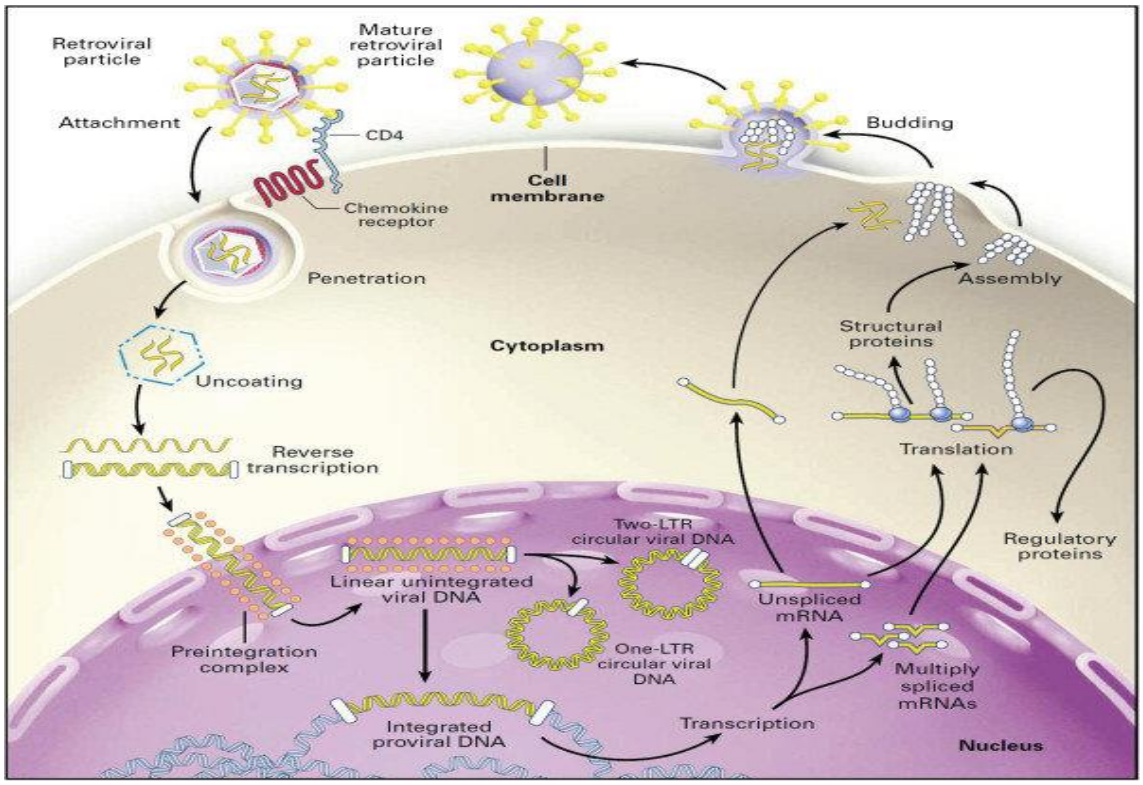


Figure 2.4: Human Immunodeficiency Virus replication cycle. HIV replication cycle begins with the interaction of retroviral particle with CD4 and CCR5 which result in fusion of particle with the host cell followed by an entrance into cytoplasm. The retroviral particle then breaks down, and the viral reverse transcriptase transcribes the viral RNA into DNA, which is then translocated to the nucleus. Within the nucleus the HIV integrase peptide incorporates the viral DNA into the host genome, and transcription can then take place. Viral transcripts are then exported from the nucleus to be translated and assembled into a mature HIV particle at the cell surface. Adapted from Pasternak ***et al***., (2013).

2.3.4 Genome organization

The genome of HIV (Figure 2.5) is designed in such a way as to enable it to efficiently adapt to its environment, and promote viral transmission and replication in its host (Anand *et al*., 2018). In addition to structural and enzymatic factors, HIV-1 produces several viral proteins coded for by the group-specific antigen (*gag*), polymerase (*pol*), and envelope (e*nv*) genes (Percario *et al*. 2015). *Gag* codes for the structural proteins nucleocapsid (NC), matrix (MA) and capsid (CA); *pol*

encodes the enzymes integrase (IN), transcriptase (RT) and protease (PR); while *env* encodes the glycoproteins gp41 and gp120 (Kirchhoff, 2013). In addition, the HIV genome codes for the transcriptional transactivator (*Tat*) and the regulator of virion gene expression (*Rev*), which are known as regulatory proteins, and four accessory proteins which includes the negative regulatory factor (*Nef*), viral infectivity factor (*Vif*), viral protein R (*Vpr*) and viral protein unique (*Vpu*) (Percario *et al*. 2015).

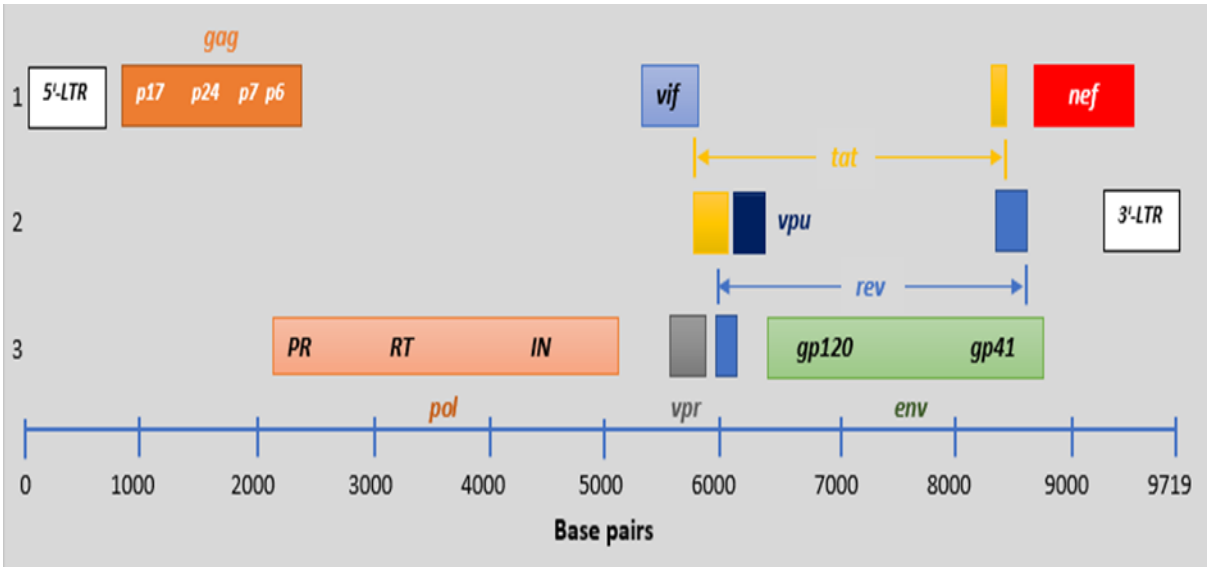


Figure 2.5: Organisation of HIV-1 genome. HIV-1 genome consists of 9 proteins: gag, pol, vif, vpr, tat, rev, vpu, env and nef, and two long terminal repeats at the 5'-end and 3'-end.

2.4 The HIV negative regulatory factor

2.4.1 Features of HIV Nef

The HIV Nef is a small viral accessory protein, 200 to 215 amino acids in length, that plays a key role in maintaining elevated viral loads during HIV infection and favours clinical progression to AIDS (Duffy *et al*., 2009). Among the accessory proteins, Nef is the largest and is expressed the most during the early stage of infection (Aqil *et al*. 2014). The HIV *nef* gene is situated at the 3'-end of the viral genome, partly overlapping the *env* gene and the unique 3'-end (U3) region in the 3'- long terminal repeat. It is a membrane-associated protein that becomes expressed following the onset of infection (Aqil *et al*. 2014).

Nef is primarily localised to the cell membranes, such as the inner surface of the plasma membrane, the perinuclear region and endosomal membrane (Lenassi *et al*., 2010). It is released from cells either as a soluble protein or in microvesicles (MVs), and negatively effects bystander cells (Raymond *et al*., 2011). Due to its ability to exert its effect on both infected and uninfected bystander cells, Nef is referred to as a shuttling molecular adaptor (Percario *et al*., 2015).

2.4.2 Biological functions of HIV Nef

Well-known functions of Nef include the downregulation of cell surface molecules through interaction with the vesicular trafficking machinery, alteration of T-cells and the macrophage activation state, perturbation of the actin cytoskeleton via interaction with cellular kinases and enhancement of virion infectivity (Rosa *et al*., 2015). Numerous viruses are known to manipulate the innate immune responses to evade immune recognition and improve viral replication and spreading and Nef is largely involved in this "hijacking" activity (Percario *et al*., 2015). It is also

responsible for protecting infected cells from recognition and lysis by cytotoxic T lymphocytes by downregulating the HLA-A and – B MHC-1 molecules (Simmons *et al*., 2001). Moreover, Nef has been reported to enhance the ability of infected cells to support viral replication (Swingle *et al*., 2003).

2.4.2.1 Downregulation of cell surface receptors

HIV-1 infects and replicates within cells that express the cell surface glycoprotein, CD4 (Wolf-Lindwasse *et al*., 2007). The latter is expressed on a subset of macrophages and T lymphocytes which are central regulators in innate and adaptive immunity, respectively (Wolf-Lindwasse *et al*., 2007). Moreover, CD4 acts as the primary co-receptor for HIV-1 and its binding with virions is the initial step in viral entry into the target cells (Chaudhuri *et al*., 2007). HIV-1 has been shown to deplete CD4+ T-cells thereby resulting in destruction of the immune system in AIDS (Wolf-Lindwasse *et al*., 2007). Remarkably, the progression of HIV infection to full-scale AIDS is critically dependent on Nef's ability to induce CD4 downregulation (Mariani *et al*., 1996). Despite extensive studies on the molecular machinery and cellular pathways involved in the downregulation of CD4 following HIV infection, the mechanism by which Nef downregulates CD4 is still not fully understood (Chaudhuri *et al*., 2007).

Nef-induced CD4 down-modulation occurs after viral infection and is referred to as an undefined post-translational event (Greenway *et al*., 2003). Further studies demonstrate that Nef leads to CD4 endocytosis in clathrin-coated pits and subsequently degradation of CD4 in lysosomes (Aiken *et al*., 1994). CD4 receptor endocytosis is a normal physiological response to T-cell activation by antigen presenting cells (Mangasarian *et al*., 1997). Nef appears to act as a connector protein

between the endocytic machinery and CD4, thereby causing CD4 internalization and by-passing normal routes of CD4 down-modulation (Mangasarian *et al*., 1999). It is suggested that rather than interacting directly with endocytic machinery through its dileucine motif, CD4 specifically interacts with Nef, which in turn, has its own dileucine motif that acts as a connector with the endocytic machinery (Craig *et al*., 1998). Nef also causes the loss of cell surface T-cell antigen coupler (Tac) and CD4 from CD4+ cell lines and PBMCs (Greenway *et al*., 1995). As both Tac and CD4 play significant roles in antigen-driven helper T-cell proliferation and T-cell signalling, respectively, the role of Nef in the viral life cycle may be to influence signalling pathways emanating from these receptors (Greenway *et al*., 1995).

2.4.2.2 Role of Nef in apoptosis

In vivo studies have shown that HIV-1 infection enhances apoptosis, which also incorporates uninfected bystander cells (Geleziunas *et al*., 2001). Elevated termination of such bystander cells is undertaken by Nef through an induction of Fas ligand (FasL) expression on the surface of the infected T cells (Geleziunas *et al*., 2001). Subsequently, the interaction of FasL with Fas (CD95) on neighbouring cells, including HIV-specific cytotoxic T lymphocytes, might potentially lead to bystander cell termination and thus immune evasion (Katsikis *et al*., 1995). Conversely, Nef inhibits apoptosis in infected host T-cells through Akt-independent phosphorylation of Bad, and via its concomitant suppressive effects on ASK1, which is a key intermediate in TNF-α and Fas death signalling cascade (Geleziunas *et al*., 2001; Wolf *et al*., 2001). Additionally, it has been outlined that Nef inhibits apoptosis mediated by p53 through binding to this tumour suppressor (Greenway *et al*., 2002).

2.4.2.3 Role of Nef in endothelial dysfunction

Among its numerous functions, Nef has been identified as a possible role player in the development of CVD via its effect on endothelial function (Duffy *et al*., 2009; Anand *et al*., 2018). Endothelial cells are in a prime position for contact with circulating HIV-infected cells and the Nef viral protein (Wang *et al*., 2014). The latter is reported to be transferred in exosomes into arterial endothelial cells, and as a consequence, results in the activation of endothelial cells and endothelial dysfunction, with potential progression to cardiovascular diseases (Wang *et al*., 2014). *In vitro* studies suggest that the Nef viral protein alters vascular homeostasis by affecting macrophages and endothelial cells through discrete pathways (Walker *et al.,* 2007).

HIV infection has been linked to increased inflammation in blood vessels (Fisher *et al*., 2006). Elevated plasma levels of interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) have been recorded in HIV-positive individuals compared to uninfected individuals (Fisher *et al*., 2006). HIV infection may increase the risk of atherosclerosis via inhibition of cellular cholesterol efflux, thereby resulting in the accumulation of cellular cholesterol, which is facilitated by the Nef viral protein (Cui *et al*., 2014). Soluble Nef released from infected cells impairs ATP binding cassette transporter A1 of the cholesterol efflux machinery in HIV uninfected cells (Asztalos *et al*., 2010). Reports from *in vivo* studies show that Nef is associated with increased plasma levels of triglycerides and total cholesterol and decreased HDL-C levels (Cui *et al*., 2014).

2.5 The HIV envelope proteins

2.5.1 Features of HIV gp160

The HIV envelope glycoprotein (env) is expressed as a precursor, gp160, which is cleaved into two fragments, gp120 and gp41, by cellular furin proteases (Anand *et al*., 2018; Zhu *et al*., 2019). These fragments, however, remain associated with each other (Zhu *et al*., 2019). Gp120 is expressed on the outer layer of the virus and on the surface of infected cells, while gp41 is a transmembrane protein (Anand *et al*., 2018; Marincowitz *et al*., 2019). Gp120 and gp41 facilitate binding of the virus to the target cells and membrane fusion, respectively, by interacting with cell surface receptors including CD4 receptor, and CXCR4 and CXR5 co-receptors (Lu *et al*., 2019; Anand *et al*., 2018; Marincowitz *et al*., 2019). Binding of gp120 to CD4 and co-receptors initiates refolding of gp41 into a trimer of hairpins with a six -helix bundle core, which induces membrane fusion and viral entry and infection (Lu *et al*., 2019).

Gp120 is divided into five loops that display a high degree of genetic variability (V1-V5), dispersed with other domains (C1-C5) that seem to be more structurally constrained and less variable. Gp41, however, is less variable (Geller *et al*., 2016). Selection pressure brought on by the immune system is believed to be the prime factor that promotes sequence diversity in gp160, thus making the V1-V5 gp120 region of the HIV-1 genome the most variable region (Geller *et al*., 2016). The HIV gp41 protein consists of three subdomains, including the conserved transmembrane domain, the ectodomain, and the cytoplasmic domain (Kafando *et al*., 2019). The latter incorporates three lentivirus lytic peptides, which contribute to the surface expression of gp160, incorporation of gp160 into viral particles, fusogenicity and its localisation in lipid rafts (Yang *et al*., 2010). The ectodomain mediates membrane fusion (Shang *et al*., 2008). The

conserved transmembrane domain inhibits gp160 from being released into the endoplasmic reticulum lumen, mediates viral envelope and host cell membrane fusion and enhances virus to cell and cell to cell fusions (Chen & Chou, 2017).

2.5.2 Role of envelope glycoproteins in endothelial dysfunction

Gp120 has been associated with endothelial dysfunction, characterized by decreased eNOS levels, elevated oxidative stress and increased endothelial barrier permeability (Marincowitz *et al.*, 2019). It mediates endothelial dysfunction either via an indirect or direct proapoptotic effect (Mazzuca *et al.*, 2018). The indirect proapoptotic effect of gp120 is based on the increased production of endothelin-1 (ET-1), elevated surface expression of endothelial monocyte-activating polypeptide II, and inhibition of NO synthase (Mazzuca *et al.*, 2018). *In vitro* studies conducted by Jiang *et al.* (2010) showed that HIV gp120 with TNF-α pre-treatment significantly reduced endothelium-dependent vasorelaxation, decreased eNOS mRNA and protein expression and significantly increased ICAM-1 expression in porcine coronary artery rings and cultured human coronary artery endothelial cells. Moreover, in *in vitro* studies performed by Price *et al.* (2005) elevated oxidative stress was observed in immortalised rat brain endothelial cells treated with gp120, while Yang *et al.* (2009) reported upregulation of IL-6 and IL-8 expression, in rat brain endothelial cells following exposure to gp120. Upregulation of these cytokines in turn enhanced the adhesion and migration of monocytes across the *in vitro* blood brain barrier (Yang *et al.*, 2009). Both soluble and membrane-bound gp120 have also been shown to significantly increase ICAM-1 expression at both the mRNA and protein levels (Ren *et al.*, 2002). The HIV-1 gp41 protein has been shown to induce ICAM-1 expression in other cell types such as leukocytes and glial cells (Chen *et al.*, 1994). Reports from Chen *et al.* (1994) show that soluble gp41 increases

expression of ICAM-1 by 70% in the monocytic cell line H9 with no effect, however, on the U937 cell line, thus suggesting a selective biological function of gp41 in modulating ICAM-1 expression. The gp41 protein is also capable of increasing ROS in bystander cells, which is crucial for ROS-dependent Env-mediated apoptosis (Couret & Chang, 2016). The process of Env-mediated cell death is poorly understood, however, Garg and Blumenthal (2008) suggest that it may start with gp120 binding to CD4 and CXCR4 and lead to gp41-mediated membrane fusion/hemifusion. The HIV gp41 fusion inhibitors, C34 and enfuvirtide (T20), inhibited apoptosis of bystander cells in models where HIV Env expressing cells were co-cultured with CD4 and CXCR4 expressing bystander cells (Garg & Blumenthal, 2006; Stocker *et al*., 2000; Blanco *et al*., 2003).

2.6 Development of CVD in the HIV-infected population

The introduction of highly active antiretroviral therapy (HAART) has increased the life span of individuals infected with HIV (Wada *et al*., 2015). Although early diagnosis and introduction of HAART have contributed to the observed decrease in HIV-related deaths, HIV infection is associated with an increased risk of non-communicable diseases (NCDs) (Nanni *et al.,* 2015). Thus, cardiovascular diseases, such as atherosclerosis and atherosclerosis-associated complications, contribute to the elevated morbidity and mortality in the HIV-infected population (Kearns *et al.,* 2017). It is evident that there is an increased rate of CVDs in the HIV-infected population compared to the uninfected population (Duprez *et al*., 2012). Several studies have shown that HIV-infected adults are more likely to be at risk of accelerated CVDs and atherosclerosis (Nix *et al.,* 2014). Moreover, studies have reporteded that both clinical cardiovascular events such as peripheral artery disease and coronary heart disease, along with subclinical cardiovascular impairment such as increased carotid intima-media thickness (CIMT),

abnormal ankle-brachial index, coronary calcification and silent myocardial ischemia, are highly prevalent in the HIV-infected population (Anand *et al.*, 2018).

The aetiology of CVDs, such as coronary artery disease (CAD), within the HIV-infected population, is likely to involve a complex interaction of factors, such as highly active antiretroviral therapy, traditional CVD risk factors, and HIV-related parameters such as inflammatory and immunological changes (Triant, 2012). Several indices reflecting accelerated and discrete stages of atherosclerosis, including coronary artery calcification, coronary plaque and endothelial dysfunction, have been observed in HIV-infected individuals (Triant, 2012).

2.6.1 Traditional risk factors of CVD in the HIV-infected population

The prevalence of traditional CVD risk factors appears to be higher among HIV-infected individuals compared to HIV-uninfected individuals (Lambert *et al.*, 2016). The National Institute of Health (NIH) reported 50-70% and less than 20% of HIV-infected and uninfected individuals, respectively, are smoking in the United States (Lambert *et al.*, 2016). A study involving 33,308 HIV-infected participants on HAART of which 21 % and 23 % were previous and current female smokers respectively compared with 35 % non-smoking females, revealed the risk of myocardial infarction was reduced with each passing year of smoking cessation for 3 years (Wang *et al.*, 2015; Petoumenos *et al.*, 2011). Alcohol abuse was linked with higher prevalence of CVDs among HIV-infected individuals versus infrequent and moderate HIV-infected drinkers in the veteran aging cohort study (Wang *et al.*, 2015). A cross-sectional study involving 189 HIV-infected females and 111 HIV-infected males, with mean age of 37.1 ± 8.6 years and mean BMI of 25.5 ± 4.8 kg/m^2, reported that obesity occurs frequently in HIV-infected individuals (Okeke *et al.*,

2017). A study involving 8610 HIV-infected individuals of which 74 % were males and 26 % were females with 10 % of the participants being older than 60, 51 % within the age range of 45 years to 60 years and 39 % within the age range of 20 years to 44 years, reported higher prevalence of diabetes mellitus in HIV-infected individuals than in HIV-uninfected individuals (Hernandez-Romie *et al.*, 2017; Noubissi *et al.*, 2018).

The relative risk of MI among HIV-infected individuals versus controls was almost double after adjustment of traditional risk factors in a large health-care system based retrospective study (Zanni *et al.*, 2014). Additionally, a higher prevalence of subclinical coronary atherosclerosis was recorded in HIV-infected individuals compared to HIV-uninfected individuals in a cross-sectional study (Zanni & Grinspoon, 2012). The prevalence of traditional risk factors of CVDs in HIV-infected individuals is important, but however, explains only a part of the burden of CVD in these individuals (Zanni & Grinspoon, 2012). Therefore, it is extremely important to consider other parameters that contribute to the development of CVDs when comparing the prevalence of CVDs between HIV-infected and HIV-uninfected individuals.

2.6.2 Contribution of HAART in the development of CVD

The role of HAART in the context of CVDs and atherosclerosis has been substantially debated (Ballocca *et al.*, 2017). Administration of HAART is linked with elevated levels of triglycerides, LDL-C and total cholesterol, and decreased levels of HDL-C, resulting in a proatherogenic lipid profile (Baekken *et a*l., 2008). Therefore, it is important that research be conducted in this area to develop new measures to alleviate the risk of CVDs in people living with HIV.

The current highly active antiretroviral drugs used in southern Africa are shown in Table 2.2 (Eggleton & Nagalli, 2020). These drugs include the nucleoside reverse transcriptase inhibitors (NRTIs), which block transcription of viral RNA to DNA; non-nucleoside reverse transcriptase inhibitors (NNRTIs), which inhibit the reverse transcriptase enzyme; protease inhibitors (PI), which deactivate the viral protease enzyme; cell-surface co-receptor-5 (CCR5) inhibitors, which block entry of HIV into the host cell; integrase strand transfer inhibitors (InSTI), which block integration of the viral DNA into the host's genome; and fusion inhibitors (FIs), which inhibit fusion of HIV with the host cell at the first point of contact (Dau & Holodniy, 2008).

Table 2.2: The current highly active antiretroviral drugs used in southern Africa

Class of drug	Examples of drugs	Side effects
NRTIs	Zidovudine, stavudine, tenofovir, lamivudine, abacavir, and didanosine	Mitochondrial toxicity, lipodystrophy, anaemia, and bone marrow suppression
NNRTIs	Efavirenz, rilpivirine, nevirapine and delavirdine	
PIs	Indinavir, darunavir and atazanavir	Hyperlipidaemia, hyperglycaemia, hepatotoxicity, insulin resistance and lipodystrophy
FIs	Enfuvirtide	Generally well tolerated
INSTIs	Elvitegravir, dolutegravir and raltegravir	Generally well tolerated
CCR5 Antagonists	Maraviroc	Generally well tolerated

Abbreviations: NRTIs, nucleoside reverse transcriptase inhibitor; NNRTIs, non-nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors; FIs, INSTIs, integrase strand transfer inhibitors; CCR5, cell-surface co-receptor-5.

Evidence associating HAART to the development of CVDs points explicitly to PIs such as abacavir and didanosine (Bavinger, 2013). Observational studies report a direct effect of some

specific antiretroviral drugs to the increased risk of CVDs (Ballocca *et al.*, 2017). The PIs, indinavir and lopinavir, have been suspected to increase the risk of CVDs by remodelling the LDL-C particle size; however, these first-generation drugs have now been replaced with less aggressive medications (Worm *et al.*, 2010). Initially, abacavir was implicated of favouring the risk of MI in the HIV-infected population, nonetheless, this association was not confirmed in subsequent studies (Young *et al.*, 2015; Ballocca *et al.*, 2017). HAART inhibits viral replication at different stages of the HIV viral cycle as shown in Figure 2.6 (Gonzalo-Gil *et al.*, 2017)

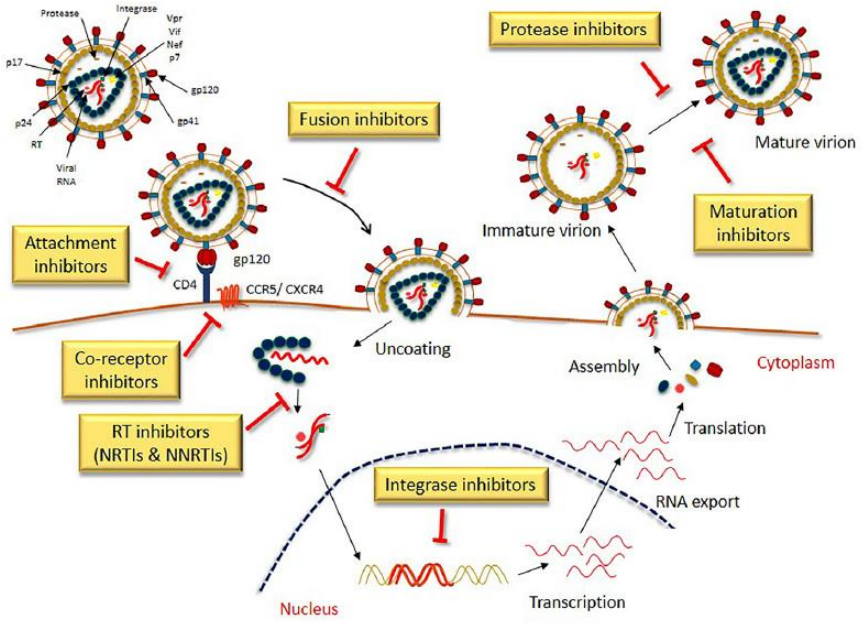


Figure 2.6: Different sites of inhibition on HIV replication cycle by HAART. Highly active antiretroviral therapy inhibits viral replication by inhibiting, attachment and fusion of the virus to the cell surface and the cell respectively, co-receptors, function of integrase, and function the viral protease. Adapted from Gonzalo-Gil et al., (2017)

2.6.3 Role of HIV infection in the development of CVD

Viral infections may play a crucial role in the pathophysiology of atherosclerosis (Vercellotti, 2001). Such viruses include cytomegalovirus (CMV), herpes simplex virus (HSV), influenza virus and hepatitis virus (Vercellotti, 2001; Shah, 2002). As an example, CMV infection has been shown to induce human vascular smooth muscle cell proliferation by inactivating a protein responsible for the accumulation of cholesterol esters in smooth muscle cells and generate a prothrombotic phenotype in human endothelial cells (Shah, 2002). This therefore provides biological plausibility to the causal correlation between proliferative atherothrombotic vascular diseases and viral infection (Shah, 2002).

Infection of individuals with HIV has thus been shown to promote endothelial dysfunction and atherosclerosis. HIV infection is associated with the increased production of pro-inflammatory cytokines such as TNF-α and IL-6, which in turn causes a build-up of plaques in the arteries through several mechanisms (see Figure 2.7) (Okeke *et al.*, 2017; Appay & Sauce, 2008). Immune mechanisms induced by HIV-infection may result in endothelial dysfunction which, in turn, triggers the expression of adhesion molecules and inflammatory and prothrombtic cytokines (Kearns *et al.,* 2017). This leads to the formation of atherosclerotic plaques which may rupture and result in thrombosis, myocardial infarction and stroke (Kearns *et al.,* 2017). Elevated serum levels of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), von Willebrand factor (vWF) and thrombomodulin have been reported in the HIV-infected population (Fourie *et al.*, 2011; Seigneur *et al.*, 1997; Calza *et al.*, 2009). These are proxy markers of endothelial dysfunction and suggest a role for the endothelial adhesion molecules in the aetiology of HIV-associated CVD risk. A South African study involving 20 HIV-infected

individuals with mean age of 51.1 $\pm$ 8.1 years, of which 65 % were males, reported that ICAM-1 and VCAM-1 were elevated compared to HIV-uninfected individuals (Vachiat *et al.*, 2020).

The virus has also been associated with pulmonary hypertension (Speich *et al.*, 1991). The prevalence of pulmonary hypertension in HIV-infected individuals is higher compared to HIV-uninfected individuals (Lambert *et al.*, 2016). The mechanism of vascular damage has been linked to the release of inflammatory cytokines induced by HIV-glycoprotein 120 (Pellicelli *et al.*, 2001). In addition, HIV-infected individuals with unregulated HIV infection are susceptible to increased pulmonary hypertension compared with HIV-infected individuals with regulated HIV infection (Kanmogne *et al.*, 2003).

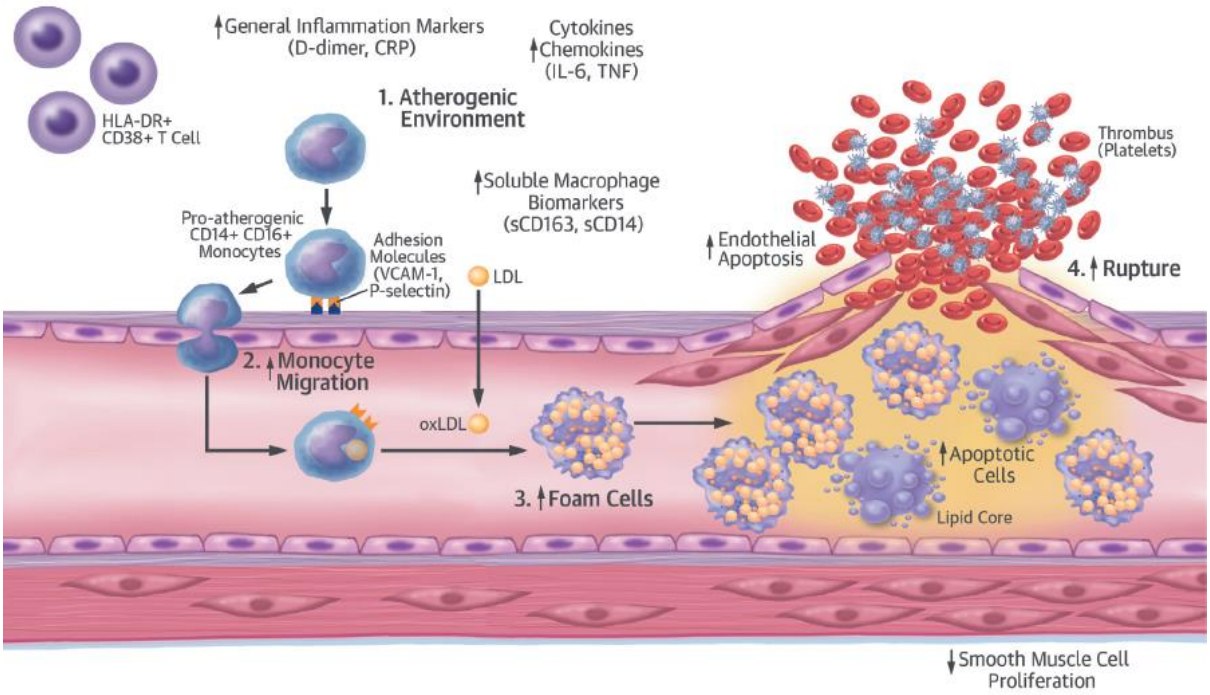


Figure 2.7: HIV-associated atherosclerosis. HIV-infection triggers expression of pro-inflammatory cytokines and cell adhesion molecules (1), which, in turn facilitates cellular transmigration of monocytes into the arterial intima (2). Monocytes differentiate into macrophages by absorbing ox-LDL to form foam cells and thus formation of the unstable lipid core which may rupture and result in thrombosis (3-4). Reproduced with permission from Kearns ***et al.***, (2017)

2.7 Thrombosis in the HIV-infected population

As stated previously, infection with HIV may result in endothelial injury (Wang *et al.*, 2007). Vascular stress and injury may lead to the disruption of the endothelial monolayer of blood vessels, thereby triggering thrombus formation, which is a well-modulated and complex cascade of events (Nording *et al.*, 2015). Thrombus formation within the vasculature leads to activation of the immune system and inflammation as part of its innate response (Rondina *et al.*, 2013). This response triggers several events which promote the healing process and protection against intruders (Nording *et al.*, 2015).

Thrombomodulin (TM) is a protein cofactor expressed on the surface of endothelial cells that re-orientates the substrate specificity of thrombin, evidently by an allosteric mechanism (Sadler, 1997). There is experimental data exhibiting TM as a crucial mediator of blood vessel wall-initiated inflammation and coagulation (Kleinegris $et\ al.$, 2012). However, the impact of TM on the development of atherosclerosis is a novel topic of investigation (Kleinegris $et\ al.$, 2012). In atherosclerotic lesions from humans and rabbits, TM can be localised to intimal macrophages and monocytes, vascular endothelial cells, and smooth muscle cells (Conway, 2012). It is downregulated in atherosclerotic coronary arteries resulting in further inflammation and thrombosis (Conway, 2012). This downregulation is mediated through the JNK and p38 pathways by free fatty acids which are known to increase cardiovascular risk (Rong, 2010). Elevated levels of soluble TM have been shown to lower the risk of coronary heart disease (Zhang $et\ al.$, 2014).

Several haemostatic abnormalities have been observed in the HIV-infected population that provide mechanisms for elevated risk of thrombosis and hypercoagulation (Shen & Frenkel, 2004). These mechanisms include decreased anticoagulant activities due to insufficient levels of antithrombin, protein C, protein S and heparin cofactor II; decreased fibrinolysis due to elevated levels of serpin E1; microangiopathic changes and endothelial-related changes that involve the presence of antiphospholipid-anticardiolipin antibodies (Saif & Greenberg, 2001). It has been documented that these alterations are largely the result of endothelial activation and disseminated intravascular coagulopathy (Shen & Frenkel, 2004).

The protein C anticoagulant pathway plays a crucial role in regulating thrombosis, decreasing cell apoptosis in response to ischemia and inflammatory cytokines, and inhibiting inflammatory responses (Esmon, 2003). The essential components of the pathway incorporate thrombomodulin, thrombin, protein C, protein S, and endothelial cell protein C receptor (EPCR) (Esmon, 2003). Protein C and protein S are significant inhibitors of physiologic coagulation (Erbe *et al.*, 2003). Although both proteins are produced by hepatocytes, protein S is additionally produced by megakaryocytes and endothelial cells (Erbe *et al.*, 2003). Approximately 40% of protein S circulates free and about 65% is bound to C4b-binding protein C (C4b-BP) (Vincenot & Gaussem, 1997).

Thrombomodulin binds to thrombin, inhibiting its clotting and cell activation ability while simultaneously augmenting protein C (Taylor *et al.*, 2001). The thrombin-thrombomodulin complex converts inactive protein C to activated protein C (APC) (Vincenot & Gaussem, 1997). The interaction of APC and protein S on the surface of phospholipids plays a crucial role in the inactivation of factor V and factor VIII by proteolysis (Erbe *et al.*, 2003). Increased levels of factor V and factor VIII are linked with elevated risk of thrombosis (Kamphuisen *et al.*, 2000). Thus, protein C and protein S deficiencies are associated with elevated risk of venous thromboembolism (Erbe *et al.*, 2003). Moreover, plasma protease inhibitors inactivate thrombin bound to thrombomodulin more than 20 times faster than free thrombin, leading to increased clearance of thrombin from blood circulation (Rezaie *et al.*, 1995). Blood coagulation is hindered when inhibited thrombin dissociates from thrombomodulin (Esmon, 2003). In addition, thrombomodulin minimizes the formation of cytokines in the endothelium and decreases leukocyte-endothelial cell adhesion (Esmon, 2003).

2.8 Association between mutations in the *nef* gene and disease outcome

The HIV genome exhibits a high degree of genetic diversity, partly due to mutational escape and its adaptation to both the host's immune response and HAART (Santoro & Perno, 2013). Although mutational escape is advantageous to the virus, some of the escape mutations can severely affect viral fitness (Santoro & Perno, 2013). The latter plays a key role in virus persistence, transmission, pathogenesis and disease progression (Song *et al*., 2012). Due to HIV-1 error prone reverse transcriptase and rapid virus turnover and immune selection pressure, a slight change in viral fitness may affect HIV-1 evolution significantly (Song *et al*., 2012). Strong pressure from cytotoxic T lymphocyte (CTL) responses leads to replacement of CTL sensitive viruses (Ganusov *et al*., 2011). Reduced viral fitness may result in decreased viral load, leading to long-term HIV-1 control and reduced probability of transmission to new hosts (Goepfert *et al*., 2008). Additionally, a better clinical outcome may be expected if less fit viruses are transmitted to new hosts even though the effect may not be sustained into chronic infection (Goepfert *et al*., 2008; Song *et al*., 2012).

HIV-1 Nef functionality *in vivo* exerts a significant effect on viral fitness and progression to AIDS (Biesinger & Kimata, 2008). A study involving 34 HIV-infected individuals with slow progression and rapid progression identified *nef* polymorphisms associated with disease stage (Walker *et al.,* 2007). This study outlined that deletions and insertions in the N-terminal variable region of *nef* were associated with slow progressing disease (Walker *et al.,* 2007). Sequences from rapid progressing disease had strong negative selection pressure across the gene, whereas those from slow progressing disease had strong positive selective pressures in sites that had known functional

importance (Walker *et al.,* 2007). Some escape mutations in Nef have been reported to decrease HLA-I downregulation activity, a Nef activity that allows evasion of CD8+ T lymphocyte response (Mann *et al.*, 2020). A cohort study involving 298 HIV-1 subtype C individuals has shown that HLA-associated mutations of Nef at residues 199Y, 108D, 105R and 102H were individually associated with HLA-I downregulation (Mann *et al.*, 2020). A study of plasma HIV-1 RNA-derived Nef clones isolated from 50 recently infected acute progressors who failed to suppress viral load to less than 2000 RNA copies/ml and 10 recently infected acute controllers who managed to suppress viral load to less than 2000 RNA copies/ml within a year reported that Nef clones from acute controllers showed a diminished ability to downregulate CD4 and HLA class I from the cell surface and decreased ability to enhance virion infectivity compared to the acute progressors (Kuang *et al.*, 2014).

Variation in the viral genome not only affects viral fitness but may also be associated with the development of co-morbid diseases. Thus, studies have shown that variants in the *nef* gene are associated with markers of endothelial function. In a study conducted by Mezoh *et al.* (2020) involving 77 HIV-infected HAART-naïve South African subjects compared with 61 HIV-uninfected healthy individuals showed evidence of endothelial dysfunction in the South African HIV-infected population. The HIV-positive cohort had higher levels of the markers of endothelial dysfunction, ICAM-1, VCAM-1, and vWF, and lower levels of HDL-C, compared to the HIV-negative cohort. Furthermore, five mutations in the HIV *nef* gene (V16I, H40Y, T50H,A, S169N, H188Q,S) were significantly associated with serum levels of ICAM-1 and one mutation (Y202F) was associated with VCAM-1 levels, suggesting a role of Nef in affecting endothelial function. A further study of 140 HIV-infected individuals on HAART has demonstrated that 5 HIV-1 *nef*

polymorphisms, K39R, H40Y, D177E, F143Y and V180T, were associated with high carotid intima-media thickness (CIMT).

2.9 Problem statement

The number of people living with HIV has dramatically increased since the emergence of the virus, with a perceived decrease in HIV-related deaths due to early diagnosis and introduction of HAART (Wada *et al.*, 2015). The new trend being observed is an increase in the incidence of non-communicable diseases in the HIV-infected population (Nanni *et al.,* 2015) of which sub-Saharan Africa accounts for up to 70 % (Nix & Tien, 2014; Shah *et al.*, 2018). The prevalence of cardiovascular disease is steadily on the rise, and is rapidly becoming the leading cause of death worldwide (Shah *et al.*, 2018). This therefore represents an overwhelming proportion of disease burden in sub-Saharan Africa, especially in subjects with HIV and CVD, which will dramatically impact individuals, households, and the economy at large.

A study conducted by Mezoh *et al.* (2020) involving HIV-infected South African subjects has demonstrated the association of HIV-1 *nef* polymorphisms with biomarkers of endothelial dysfunction and with high CIMT. We therefore hypothesize that HIV-1 *nef* polymorphisms accelerate the development of atherosclerotic disease in HIV-infected patients by triggering endothelial dysfunction, and that HIV-infected subjects with coronary artery disease (CAD) and those with peripheral arterial disease have a higher prevalence of specific HIV-1 *nef* polymorphisms. This study seeks to contribute to the knowledge pool that aims to elucidate the aetiology of HIV-associated CVD in the HIV-infected population.

2.10 Aim and objectives of the study

The general aim of the study was to determine whether HIV-1 subtype C *nef* polymorphisms in HIV-infected patients are associated with CAD and with biomarkers of endothelial dysfunction. To achieve this, the objectives of the project were as follows:

- Recruit HIV-infected and non-infected individuals with and without CAD.
- Quantify plasma levels of biomarkers of endothelial dysfunction i.e., ICAM-1, VCAM-1, vWF and thrombomodulin in all subjects using a Human Magnetic Luminex Screening Assay kit.
- Sequence the HIV-1 *nef* gene from the HIV-infected participants by Sanger sequencing.
- Study the association of *nef* variants with CAD and with levels of ICAM-1, VCAM-1, vWF and thrombomodulin.

CHAPTER THREE – METHODS AND MATERIALS

3.1 Study design and patient population

3.1.1 Ethical clearance

Ethical clearance for this study was obtained from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (M190714) (Appendix A). Participants were recruited from the Cardiology Unit and Vascular Unit of the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). Permission to recruit participants from the Cardiology Unit and Vascular Surgery Unit were granted by respective Departments' Academic Heads, Professor N Tsabedze and Professor ATO Carrim, respectively (Appendix B). Remnant blood samples of recruited participants were retrieved from the NHLS Chemical Pathology routine laboratory of the CMJAH. Additional blood samples for control groups were obtained from a previous PhD study conducted by Mezoh $et\ al.$ (2020). Participants were recruited and blood samples collected by Dr Alinda G. Vos and Dr Nereshni Lutchman. Dr Genevieve Mezoh was granted ethical clearance by the Medical Human Research Ethics Committee of the University of the Witwatersrand, under the clearance number M150979 (Appendix C), to carry out further analyses on these blood samples.

3.1.2 Recruitment of study participants

3.1.2.1 HIV-infected and HIV-uninfected participants with coronary artery disease

The HIV-infected participants were recruited alongside HIV-uninfected subjects from the Cardiology Unit of the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), with both groups having confirmed cases of atherosclerotic cardiac events. These subjects had angiogram-confirmed coronary artery disease (CAD). Patients who were scheduled for an angiogram were

invited to participate in the study before they left the hospital wards and within 1 to 3 days after they had an angiogram. All patients scheduled for an angiogram were offered HIV pre-counseling prior to testing for HIV using a rapid testing kit (ADVANCED QUALITY™ Rapid HIV Test [InTec Products, China]) and were offered counseling before the test. Positive results were confirmed with a second rapid test kit (ABON™ HIV 1/2/O Tri-Line HIV Rapid Test Device [ABON Biopharm Hangzhou, China]). Counseling was offered to patients who tested positive with both test kits, of which confirmed positive results were then validated by ELISA within the NHLS Chemical Pathology routine laboratory of the CMJAH. Regardless of their HIV status, these patients were interviewed and CAD and HIV status of those who gave consent to take part in the study were then confirmed from patients' files. This was followed by measurements of anthropometric data and collection of remnant samples (EDTA plasma and serum tubes) from the NHLS Chemical Pathology routine laboratory of the CMJAH. Participants were recruited for a period of 24 months and patient's informed consent was obtained from all participants (Appendices D, E and F). Only pregnant females were excluded from the study.

A total of 30 subjects with confirmed HIV-positive status and CAD were recruited. All 30 subjects had remnant blood samples in EDTA plasma tubes and a total of 26 subjects had remnant serum samples in serum-separating tubes. Therefore, all 30 subjects were included in the HIV-positive/CVD-positive (HIV+/CVD+) group of this study.

A total of 115 HIV-uninfected subjects with coronary artery disease were recruited of which all 115 subjects had remnant serum in serum-separating tubes and a total of only 30 subjects had additional EDTA plasma tubes. A total of 65 subjects were selected and included in the HIV-

negative/CVD-positive (HIV –/CVD+) group of this study based on availability of anthropometric data and subjects' lipid profile. Anthropometric data for subjects that were not selected for this study was missing because of the inability of these patients to stand up and allow measurement of these variables due to their critical condition.

3.1.2.2 HIV-infected participants with peripheral artery disease and coronary artery disease

HIV-infected participants presenting with peripheral artery disease (PAD) were recruited from the Vascular Surgery Unit of the CMJAH. These subjects had angiogram confirmed PAD. These patients were identified by vascular surgeons and were referred to us and were invited and interviewed for participating in the study. The HIV status of these patients were confirmed as described in section 3.1.2.1. A total of 10 subjects with confirmed PAD were recruited of which 3 were previously diagnosed with CAD based on data obtained from patients' files. Seven (7) of these participants were excluded from the study due to absence of data on CAD history and the remaining 3 were added to the HIV+/CVD+ group. Participants were recruited over a period of 12 months. Patient's informed consent was obtained from all participants and only pregnant females were excluded due to low number of HIV-infected patients who visit the Vascular Surgery Unit of CMJAH.

3.1.2.3 Recruitment of HIV-infected and HIV-uninfected participants without history of cardiovascular disease

Control groups of HIV-infected subjects (60) and HIV-uninfected subjects (60) without CAD and PAD were obtained from the Ndlovu study (Vos *et al.*, 2017) and a previous PhD conducted by

Mezoh *et al*. (2020), respectively. The HIV-infected subjects were recruited from the Wits Reproductive Health and HIV Institute (WHRI) in Johannesburg whereas HIV-uninfected subjects were recruited from the Zazi Clinic, Chris Hani Baragwanath Hospital. The HIV-infected participants recruited for the Ndlovu study consisted of a mixture of ART-naïve individuals attending the WRHI for follow-up visits (4, 12, 24 or 36 weeks), individuals who tested HIV-infected at time of recruitment, individuals receiving first-line tenofovir-containing ART regimen (Tenofovir/Lamivudine/Efavirenz) for at least 2.5 years, and individuals receiving second-line ART treatment (Darunavir/Ritonavir and Lopinavir/Ritonavir) for at least six months. Only individuals with an unknown HIV status were excluded from the study. The HIV status of both HIV-infected and HIV-uninfected participants recruited were confirmed as described in section 3.1.2.1. Moreover, exclusion criteria for recruiting these participants was designed to exclude subjects who may have clinical conditions known to influence endothelial function, or potentially received drugs that influence their lipid profile, or glucose and blood pressure levels. The exclusion criteria were therefore as follows:

- Current AIDS-defining illness or opportunistic infection or neoplasm
- Personal history of coronary artery disease, peripheral artery disease, diabetes mellitus, cerebrovascular accident
- Patient drug history e.g., statin or anti-hypertensive therapy
- Active drug abuse
- Pregnant females

The HIV-infected and HIV-uninfected subjects without a history of CVD were divided into the following groups: HIV-positive/CVD-negative (HIV+/CVD–) and HIV-negative/CVD-negative

(HIV−/CVD−), respectively. See Figure 3.1 for an overview of the selection process used for recruiting subjects into this study.

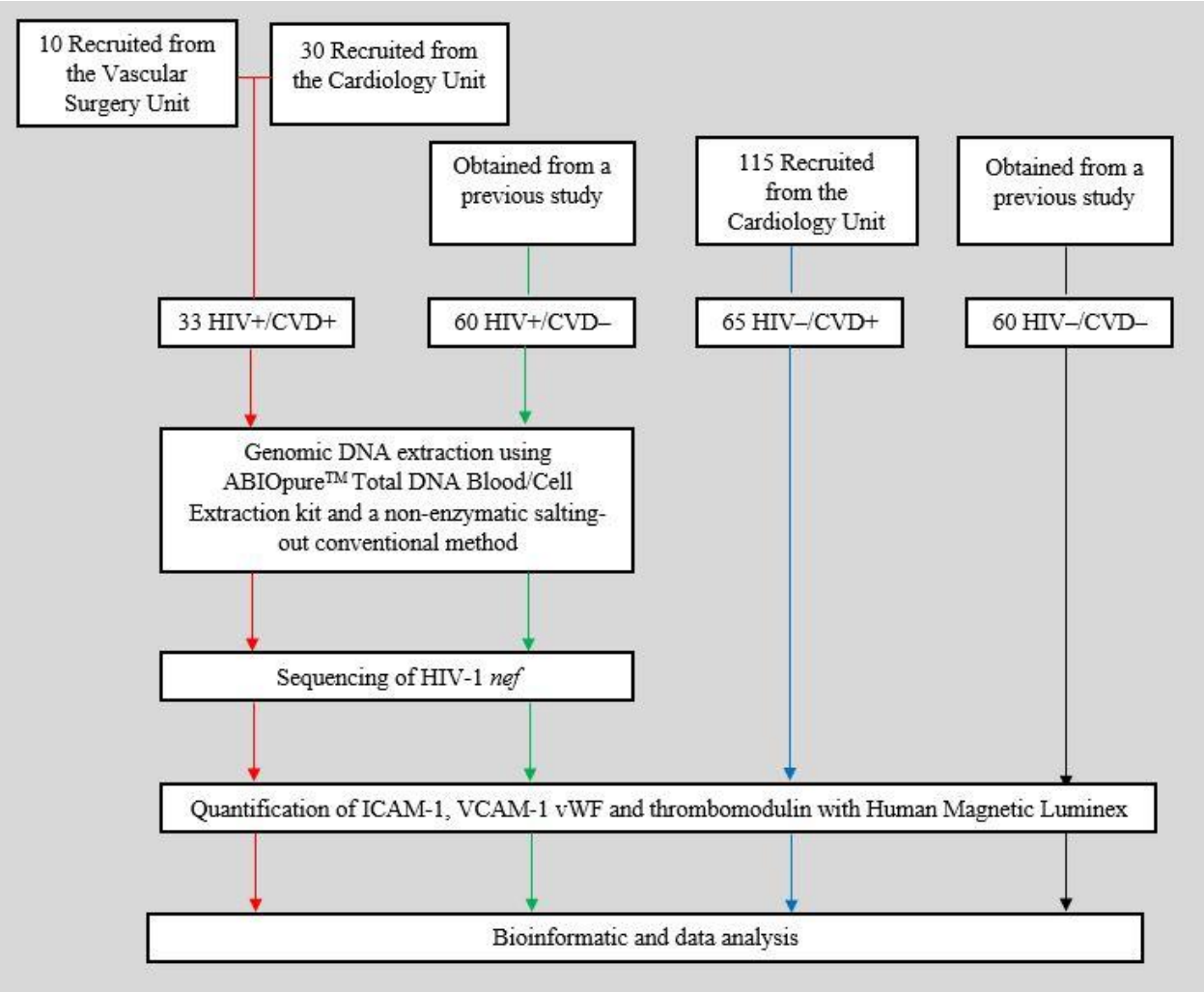


Figure 3.1: Study design. HIV+/CVD+, HIV−/CVD+, HIV+/CVD− and HIV−/CVD− represents HIV-positive/CVD-positive, HIV-negative/CVD-positive, HIV-positive/CVD-negative, and HIV-negative/CVD-negative subjects groups, respectively**.**

3.2 Data and sample collection

Fasting morning blood samples were collected from all participants into EDTA plasma and serum tubes, 5ml per tube. The serum and plasma samples were divided into 500 µL aliquots and stored at -80^{o}C until further analysis. Measurements of high-density lipoprotein-cholesterol (HDL-C), total cholesterol, low-density lipoprotein-cholesterol (LDL-C), and triglycerides (TGs) were quantified using the Siemens ADVIA 1800 Chemistry Immunoassay System within the NHLS Chemical Pathology routine laboratory of the CMJAH.

Total cholesterol is quantified enzymatically in serum in a series of reactions that hydrolyze cholesteryl esters and oxidize the 3-hydroxyl (3-OH) group. Oxidation of the latter produces a byproduct of hydrogen peroxide (H_2O_2) which is measured quantitively in a reaction catalyzed by peroxidase. This reaction produces colour that has intensity proportional to cholesterol concentration. The absorbance of this is measured at 500 nm (Li *et al.*, 2011; Shih *et al.*, 2000).

The TGs are also quantified enzymatically in serum following a series of coupled reactions in which TGs are hydrolyzed to produce glycerol. The latter is then oxidized by glycerol oxidase to produce a byproduct of H_2O_2 which is quantified as mentioned above at 500 nm (Li *et al.*, 2011).

The HDL-C is quantified in serum by reacting the apoB containing lipoproteins in the specimen with a blocking reagent (sulfated alpha-cyclodextrin in the presence of Mg^{2+}) that renders them non-reactive. This allows apoB containing lipoproteins to be effectively excluded from the assay allowing only HDL-C to be detected. HDL-cholesteryl esters are hydrolyzed by cholesterol

esterase to produce unesterified cholesterol which is then oxidized to produce a byproduct H_2O_2. The latter is then quantified as mentioned above at 600 nm (Bachorik *et al.*, 1994; Li *et al.*, 2011).

Circulating major lipoproteins are mostly found in three major lipoproteins fractions, LDL-C, HDL-C, and very-low density lipoprotein-cholesterol (VLDL-C) and thus concentration of total cholesterol is given by the equation: [total cholesterol] = [LDL-C] + [HDL-C] + [VLDL-C]. The LDL-C concentration is therefore calculated from measured concentrations of total cholesterol, TGs, and HDL-C according to the following equation: [LDL-C] = [total cholesterol] - [HDL-C] - [TGs]/5, where [TGs]/5 is an estimated value of VLDL-C, and all values are given in mg/dl (Friedewald *et al.*, 1972 ;Li *et al.*, 2011).

The coefficient of variations (CVs) of enzymatic quantification of total cholesterol, TGs, and HDL-C were 1.5%, 1.8 and 3.5% respectively. Moreover, total cholesterol, TGs, and HDL-C had measuring ranges of 0.1 – 20.7 mmol/l, 0.1– 10.0 mmol/l and 0.08– 3.12 mmol/l, respectively.

Data in relation to patients' family and personal history of coronary artery disease, hypertension, diabetes mellitus, cerebrovascular events, drug and alcohol history, and recent infections or recently diagnosed malignancies were obtained from the patient's file and by talking to the subjects and collecting this data using a data collection sheet (Appendix G). Anthropometric data such as weight, height, hip and waist circumference were measured at the time of recruitment as described by Crowther & Norris (2012). Briefly, height and weight were measured by using a wall-mounted stadiometer and an electronic scale, respectively. Subjects were asked to remove their shoes and heavy clothing prior to taking these measurements. Subjects' height and weight were then used to

compute each subject's body mass index (BMI). A soft measuring tape was used to measure subjects' hip and waist circumference by placing a tape around patients' widest part of the gluteal region and around the waist above the umbilicus in standing position, respectively.

3.3 HIV-1 Nef sequencing

3.3.1 Genomic DNA extraction

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using either the ABIOpure^{TM} Total DNA Extraction kit or a non-enzymatic salting-out conventional method as described by Suguna $et\ al.$, (2014). The extraction kit was used according to the manufacturer's protocol (AllianceBio, Bothell, WA, USA). Briefly, 20 µl of Proteinase K solution was pipetted into a 1.5 ml tube following which 200 µl of blood sample was added and the mixture vortexed. Subsequently, 200 µl of Buffer BL was added to the tube and the mixture thoroughly vortexed and incubated at 56°C for 10 minutes. The tube was then briefly spun to remove any drops from inside the lid followed by addition of 200 µl of absolute ethanol. Subsequently, the mixture was carefully transferred to a mini column and centrifuged for 1 minute at 6500 × g. Thereafter, 600 µl of Buffer BW was added to the column and centrifuged for 1 minute at 6500 × g. Subsequently, 700 µl of Buffer TW was applied to the mini column and centrifuged for 1 minute at 6500 × g. The flow-through was discarded and the column centrifuged at full speed for 1 minute to remove residual wash buffer. The mini column was then placed in a clean 1.5 ml tube following which 50 µl of RNAse-free water was added to the center of the column and the tube incubated for 1 minute at room temperature. This was then centrifuged at full speed for 1 minute and the extractions stored at –20^{o}C until downstream processing.

The method for DNA extraction according to Suguna *et al*., (2014), was as follows: firstly, 300 µl of blood sample was added into a 2 ml tube and mixed with 900 µl of TKM-1 buffer (10 mM Tris–HCl pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, 2 mM EDTA) followed by the addition of 50 µl of 1X Triton-X. The mixture was then incubated for 3 minutes at 37°C followed by centrifugation at 7200 × g for 3 minutes. The supernatant was discarded, and the incubation and centrifugation steps were repeated four times with decreasing volume of 1X Triton-X. Subsequently, 300 µl of TKM-2 buffer (10 mM Tris–HCl pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, 2 mM EDTA, 0.4 M NaCl) and 40 µl of 10% SDS were added to the pellet and mixed thoroughly by vortexing, followed by incubation for 5 minutes at 37°C. Thereafter, 100 µl of 6 M NaCl was added and the mixture was vortexed to precipitate the proteins. The mixture was then centrifuged at 7200 × g for 5 minutes and the supernatant transferred into a clean 2 ml tube followed by addition of 300 µl of isopropanol. The tube was then inverted slowly to mix and centrifuged at 7200 × g for 10 minutes. The supernatant was discarded and 100 µl of 70% ethanol added to remove any excess salts, followed by centrifugation at 7200 × g for 5 minutes. The supernatant was then discarded, and the pellet air-dried followed by addition of 50 µl of RNAse-free water to dissolve the DNA. The concentration of extracted DNA was determined by using the Nanodrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

3.3.2 Nested polymerase chain reactions

The HIV-1 subtype C *nef* from HIV+/CVD+ and HIV+/CVD– cohorts was amplified by nested polymerase chain reactions (PCRs), as illustrated in Figure 3.2, using high-fidelity Platinum *Taq* polymerase (Invitrogen, CA, U.S.A) according to the manufacturer's protocol. This technique was used to increase sensitivity by re-amplifying targeted DNA amplified with the first PCR reaction.

The HIV-1 *nef* primers were designed using the Primer Design-M tool of the Los Alamos HIV database (Table 3.1). The master mix was prepared by adding the components in Table 3.2. Primer set 2 (nested PCR outer primers), and set 1 (nested PCR inner primers) were used in the first and second run of nested PCR reactions, respectively. Samples with unsuccessful amplification of the target of interest were subjected to another nested PCR reaction with different sets of outer and inner primers (Primer set 4 and 3, respectively). Cycling conditions for the thermal cycler was set according to the programme in Table 3.3. In each PCR tube, 19 µl of master mix was added followed by addition of 1 µl of sample (>50 ng/µl). The tubes were then placed in a thermal cycler and the program started.

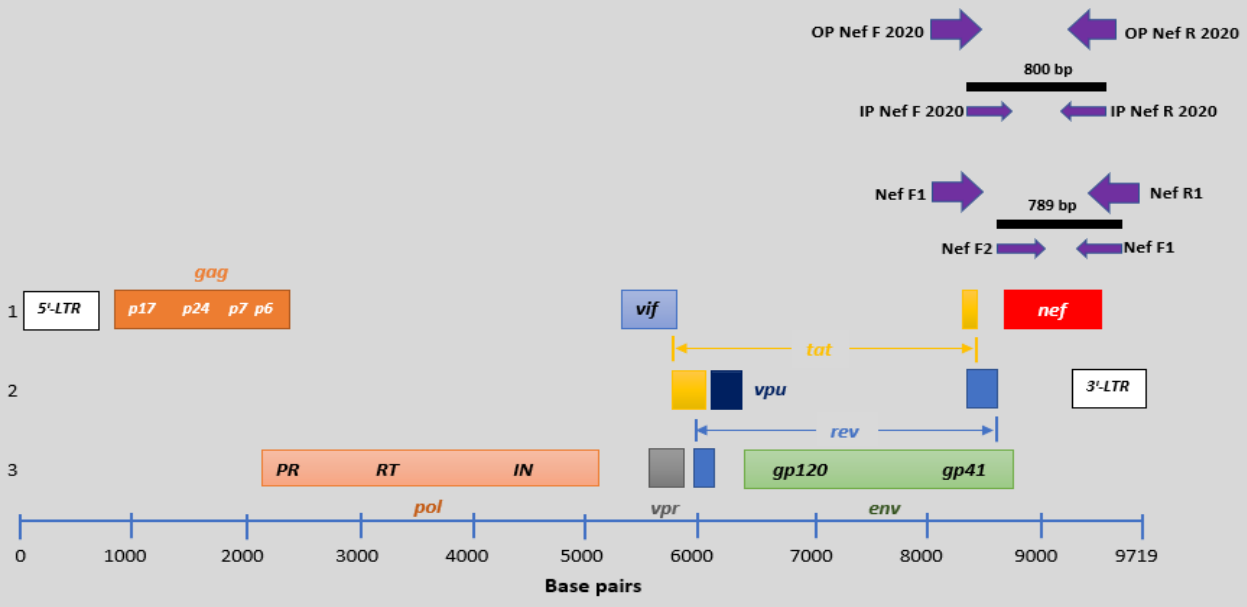


Figure 3.2: Schematic diagram showing where HIV-1 *nef* primers bind on HIV-1 genome.

Table 3.1: HIV-1 *nef* nested PCR primers

Set	Type	Primer	Sequence	Product size
1	Inner	IP Nef F 2020	5'TARYARYAGCTGARGGAACAGATAG3'	800 bp
	Inner	IP Nef R 2020	5'GAACGCCCCRGWGGAAAGTC3'	
2	Outer	OP Nef F 2020	5'AGAGTYAGGCAGGGATACTCAC3'	1224 bp
	Outer	OP Nef R 2020	5'CWGTACARGCRMAAAGCWGC3'	
3	Inner	Nef F2	5'AAGAATAAGACARGGCTTYG3'	789 bp
	Inner	Nef R2	5'CAGCTGCTTATATGCAGCATC3'	
4	Outer	Nef F1	5'CAGTAGCTGARGGRACAGATAGG3'	957 bp
	Outer	Nef R1	5'CTTTATTGAGGCTTAAGCAGTGGGTTC3'	

Table 3.2: Components of master mix

Component	Volume (µl)
10X High Fidelity PCR Buffer	12.4
$MgSO_4$	4.00
10 mM dNTP mix	0.40
Forward primer	0.20
Reverse primer	0.20
Platinum® Taq DNA High Fidelity Polymerase (5 U/µl)	1.00
Autoclaved, distilled water	15.3

Table 3.3: Nested PCR thermal cycler program conditions for amplification of HIV-1 *nef*

Steps		Temperature (°C)	Time
Initial denaturation		94.0	2 minutes
35 PCR Cycles	Denature	94.0	15 seconds
	Anneal	55.0	30 seconds
	Extend	68.0	4 minutes
Final annealing		68.0	15 minutes
Hold		4.00	∞

3.3.3 Agarose gel electrophoresis

The DNA was separated by gel electrophoresis using 1.0 % agarose gel according to Tang *et al* (2007). This technique was used to confirm successful amplification and size of PCR products. Briefly, 0.5 g of agarose powder was mixed with 50 ml of Tris Borate EDTA (TBE) buffer and boiled to dissolve. The solution was then cooled down to 60^{o}C at room temperature followed by addition of 2.5 µl Gel-Red. The gel solution was then poured onto agarose gel plates and left to solidify. Subsequently, 5 µl of each sample was mixed with 1 µl of 6 X loading dye and the mixture added into the wells of the gel. The gel was then allowed to run at 100 V for 40 minutes.

3.3.4 Purification of HIV-1 subtype C *nef* amplicons

The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. This technique is based on binding of DNA to a silica membrane in a high-salt buffer and elution with a low-salt buffer. Briefly, 75 µl of Buffer PB was added to 15 µl of the PCR reaction and mixed by shaking gently. The mixture was then transferred into a QIAquick column and placed in a 2 ml collection tube followed by centrifugation for 1 minute at 18894 × g. This allows amplified DNA to bind to a silica membrane in a high-salt condition provided by Buffer PB and centrifugation allows all impurities such as salt, primers, and nucleotides to be washed away. Subsequently, 750 µl of Buffer PE was added to the QIAquick column and centrifuged for 1 minute at 13000 rpm. This permits removal of excess impurities from the membrane. The QIAquick was then centrifuged further for 1 minute at 18894 × g to remove any residual buffer. Thereafter, the QIAquick column was placed in a clean 1.5 ml microcentrifuge tube and 25 µl of RNAse-free water added to the centre of the QIAquick column. The tube was incubated for 1 minute at room temperature and centrifuged for 1 minute at 18894 ×

g to elute the DNA. The low-salt condition provided by RNAse-free water allows DNA to detach from the silica membrane for elution. The purified amplicons were then stored at –80^{o}C until further analysis.

3.3.5 Cycle sequencing

3.3.5.1 Cycle sequencing of HIV-1 subtype C *nef* gene

Purified PCR amplicons were sequenced using the ABI PRISM Big Dye Sequencing Ready Reaction kit (Applied Biosystems, CA, U.S.A) according to the manufacturer's protocol with HIV-1 subtype C *nef* sequencing primers as illustrated in Figure 3.3. Cycle sequencing involves cycles of denaturation of the template PCR product, followed by annealing of the sequencing primer and extension of the primer with random incorporation of dideoxynucleotide triphosphate (ddNTPs) by DNA polymerase. Briefly, the master mix was prepared by mixing the components shown in Table 3.4. Subsequently, 2.8 µl of the master mix was added into each well of a 96-well plate, followed by addition of 3.2 µl of 20 µM forward or reverse sequencing primers (Table 3.5) and 4 µl of template DNA PCR product. The DNA PCR products were blindly used for sequencing reactions without quantification of each product's concentration. Sequencing primers were designed using the Primer Design-M tool of the Los Alamos HIV database (Los Alamos National Laboratory, 2019). Cycling conditions for the thermal cycler was set according to the manufacturer's instruction (Table 3.6). The tubes were subsequently placed in a thermal cycler and the program started.

Table 3.4: Components of reaction mixtures

Component	Volume (µl)
BDX 64 Buffer	0.875
Big Dye Sequencing Reaction Mix ver. 3.1	0.125
5X Sequencing Buffer	1.5
RNAse free water	0.3

Table 3.5: HIV-1 *nef* sequencing primers

Primer	Sequence
IP Nef F 2020	5'TARYARYAGCTGARGGAACAGATAG3'
IP Nef R 2020	5'GAACGCCCCRGWGGAAAGTC3'
NefIF	5'CCTAGAAGAATAAGACAGGGC3'
NefIR	5'CTTATATGCAGCATCTGAGG-3'

Table 3.6: Cycle sequencing thermal cycler program conditions

Steps		Temperature (ºC)	Time
25 cycles	Denature	96	30 seconds
	Anneal	50	15 seconds
	Extend	60	4 minutes
Hold		4	∞



Figure 3.3: Illustration of binding of sequencing primers. Binding of IP Nef F 2020 (light blue) and IP Nef R 2020 (pink) in HCV0003 raw *nef* sequence alignment.

3.3.5.1 Purification of sequencing reactions with sodium acetate

Following cycle sequencing, the sequencing reaction mixtures were cleaned up using the ethanol/sodium acetate method. Briefly, 50 µl of ethanol/sodium acetate solution was added to 10 µl of the sequencing reaction mixture. This allows the DNA to be neutralised by a mono-cation (Na^+) from sodium acetate and be precipitated by ethanol. The 96-well plate was then centrifuged at 2000 × *g* for 30 minutes. Subsequently, the plate was inverted onto a paper towel to discard the ethanol/sodium acetate solution and centrifuged, inverted on a paper towel, for 1 minute at 150 × *g*. The pellets were rinsed with 100 µl of 70 % cold ethanol by carefully pipetting up and down. Thereafter, the plate was centrifuged at 2000 × *g* for 30 minutes, followed by inversion onto a paper towel to discard the 70 % ethanol. The plate was then centrifuged inverted on a paper towel for 1 minute at 150 × g. The pellet in each well was dried by placing the opened plate in a thermocycler for 5 minutes at 45°C.

3.3.5.2 Sanger sequencing of HIV-1 subtype C *nef*

Sanger sequencing of HIV-1 subtype C *nef* was performed with the automated Genetic Analyser 3100 (ABI prism Model 3100, Perkin Elmer, Norwalk, CT) at the National Institute for Communicable Diseases (NICD). This genetic analyser can separate, detect and analyse a maximum of 16 capillaries (long and thin acrylic-fibre) of fluorescently labelled DNA fragments in a single run. This allows ddNTPs used for sequencing reactions in section 3.3.5.1 to be detected and to generate the DNA sequence of the fragment of interest. The ddNTPs are fluorescently labelled nucleotides with distinctive dye which allows detection of four different ddNTPs (ddATP, ddGTP, ddCTP and ddTTP).

3.4 Measurements of biomarkers of endothelial function

3.4.1 Human Magnetic Luminex Screening Assay

Plasma levels of ICAM-1, VCAM-1, vWF and thrombomodulin in all cohorts were measured using a customised Human Magnetic Luminex Screening Assay kit (R&D Systems, Minneapolis, U.S.A) on the Bio-Plex® Multiplex System with Luminex xMAP Technology, according to the manufacturer's instruction. This assay is based on simultaneous detection and quantification of multiple analytes in one sample. The assay uses superparamagnetic beads or microspheres laminated with analyte-specific capture antibodies. On that account, human ICAM-1, VCAM-1, vWF, and thrombomodulin were multiplexed using the online R&D Systems Luminex Assay Customization Tool (https://www.rndsystems.com/luminex/analytes). Briefly, plasma sample were centrifuged at 16 000 × *g* for 4 minutes and 75 µl of the sample diluted (2-fold) with 75 µl of the Calibrator Diluent RD6-52. Standard Cocktails (10X) were reconstituted with Calibrator Diluent RD6-52 and gently agitated for 15 minutes, followed by preparation of 1X Standard

Cocktails. Preparation of working standards was done by diluting the 1X Standard according to Table 3.7. Fifty (50) µl of standard or sample was added into each well of a microplate, followed by 50 µl of diluted Microparticle Cocktail (prepared by adding 5 ml of Diluent RD2-1 into 500 µl of Microparticle Cocktail). The microplate was incubated for 2 hours at room temperature (RT) on a shaker. This allows capture antibodies to bind to the analytes of interest. The microplate was then washed thrice with wash buffer to remove unbound material. Diluted Biotin-Antibody Cocktail (prepared by adding 5 ml of Diluent RD2-1 into 500 µl of Biotin-Antibody Cocktail) was then added into each well and incubated for 1 hour at RT with shaking to allow analyte-specific detector antibodies to bind to the analytes of interest. The washing step was then repeated, and diluted Streptavidin-PE (prepared by adding 5.35 ml of Wash Buffer into 220 µl of Streptavidin-PE) was added into each well and incubated for 30 minutes at RT on the shaker. Streptavidin-PE is a fluorescent protein that will bind to detector antibodies thereby enabling analytes to be detected with its capability to emit light. The washing process was then repeated, followed by re-suspension of microparticles with 100 µl of wash buffer by shaking at 800 rpm for 2 minutes. The plate was then read using the Bio-Plex® Multiplex System with Luminex xMAP Technology (Bio-Rad, Hercules, CA, U.S.A) and Luminex Xponent Software v3.1 where beads were excited using one laser to determine bead regions and assigned analytes, and another laser to determine the magnitude of the PE-derived signal. The latter is proportional to the quantity of the analyte bound.

Table 3.7: Dilutions of working Standard solutions

Standard	Volume of Standard	Volume of Calibrator Diluent RD6-52
Standard 1	100 µl of 1X Standard Cocktail 1	-
Standard 2	100 µl of Standard 1	200 µl
Standard 3	100 µl of Standard 2	200 µl
Standard 4	100 µl of Standard 3	200 µl
Standard 5	100 µl of Standard 4	200 µl
Standard 6	100 µl of Standard 5	200 µl
Blank	-	200 µl

3.5 Data analysis

3.5.1 Bioinformatic analysis of sequencing data

Sequencing data was analysed using BioEdit (ver. 5.0.9). The HIV-1 n*ef* sequences from the HIV-infected groups were aligned using published HIV-1 subtype C consensus *nef* sequences and HXB2 *nef* obtained from the Los Alamos HIV Sequence Database. Nucleotide and amino acid sequences of HIV-1 subtype C *nef* were analysed using tools available on the Los Alamos HIV Sequence Database. The frequency of HIV-1 *nef* variants in the HIV+/CVD+ group was compared with those in the HIV+/CVD– group using the VESPA software (http://www.hiv.lanl.gov/content/sequence/VESPA/vespa.html). The VESPA MEME suite evaluates the frequency of each amino acid at each location in an alignment for the query and reference sets, then picks the positions where the query set's most common character differs from the backdrop set. Character frequencies at differentiating positions are also calculated (Korber & Myers, 1992). The variants identified to be more prevalent in the HIV+/CVD+ group compared to the HIV+/CVD– group were then tested for significant differences using a two-tailed Fisher's exact test. Variants that were statically significantly different between two HIV-infected groups

were individually included in multivariable logistic regression models in which the dependent (output) variable was confirmed CAD to which possible confounding variables were then added. The number of confounding variables added to the models was kept as low as possible due to a small sample size (n = 63), which may result in overfitting. The chosen variables were age, gender, waist-to-hip ratio and BMI. Lipids were not included as it was theorised that they would be unlikely to act as confounders. Both BMI and WHR could be included in the same model because there was little collinearity between these variables as shown by a variance inflation factor of < 5.0.

3.5.2 Statistical analysis

Continuous variables were represented as median [interquartile range] or mean with SD, for data that was non-Gaussian or Gaussian, respectively. Skewness of data was used to evaluate the distribution and data that were non-Gaussian were transformed to a normal distribution by either logging and/or square rooting the data. These variables were compared between the groups using a one-way ANOVA and a Tukey post-hoc test. Allele, genotype, and amino acid variants between HIV-infected groups were analysed using a χ^2 test. Associations between amino acid sequence variation and plasma levels of biomarkers of endothelial dysfunction was analysed using either ANOVA or a Student's unpaired t test. P values less than 0.05 were regarded as significant. Multivariable logistic regression analysis was used to investigate the association of Nef amino acid sequence variation with CVD. Unadjusted models included presence/absence of CVD as the dependent variable with each individual variant, or the variants combined, as the independent variable. Possible confounding variables i.e. age, gender, BMI and waist-to-hip ratio were then added to the model. Finally, plasma levels of thrombomodulin, vWF and ICAM-1 were included

in the model to determine whether any of the associations of the Nef amino acid sequence variants with CVD were modified by these endothelial biomarkers.

Sample size calculations were not undertaken for the *nef* gene association studies because we had no prior knowledge of what *nef* polymorphisms would associate with the phenotype data. The *nef* gene is very polymorphic. With regards to the comparison of markers of endothelial dysfunction between patient groups, a previous study by Francisci *et al* (2009) demonstrated significant differences in these biomarkers between subject groups comprising of 28 individuals. We therefore attempted to ensure that each of our subject groups was greater than 28. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, Inc.) and Statistica version 14.0.0.15 (TIBCO Software, Inc).

CHAPTER FOUR – RESULTS

4.1 Comparison of demographic, anthropometric, cardiovascular risk factors and cardiometabolic variables between study groups

The mean age of participants in the HIV+/CVD– and HIV–/CVD– groups were significantly lower than that of HIV+/CVD+ group. In addition, participants in HIV–/CVD+ had significantly higher mean age in comparison to other study groups. The proportion of male participants in the HIV+/CVD+ was significantly higher than in the HIV+/CVD– and HIV–/CVD– groups, and significantly lower than those in the HIV–/CVD+ group. There was no significant difference in the distribution of black subjects across the study groups, except for HIV–/CVD+ group which was composed of a significantly lower proportion of black subjects in comparison to other study groups (Table 4.1).

Body mass index (BMI) in the HIV+/CVD+ group was significantly lower than that of the HIV+/CVD– and HIV–/CVD+ groups. The HIV–/CVD– group had significantly lower waist circumference than HIV+/CVD+ and HIV–/CVD– groups, while HIV+/CVD– had significantly lower waist circumference than HIV–/CVD+ group. Hip circumference of HIV+/CVD+ group was significantly lower than that of HIV+/CVD– and HIV–/CVD groups. A significantly higher waist-to-hip ratio was observed in HIV+/CVD+ compared to the HIV+/CVD– and HIV–/CVD– groups, as well as in HIV–/CVD+ versus HIV+/CVD– and HIV–/CVD– groups.

Systolic blood pressure in the HIV–/CVD– was significantly higher than that of HIV+/CVD+ and HIV–/CVD+ groups. There was no significant difference regarding diastolic blood pressure across the study groups. Data on, drinking of alcohol, smoking, CVD family history and diabetes family history was not available for the HIV+/CVD– and HIV–/CVD– groups. Significant differences

were observed for CVD family history and smoking which were both higher in the HIV–/CVD+ than the HIV+/CVD+ group.

The HIV–/CVD– group had significantly lower levels of triglycerides in comparison with the other study groups. Total cholesterol levels in the HIV+/CVD– group were significantly higher than in the HIV+/CVD+ and HIV–/CVD– groups. Levels of HDL-C in the HIV–/CVD– group were significantly higher than the other study groups whilst HDL-C levels in the HIV+/CVD– group were significantly higher than the HIV+/CVD+ and HIV–/CVD+ groups. Plasma levels of LDL-C in the HIV–/CVD– group were significantly lower in comparison to all the other study groups. In addition, LDL-C levels in the HIV+/CVD– group were significantly higher than in the HIV+/CVD+ group. The CD4 count of the HIV+/CVD+ group was significantly lower than that of the HIV+/CVD– group. The viral load of the HIV-infected groups was not assessed due to the high usage of HAART in these participants.

Table 4.1: Participant demographics, anthropometrics, risk factors and cardiometabolic variables

	HIV+/ CVD+	HIV+/CVD–	HIV–/CVD+	HIV–/CVD–
	n = 33	*n* = 60	*n* = 65	*n* = 60
Demographic factors				
Age (years)	46.63 ± 10.91	39.20 ± 4.58***	53.17 ± 9.36**†††	36.48 ± 4.80***$^{\#\#\#}$
Ethnicity (black), *n* (%)	31 (93.94)	60 (100)	34 (52.31) *** †††	60 (100) $^{\#\#\#}$
Male, *n* (%)	18 (54.55)	15 (25) **	51 (78.46) **†††	25 (41.67) **$^{\#\#\#}$
Anthropometric data				
BMI[a]	23.97 ± 11.79	27.38 ± 6.21**	27.33 ± 6.10**	26.02 ± 5.27
Waist circumference[a] (cm)	95.36 ± 16.46	94.13 ± 12.52	104.65 ± 15.69†††	88.20 ± 12.20*$^{\#\#\#}$
Hip circumference[a] (cm)	97.00 ± 18.64	107.82 ± 12.35**	103.60 ± 14.83*	100.57 ± 11.43
Waist-to-hip ratio[a]	1.00 ± 0.04	0.87 ± 0.06***	1.01 ± 0.06†††	0.88 ± 0.08***$^{\#\#\#}$
CVD risk factors, *n* (%)				
On HAART	31 (93.94)	60 (100)	-	-
Diabetes mellitus	10 (30.30)	0 (0)	23 (35.38)	0 (0)
CVD family history	6 (18.18)	-	25 (38.46)*	-
Diabetes family history	5 (15.15)	-	20 (30.77)	-
Systolic BP[b] (mmHg)	118.97 ± 19.60	124.26 ± 18.59	121.11 ± 19.93	130.22 ± 16.88*$^{\#}$
Diastolic BP[b] (mmHg)	78.28 ± 17.47	79.83 ± 11.48	75.98 ± 14.59	80.37 ± 11.25
Alcohol	14 (42.42)	-	21 (32.31)	-
Smoking	11 (33.33)	-	36 (55.38)*	-
Biochemical & immunological factors				
Triglycerides[b] (mmol/l)	1.10 [0.68, 1.63]	1.08 [0.79, 1.39]	1.25 [0.97, 1.96] †	0.74 [0.42, 0.99]*** $^{\dagger\dagger\dagger\#\#\#}$
T-Chol[b] (mmol/l)	3.89 ± 1.54	4.58 ± 0.99*	4.14 ± 1.17	3.84 ± 0.85††
HDL-C[b] (mmol/l)	1.00 ± 0.52	1.34 ± 0.36***	1.19 ± 0.75†	1.91 ± 0.93***$^{\dagger\dagger\#\#\#}$
LDL-C[b] (mmol/l)	2.10 [1.48, 2.85]	2.76 [2.27, 3.59] ***	2.50 [1.85, 3.08]	1.47 [1.06, 1.96] $^{\dagger\dagger\dagger\#\#\#}$
CD4[b] (cells/mm^3)	371.99 ± 278.79	518.37 ± 278.73**	-	-

Data is presented as mean ± standard deviation or median [lower quartile, upper quartile] or n (%); *p < 0.05, **p < 0.005 and ***p < 0.0005 versus HIV+/CVD+; $^{\dagger}p$ < 0.05, $^{\dagger\dagger}p$ < 0.005 and $^{\dagger\dagger\dagger}p$ < 0.005 versus HIV+/CVD–; $^{\#}p$ < 0.05 and $^{\#\#\#}p$ < 0.0005 versus HIV–/CVD+; *n* represents number of participants; [a]adjusted for age, ethnicity, and gender; [b]adjusted for BMI, waist-to-hip, age, ethnicity, and gender.

4.2 Comparison of markers of endothelial dysfunction between study groups

Biomarkers of endothelial dysfunction were quantified from only 38, 33, and 30 ETDA plasma samples of the HIV+/CVD–, HIV–/CVD+ and HIV–/CVD– groups, respectively. All 33 HIV+/CVD+ EDTA plasma samples were used for quantification of biomarkers of endothelial dysfunction. These assays were performed on a sub-group of subjects due to financial constraints. The subjects were chosen randomly from each of the study groups.

Plasma levels of ICAM-1 and VCAM-1 in the HIV–/CVD+ group was significantly higher in comparison with all the other groups (Table 4.2). Significantly lower levels of VCAM-1 were observed in the HIV+/CVD– versus the HIV+/CVD+ and HIV–/CVD–groups. The HIV+/CVD+ group had significantly higher vWF levels in comparison to the HIV+/CVD– and HIV–/CVD– groups while vWF levels of HIV–/CVD+ group were significantly higher in comparison to the HIV+/CVD– group. Significantly higher levels of thrombomodulin were observed in the HIV–/CVD–group in comparison to other study groups and the HIV+/CVD–group had significantly higher thrombomodulin levels in comparison to the HIV+/CVD+ and HIV–/CVD+ groups.

Table 4.2: Biomarkers of endothelial dysfunction

	HIV+/ CVD+	HIV+/CVD–	HIV–/CVD+	HIV–/CVD–
	n = 33	*n* = 38	*n* = 33	*n* = 30
ICAM-1 (ng/ml)	239.75 [154.59, 308.14]	160.04 [89.42, 343.67]	579.07 [413.53, 922.87] *** †††	235.64 [157.76, 375.97] $^{\#\#\#}$
VCAM-1 (ng/ml)	1048.77 [738.44, 1687.27]	712.43 [483.32, 867.30]	1789.63 [712, 11135.14] *** †††	933.92 [714.64, 1215.87] $^{\dagger\#\#}$
vWF (units/ml)	25.96 [17.52, 38.44]	13.26 [9.00, 20.88] **	29.99 [23.94, 41.73] †††	13.89 [9.69, 16.66] *$^{\#\#}$
Thrombomodulin (ng/ml)	10.49 [7.38, 16.87]	31.33 [15.67, 40.47] ***	6.41 [4.65, 7.75] †††	43.63 [36.31, 54.91] *** $^{\dagger\dagger\dagger\#\#\#}$

Data is presented as mean ± standard deviation or median [lower quartile, upper quartile]; *p < 0.05, **p < 0.005 and ***p < 0.0005 versus HIV+/ CVD+; $^{\dagger}p$ < 0.05 and $^{\dagger\dagger\dagger}p$ < 0.005 versus HIV+/CVD–; $^{\#\#}p$ < 0.005 and $^{\#\#\#}p$ < 0.0005 versus HIV–/CVD+; *n* represents number of participants. All data adjusted for BMI, waist-to-hip ratio, age, ethnicity, and gender.

In order to determine whether the measurement of biomarkers of endothelial dysfunction in a restricted number of study subjects lead to any bias, demographic, anthropometric and cardiometabolic variables and CVD risk factors were compared between subjects who did and did not have the biomarkers measured within each of the HIV+/CVD–, HIV–/CVD+ and HIV–/CVD– groups (Table 4.3). Within any of these three study groups no significant difference was observed regarding age, ethnicity, gender, BMI, waist-to-hip ratio, any of the CVD risk factors, total cholesterol and HDL-C between participants who did or did not have the endothelial biomarkers measured. However, in the HIV+/CVD- group, the CD4 counts were significantly higher in the subjects in whom the endothelial biomarkers were measured. With regards to the HIV-/CVD+ group, both the waist and hip circumference measurements were higher in those in whom the endothelial biomarkers were assayed, but BMI did not differ across the sub-groups. In the HIV-/CVD- group, hip circumference and triglyceride levels were lower but LDL-C levels were higher in the sub-group in which the endothelial biomarkers were measured.

Table 4.3: Comparison of variables between subjects in whom biomarkers of endothelial dysfunction were or were not quantified

	HIV+/CVD–	**HIV+/CVD–[@]**	**HIV–/CVD +**	**HIV–/CVD+[@]**	**HIV–/CVD–**	**HIV–/CVD–[@]**
	n = 22	*n* = 38	*n* = 32	*n* = 33	*n* = 30	*n* = 30
Demographic factors						
Age (years)	39.48 ± 4.73	39.14 ± 4.54	54.5 ± 8.26	51.88 ± 10.29	36.21 ± 5.10	36.80 ± 4.63
Ethnicity (black), *n* (%)	22 (100)	38 (100)	14 (43.75)	20 (60.61)	30 (100)	30 (100)
Male, *n* (%)	8 (34.79)	7 (18.42)	23 (71.88)	28 (84.85)	12 (40)	13 (43.33)
Anthropometric data						
BMI	26.79 ± 5.77	28.02 ± 6.64	27.43 ± 5.90	27.22 ± 6.42	26.69 ± 5.78	25.11 ± 4.57
Waist circumference (cm)	93.26 ± 10.91	95.18 ± 13.72	99.09 ± 10.99	111.00 ± 17.91**	90.38 ± 11.96	85.53 ± 11.95
Hip circumference (cm)	106.35 ± 11.07	108.97 ± 13.06	99.53 ± 11.20	108.25 ± 17.17**	104.55 ± 11.95	95.90 ± 8.31*
Waist-to-hip ratio	0.88 ± 0.06	0.87 ± 0.06	1.00 ± 0.07	1.03 ± 0.04	0.86 ± 0.07	0.89 ± 0.08
CVD risk factors, *n* (%)						
Diabetes mellitus	0 (0)	0 (0)-	9 (28.13)	14 (42.42)	0 (0)	0 (0)
CVD Family history	-	-	12 (37.5)	13 (39.39)	-	-
Diabetes Family history	-	-	10 (31.25)	10 (30.30)	-	-
Systolic BP (mmHg)	122.52 ± 17.89	125.75 ± 19.05	123.97 ± 20.76	118.42 ± 19.05	128.61 ± 18.33	131.47 ± 15.84
Diastolic BP (mmHg)	79.26 ± 9.56	70.34 ± 12.51	75.55 ± 17.50	76.39 ± 11.47	80.03 ± 13.05	80.33 ± 9.44
Alcohol	-	-	7 (21.88)	14 (42.42)	-	-
Smoking	-	-	20 (62.50)	16 (48.48)	-	-
Biochemical and immunological factors						
Triglycerides (mmol/l)	1.15 [0.08, 1.39]	1.04 [0.78, 1.32]	1.38 [0.97, 2.06]	1.22 [0.92, 1.82]	0.91 [0.71, 1.09]	0.52 [0.35, 0.81]*
T-Chol (mmol/l)	4.56 ± 0.82	4.59 ± 1.08	3.92 ± 1.01	4.37 ± 1.29	3.80 ± 0.91	3.87 ± 0.82
HDL-C (mmol/l)	1.36 ± 0.40	1.33 ± 0.34	1.15 ± 0.66	1.23 ± 0.85	2.00 ± 0.97	1.71 ± 0.87
LDL-C (mmol/l)	2.71 [2.29, 3.45]	2.76 [2.15, 3.60]	2.42 [1.88, 3.07]	2.64 [1.70, 3.37]	1.06 [0.86, 1.49]	1.85 [1.43, 2.32]*
CD4 (cells/mm^3)	397.58 ± 193.37	595.91 ± 296.09*	-	-	-	-

Data is presented as mean ± standard deviation, median [lower quartile, upper quartile] or n (%); *$p < 0.05$ and **$p < 0.005$ versus subjects in same study group in whom endothelial biomarkers were not measured; *n* represents number of participants; [@]sub-group in which endothelial biomarkers were quantified

4.3 Clinical and virological characteristics of subjects in whom *nef* was sequenced

Genomic DNA of all HIV-infected participants in this study was extracted from peripheral blood mononuclear cells (PBMCs) followed by amplifications of HIV-1 *nef* and assessment of successful amplification by agarose gel electrophoresis (Figure 4.1). Among 33 HIV+/CVD+ samples, HIV-1 *nef* was successfully sequenced in a total of 25 samples whilst sequencing was unsuccessful in 8 samples (see Discussion for possible explanations for unsuccessful sequencing). Data from the 25 subjects in whom *nef* sequencing was successful was compared with data from the 8 subjects in whom HIV-1 *nef* was not successful sequenced (Table 4.4). The only observed significant difference between the two sub-groups was the level of vWF which was lower in the non-sequenced HIV-1 *nef* group.

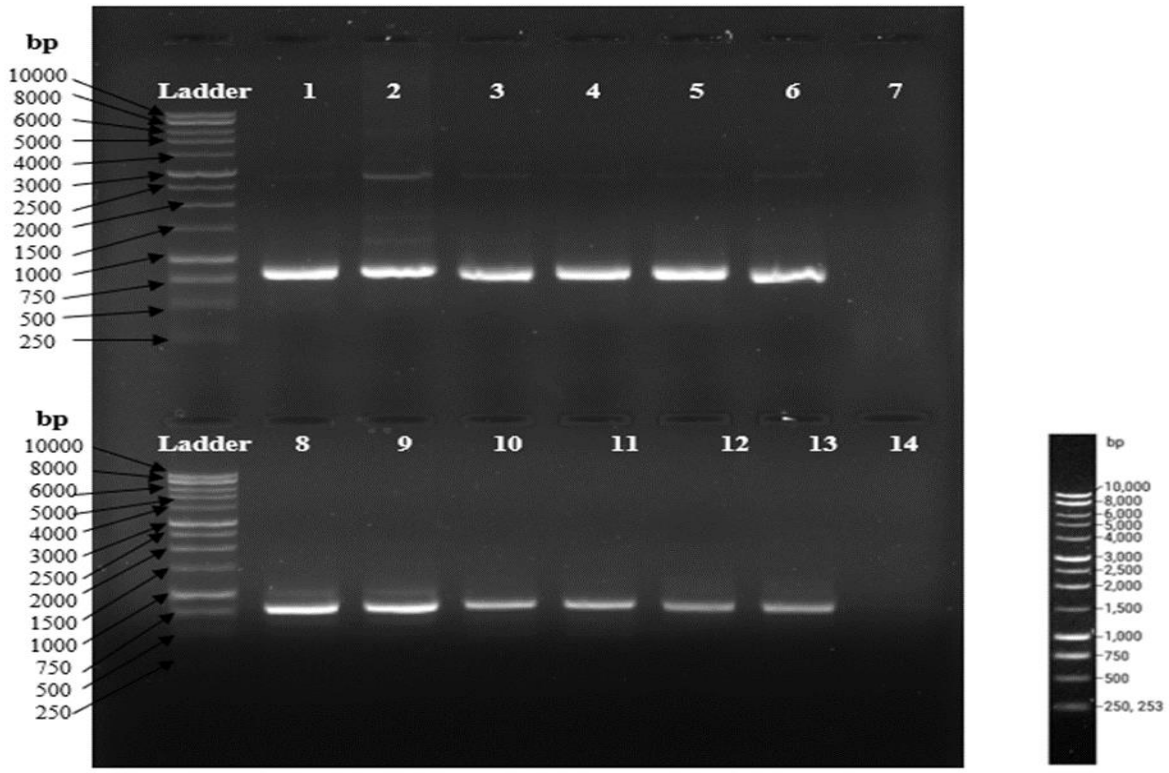


Figure 4.1: Agarose gel image showing PCR products of HIV *nef* in HIV+/CVD+ group. Products on lanes 1-6 and 8-13 were amplified with primer set 1 and 2, and 3 and 4, respectively (see section 3.3.2 and Table 3.1). Lanes 7 and 14 had blanks (negative controls).

Table 4.4: Comparison of data between subjects in whom HIV-1 *nef* was successfully sequenced or was not sequenced

	HIV+/CVD+ successfully sequenced *nef* group	**HIV+/CVD+ non-sequenced *nef* group**
	n = 25	*n* = 8
Demographic factors		
Age (years)	46.20 ± 10.95	48.00 ± 11.41
Ethnicity (black), *n* (%)	23 (92)	8 (100)
Male, *n* (%)	14 (56)	4 (50)
Anthropometric data		
BMI	24.62 ± 13.67	22.10 ± 1.80
Waist circumference (cm)	97.04 ± 18.34	95.14 ± 18.34
Hip circumference (cm)	97.59 ± 18.32	95.14 ± 21.00
Waist-to-hip ratio	1.00 ± 0.04	1.00 ± 0.03
CVD risk factors, *n* (%)		
On HAART	23 (92)	8 (100)
Diabetes mellitus	9 (36)	1 (12.50)
CVD family history	5 (20)	1 (12.50)
Diabetes family history	4 (16)	1 (12.50)
Systolic BP (mmHg)	120.25 ± 20.00	115.13 ± 19.08
Diastolic BP (mmHg)	80.21 ± 18.01	72.50 ± 15.32
Alcohol	12 (48)	2 (25)
Smoking	10 (40)	1 (12.50)
Blood analytes		
Triglycerides (mmol/l)	1.10 [0.75, 1.59]	1.60 [0.66, 2.51]
T-Chol (mmol/l)	4.07 ± 1.68	3.49 ± 1.18
HDL-C (mmol/l)	1.09 ± 0.54	0.80 ± 0.44
LDL-C (mmol/l)	2.26 [1.48, 3.05]	1.68 [1.00, 2.34]
CD4 (cells/mm^3)	414.33 ± 278.66	240.83 ± 340.32
ICAM-1 (ng/ml)	239.755 [158.37, 297.88]	250.31 [126.33, 373.51]
VCAM-1 (ng/ml)	1048.77 [762.71, 1687.27]	1355.76 [700.98, 1841.43]
vWF (units/ml)	30.76 [24.18, 48.51]	15.06 [9.53, 20.69]**
Thrombomodulin (ng/ml)	10.49 [7.63, 16.87]	13.56 [7.01, 18.41]

Data is presented as mean ± standard deviation, median [lower quartile, upper quartile] or n (%); ** $p < 0.005$; n represents number of participants.

Table 4.5 shows comparisons of all study variables between the 2 different HIV-infected population groups i.e. HIV+/CVD+ and HIV+/CVD-, for subjects in whom HIV-1 *nef* was successfully sequenced and endothelial biomarkers were quantified. The statistical differences observed between these 2 groups and shown in Table 4.5, were the same as those that were observed in Table 4.1 when the main study population of HIV+/CVD+ and HIV+/CVD– (n=33 versus n=60, respectively) were compared to each other. The only exception was total cholesterol, which although higher in the HIV+/CVD- groups as shown in both tables was only statistically significantly higher in the analysis performed for the data in Table 4.1. In addition, similar statistical differences for the biomarkers of endothelial dysfunction observed in Table 4.5 were observed in Table 4.2 for the full populations.

Table 4.5: Comparison of HIV-infected groups from which HIV-1 *nef* was sequenced

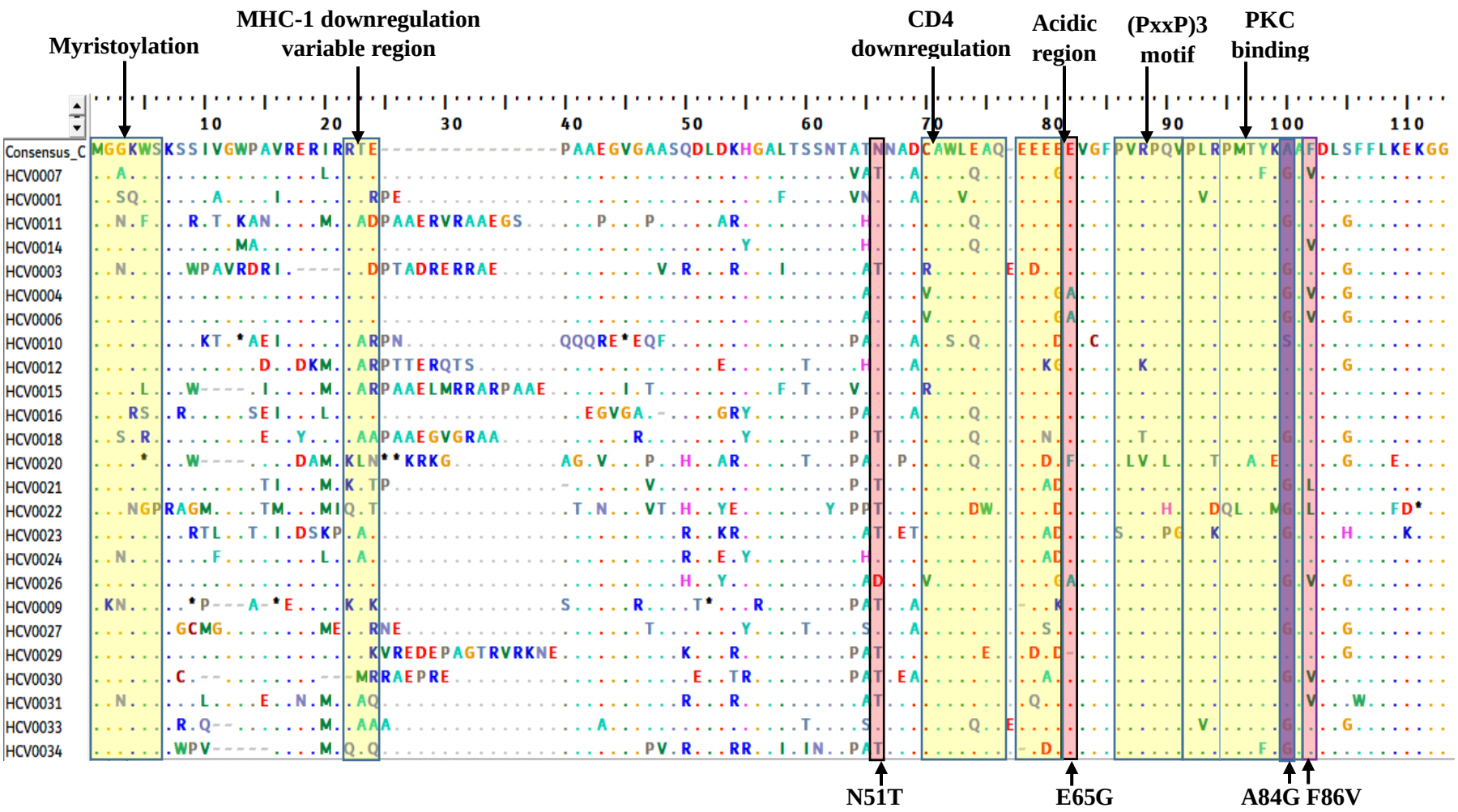
	HIV+/CVD+	**HIV+/CVD–**
	n = 25	*n* = 38
Demographic factors		
Age (years)	46.20 ± 10.95	39.14 ± 4.54**
Ethnicity (black), *n* (%)	23 (92)	38 (100)
Male, *n* (%)	14 (56)	13 (43.33) *
Anthropometric data		
BMI[a]	24.62 ± 13.67	28.02 ± 6.64*
Waist circumference[a] (cm)	97.04 ± 18.34	95.18 ± 13.72
Hip circumference[a] (cm)	97.59 ± 18.32	108.97 ± 13.06**
Waist-to-hip ratio	1.00 ± 0.04	0.87 ± 0.06***
CVD risk factors, *n* (%)		
On HAART	23 (92)	38 (100)
Diabetes mellitus	9 (36)	0 (0)
CVD Family history	5 (20)	-
Diabetes Family history	4 (16)	-
Systolic BP[b] (mmHg)	120.25 ± 20.00	125.75 ± 19.05
Diastolic BP[b] (mmHg)	80.21 ± 18.01	70.34 ± 12.51
Alcohol	12 (48)	-
Smoking	10 (40)	-
Blood analytes		
Triglycerides[b] (mmol/l)	1.10 [0.75, 1.59]	1.04 [0.78, 1.32]
T-Chol[b] (mmol/l)	4.07 ± 1.68	4.59 ± 1.08
HDL-C[b] (mmol/l)	1.09 ± 0.54	1.33 ± 0.34*
LDL-C[b] (mmol/l)	2.26 [1.48, 3.05]	2.76 [2.15, 3.60] *
CD4[b] (cells/mm^3)	414.33 ± 278.66	595.91 ± 296.09*
ICAM-1[b] (ng/ml)	239.755 [158.37, 297.88]	160.04 [89.42, 343.67]
VCAM-1[b] (ng/ml)	1048.77 [762.71, 1687.27]	712.43 [483.32, 867.30] **
vWF[b] (units/ml)	30.76 [24.18, 48.51]	13.26 [9.00, 20.88] ***
Thrombomodulin[b] (ng/ml)	10.49 [7.63, 16.87]	31.33 [15.67, 40.47] ***

Data is presented as mean ± standard deviation, median [lower quartile, upper quartile] or n (%); *$p < 0.05$, **$p < 0.005$ and ***$p < 0.0005$; *n* represents number of participants; [a]adjusted for age and gender; [b]adjusted for BMI, waist-to-hip ratio, age, and gender.

4.4 Association of *nef* sequence variants with biomarkers of endothelial dysfunction

The HIV-1 *nef* sequence analysis of amino acid residues displayed statistically significant associations of particular *nef* polymorphisms with plasma levels of the biomarkers of endothelial dysfunction and with the presence of CVD. Figure 4.2 illustrate positions of all HIV-1 *nef* variants identified in the clinical isolates in this study aligned with HIV-1 HXB2 and consensus C sequences obtained from Los Alamos HIV Sequence Database. Amino acid residues were numbered following the HIV-1 HXB2 sequence, which differs in number from the consensus C sequence. The latter has an additional amino acid at position 62 which results in a frameshift that alters numbering by 1 amino acid from position 61. The alignment numbering system in Figure 4.2 includes numbering of each amino acid residue and alignment gaps while numbering using consensus C includes only the amino acid residues and not the sequence gaps. Figure 4.2 therefore indicates where each of the variants associated with CVD or with biomarkers of endothelial dysfunction lies along with the functional regions of the Nef protein. The Nef functional regions shown in Figure 4.2 were annotated using the HIV-1 proteomics resource (Doherty *et al.*, 2005).

Variants that were associated with biomarkers of endothelial dysfunction are shown in Tables 4.6 and 4.7. These data show that the consensus sequence amino acid at N51T, E65G, and F86V were each associated with higher levels of vWF, whilst at K185Q the most frequent non-consensus sequence amino acid i.e. Q, was associated with the highest vWF level. It should be noted that the nomenclature of these variants involves the consensus HIV-1 subtype C amino acid followed by the position number and then the most frequent non-consensus sequence amino acid. At F86V, the V residue was associated with a higher ICAM-1 level. With regards thrombomodulin, non-consensus sequence amino acids at positions N51T, E175D and K185Q were each associated with lower levels of this molecule. No variants were observed to be associated with VCAM-1.


Myristoylation
MHC-1 downregulation variable region
CD4 downregulation
Acidic region
(PxxP)3 motif
PKC binding
N51T
E65G
A84G F86V

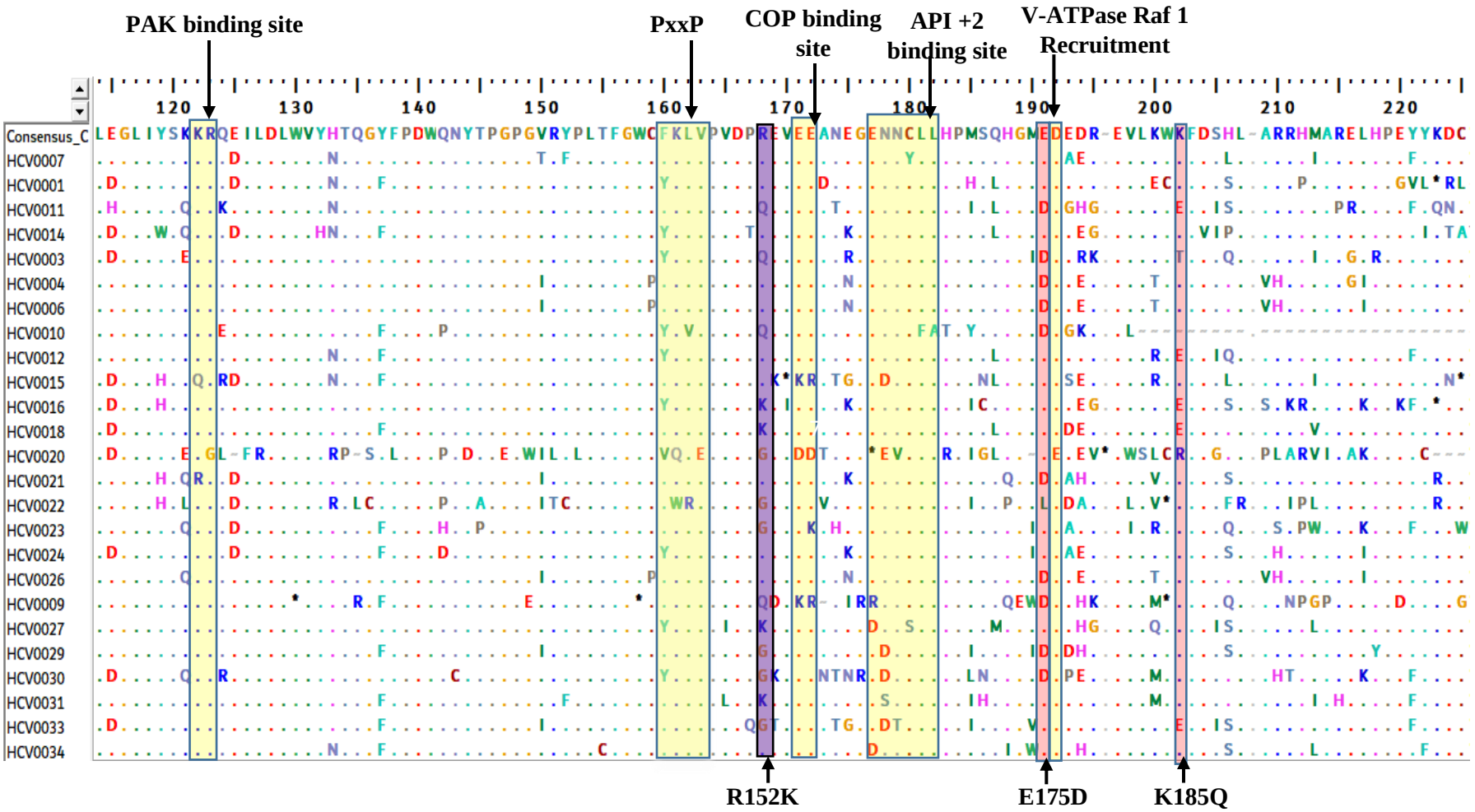


Figure 4.2: Multiple sequence alignment of HIV-1 *nef* amino acid sequences. HIV-1 *nef* functional regions are all boxed and shaded in yellow. Variants of HIV-1 *nef* identified by means of a contingency table and Fisher's Exact two tailed test to be associated with both biomarkers of endothelial dysfunction and CVD are all boxed and shaded in pink, while amino acid residues that were only associated with CVD are boxed and shaded in purple. Amino acids that are the same as those in the HIV-1 Consensus C sequence are shown as coloured dots (.), while tildes (~) represent alignment gaps.

Table 4.6: Association of HIV-1 *nef* variants with vWF

Biomarker	Variant		Number of amino acids	Biomarker level	*p*-value (*p*-value if rare amino acids are combined)
vWF	N51T	N	42	11.22 [3.86, 16.83]	0.018 (0.005)
		T	15	5.03 [3.13, 5.61]	
		D	1	2.56	
		S	2	36.87 [9.84, 63.89]	
		None	3	6.49 [1.81, 28.21]	0.032 (0.093)
	E65G	E	42	9.72 [3.39, 15.38]	
		G	12	6.97 [3.23, 16.11]	
		D	7	4.85 [2.49, 5.22]	
		K	1	6.49	
		S	1	3.72	0.015 (0.213)
	F86V	F	47	9.61 [4.53, 16.34]	
		V	9	3.19 [2.56, 3.84]	
		L	3	5.28 [3.08, 40.07]	
		None	4	16.79 [5.47, 16.44]	
	K185Q	K	35	5.33 [2.96, 14.67]	0.020 (0.009)
		Q	9	13.52 [9.94, 22.79]	
		E	6	3.33 [2.30, 4.60]	
		R	3	14.79 [5.03, 40.6]	
		T	1	9.38	
		V	4	8.07 [5.68, 15.59]	
		F	3	5.47 [1.81, 5.67, 15.59]	
		None	2	5.67 [4.58, 6.49]	

Data is presented as median [lower quartile, upper quartile]. Kruskal-Wallis ANOVA test was used to generate *p* values (shown without brackets) where biomarker levels of all variants were compared, e.g., for N51T biomarker level of variant N, T, D, S and none were compared. Another Kruskal-Wallis ANOVA test was used to generate *p* values in brackets where rare variants are combined e.g., for N51T biomarker levels of variant N, T and D combined with S and none were compared. Rare amino acid residues were defined as those that occurred ≤ 5 times for each variant.

Table 4.7: Association of HIV-1 *nef* variants with endothelial dysfunction

Biomarker	Variant		Number of amino acids	Biomarker level	*p*-value (*p*-value if rare amino acids are combined)
ICAM-1	F86V	F	47	174.23 [102.31, 330.35]	0.228 (0.042)
		V	9	266.42 [240.58, 415.00]	
		L	3	124.10 [99.02, 161.58]	
		None	4	172.14 [120.72, 294.34]	
Thrombomodulin	N51T	N	42	22.25 [13.14, 35.07]	0.011 (0.015)
		T	15	14.55 [7.20, 18.06]	
		D	1	23.44	
		S	2	44.81 [44.32, 45.29]	
		None	3	28.34 [5.90, 43.95]	
	E175D	E	47	23.54 [12.55, 35.37]	0.022 0.214
		D	14	12.32 [8.68, 20.30]	
		L	1	15.86	
		None	1	42.65	
	K185Q	K	35	20.76 [12.35, 25.81]	0.001 (0.001)
		Q	9	41.86 [33.53, 44.81]	
		E	6	7.88 [5.36, 14.96]	
		R	3	20.26 [10.21, 28.24]	
		T	1	10.07	
		V	4	25.18 [13.99, 37.18]	
		F	3	7.38 [5.90, 8.86]	
		None	2	20.22 [14.56, 25.89]	

Data is presented as median [lower quartile, upper quartile]. Kruskal-Wallis ANOVA test was used to generate *p* values (shown without brackets) where biomarker levels of all variants were compared, e.g., for F86V biomarker level of variant F, V, L and none were compared. Another Kruskal-Wallis ANOVA test was used to generate *p* values in brackets where rare variants are combined e.g., for F86V biomarker levels of variant F, V and L combined with none were compared. Rare amino acid residues were defined as those that occurred ≤ 5 times for each variant.

Further analysis of the combined effect of HIV-1 *nef* variants from Table 4.6 and 4.7 on biomarkers of endothelial dysfunction was carried out by counting the number of amino acid residues present in each HIV-infected participant that was associated with plasma levels of the biomarker. Two separate analyses were carried out: one for amino acid residues associated with higher vWF levels, and another for residues associated with lower thrombomodulin levels. Based on the data shown in Tables 4.6 and 4.7 these residue counts should vary from 0 to 4 for vWF and 0 to 3 for thrombomodulin. However,

no subjects were found with a virus harbouring all 4 or all 3 of the variants that are associated with vWF and thrombomodulin, respectively. Due to the low number of participants in this study, counts were split into 3 groups for improvement of the statistical power of the analysis. The 3 groups included a group that had individuals with 0 *nef* variants that associated with the biomarker under investigation; a group with 1 *nef* variants; and a group with 2 or 3 *nef* variants. Analysis of thrombomodulin showed that were 14, 32 and 17 subjects for groups with 0, 1, and 2 or 3 variants, respectively, whilst analysis for vWF counted 15, 30 and 18 subjects for groups with 0, 1, and 2 or 3 variants, respectively. Thrombomodulin levels in group with 2 or 3 *nef* variants were significantly lower than the other two variant groups. Although no statistically significant difference was observed with regard to vWF (p = 0.234), levels of vWF were shown to increase with an increase in number of *nef* variants (Figure 4.3). Such analysis was not carried out for ICAM-1 since plasma levels of this molecule were associated with only one *nef* variant.

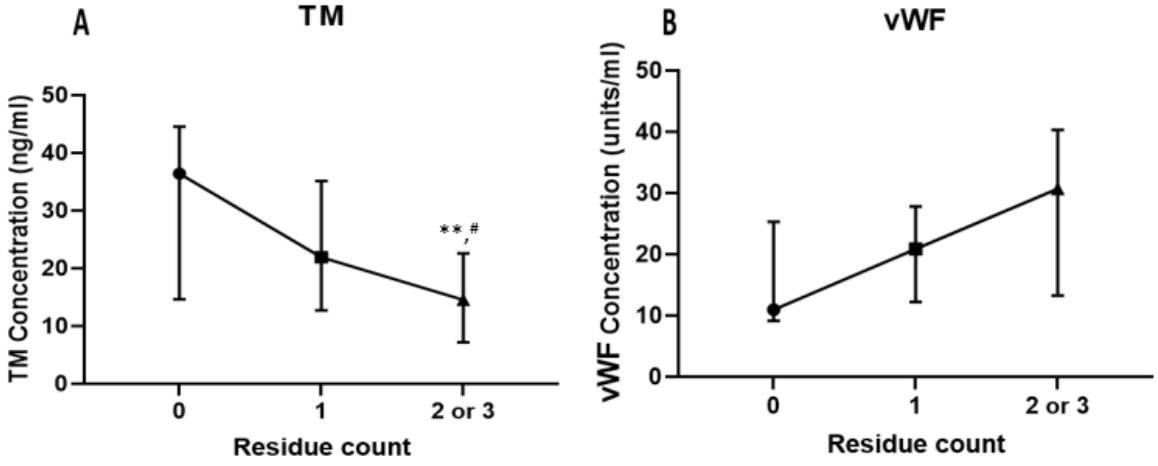


Figure 4.3: Concentration of biomarkers of endothelial dysfunction across HIV-infected groups harbouring genome-integrated HIV DNA coding for varying numbers of Nef risk residues associated with plasma levels of each endothelial biomarker. Figure 4.3A, and 4.3B, shows thrombomodulin and vWF, respectively versus residue count. $^{**}p < 0.005$ versus 0 residues group, $^{\#}p < 0.05$ versus 1 residue group.

4.5 Association of *nef* variants with CVD

The HIV-1 *nef* sequence analysis of amino acid residues displayed statistically significant differences in the frequency of particular *nef* polymorphisms between subjects with and without CVD. Table 4.8 shows the results from contingency table which revealed that variants N51T, E65G, A84G, F86V and E175D had a significantly higher frequency of the consensus sequence amino acid in the HIV+/CVD– group, whilst variants R152K and K185Q had a significantly higher frequency of the consensus sequence in the HIV+/CVD+ group. Two separate statistical analyses of these variants were performed where all the amino acids for each variant were included in the contingency table while the other analysis combined rare amino acid residues. The rare amino acid residues were defined as those that occurred ≤ 5 times in both of the study populations (see Table 4.8).

Table 4.8: Nef variant frequencies in CVD+ and CVD– groups

Variant	Amino acid	HIV+/CVD+ (*n*)	HIV+/CVD– (*n*)	HIV+/ CVD+ Frequency (%)	HIV+/CVD– Frequency (%)	*p*-value (*p*-value if rare amino acids are combined)[a]
N51T	N	13	29	52.00	76.32	0.010
	T	11	4	44.00	10.53	(0.008)
	D	1	0	4.00	0.00	
	S	0	2	0.00	5.26	
	None	0	3	0.00	7.89	
E65G	E	13	29	52.00	76.32	0.045
	G	5	7	20.00	18.42	(0.054)
	D	6	1	24.00	2.63	
	K	1	0	4.00	0.00	
	S	0	1	0.00	2.63	
A84G	A	10	23	40.00	60.53	0.081
	G	14	10	56.00	26.32	(0.049)
	S	1	2	4.00	5.26	
	None	0	3	0.00	7.89	
F86V	F	16	31	64.00	81.58	0.022
	V	7	2	28.00	5.26	(0.040)
	L	2	1	8.00	2.63	
	None	0	4	0.00	10.53	
R152K	R	11	7	44.00	18.42	0.002
	K	4	9	16.00	23.68	(0.026)
	Q	4	0	16.00	0.00	
	G	6	5	24.00	13.16	
	E	0	2	0.00	5.26	
	N	0	1	0.00	2.63	
	S	0	5	0.00	13.16	
	None	0	9	0.00	23.68	
E175D	E	14	33	56.00	86.84	0.019
	D	10	4	40.00	10.53	(0.019)
	L	1	0	4.00	0.00	
	None	0	1	0.00	2.63	
K185Q	K	17	18	68.00	47.37	0.009
	Q	0	9	0.00	23.68	(0.029)
	E	5	1	20.00	2.63	
	R	1	2	4.00	5.26	
	T	1	0	4.00	0.00	
	V	0	4	0.00	10.53	
	F	0	3	0.00	7.89	
	None	1	1	4.00	2.63	

[a]*p* values in brackets are generated from contingency table where rare variants are combined e.g. for N51T a 5×2 contingency table was used to produce a *p* value (shown without brackets) for all residues and none, and a 3×2 contingency table was used to generate a *p* value (enclosed in brackets) for residues N and T and D, S and none combined; *n* represents number of clinical isolates containing that residue

In order to determine the combined effect of the 7 *nef* sequence variants on risk for CVD, the number of viral Nef amino acid residues associated with a higher prevalence of CVD (documented in Table 4.8) was determined for each HIV-infected individual to give a count ranging from 0 to 5 (no individuals were found with a virus harbouring 6 or 7 of the high CVD risk residues). The prevalence of CVD was then determined in each of these 6 subject groups and the results are shown in Figure 4.4A. Due to the low number of subjects in some of these groups (see legend to Figure 4.4) we combined subjects with 0 or 1 risk residue into a single group (n=28) and subjects with ≥2 risk residues into a second group (n=35) and compared the prevalence of CVD between these 2 groups (see Figure 4.4B). The results in Figure 4.4 clearly show that the prevalence of CVD rises significantly with increasing numbers of risk residues present in the virus Nef protein.

The association of the 7 risk residues with CVD was analyzed further using multivariable logistic regression models (Table 4.9), which allows for adjustment for possible confounding variables. Thus, adjustment was made for age, gender, waist-to-hip ratio, BMI and for the biomarkers of endothelial dysfunction. The regression models revealed that without adjustment, each of the 7 risk residues was significantly associated with CVD risk when compared to the non-risk residues. Additionally, comparison of individuals with ≥2 risk residues with those with <2 risk residues resulted in highly significant association with CVD risk. Adjustment for age, gender, waist-to-hip ratio and BMI resulted in a decrement of the odds ratio of N51T, E65G, A84G and F86V with *p*-values rising above 0.05 whilst for E175D and K185Q and ≥2 residues the significant associations were maintained. When the three biomarkers of endothelial dysfunction were added to the model the odds ratio for N51T, E65G, A84G, R152K and E175D were decreased further with large increases in the associated *p*-values, whilst for F86V, K185Q and ≥2 residues the odds ratios were elevated but with *p*-values rising above 0.05 for F86V and ≥2 residues. With K185Q, the *p*-value did increase but stayed below 0.05. It should

be noted that VCAM-1 was not added to the regression models due to non-association with the *nef* variants from the HIV clinical isolates.

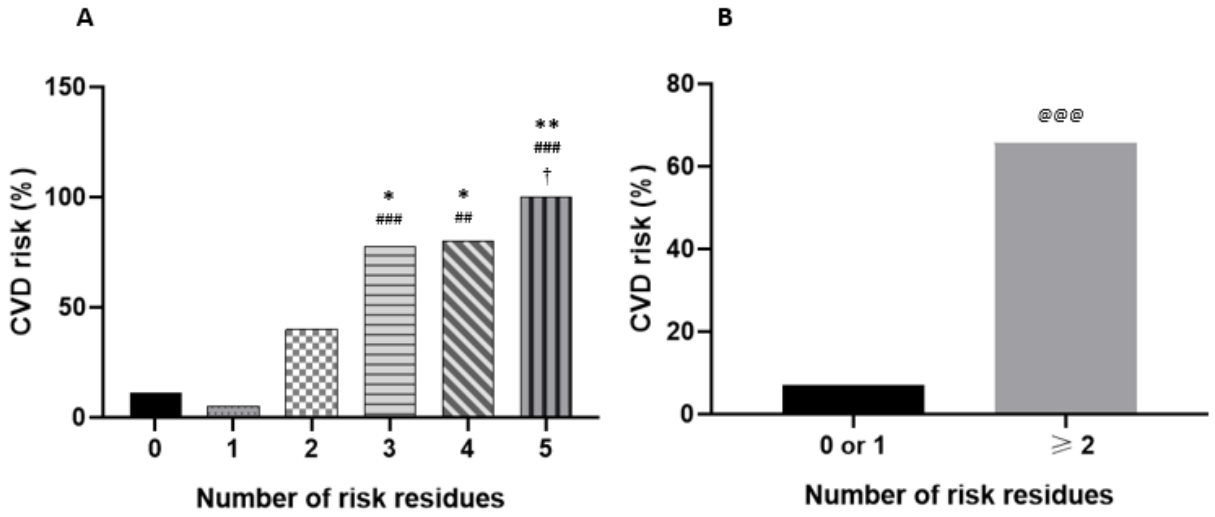


Figure 4.4: Prevalence of CVD across viral Nef risk residue count groups. Figure 4.4A shows prevalence of CVD across 6 groups with different number of risk residues. Groups with 0, 1, 2, 3, 4 and 5 risk residues had 9, 19, 15, 9, 5 and 6 participants, respectively. Figure 4.4B shows prevalence of CVD between 2 groups with different number of risk residues. Groups with 0 or 1 and ≥ 2 risk residues had 28 and 35 subjects, respectively. In Figure 4.4A, $^{*}p < 0.05$ and $^{**}p < 0.005$ versus 0 residue group, $^{\#\#}p < 0.005$ and $^{\#\#\#}p < 0.0005$ versus 1 residue group, $^{\dagger}p < 0.05$ versus 2 residues group. In Figure 4.4B $^{@@@}p < 0.0005$ versus 0 or 1 residue group.

Table 4.9: Logistic regression models analysing relationship of risk residues with CVD risk with and without adjustment for possible confounding variables

Model	*Nef* variant with OR (95% CIs) for CVD; *p*-value (unadjusted)	*Nef* variant with OR (95% CIs) for CVD; *p*-value (adjusted)*	*Nef* variant with OR (95% CIs) for CVD; *p*-value (adjusted)[#]
1	N51T: 6.68 (1.78, 25.23); 0.004	N51T: 4.63 (0.94, 22.86); 0.055	N51T: 3.8 (0.16, 93.55); 0.401
2	E65G: 11.68 (1.25, 108.93); 0.028	E65G: 7.93 (0.69, 91.57); 0.090	E65G: 2.19 (0.04, 100.25); 0.680
3	A84G: 4.10 (1.35, 12.44); 0.011	A84G: 3.43 (0.93, 12.72); 0.059	A84G: 2.89 (0.29, 29.22); 0.358
4	F86V: 7.00 (1.27, 38.47); 0.022	F86V: 6.07 (0.81, 45.36); 0.072	F86V: 13.91 (0.74, 262.90); 0.072
5	R152K: 4.19 (1.26, 13.92); 0.017	R152K: 15.43 (2.43, 97.95); 0.003	R152K: 3.84 (0.10, 143.09); 0.455
6	E175D: 5.67 (1.49, 21.55); 0.009	E175D: 5.24 (1.14, 23.98); 0.029	E175D: 0.88 (0.03, 26.70); 0.94
7	K185Q: 6.60 (1.64, 26.54); 0.007	K185Q: 42.80 (3.38, 542.33); 0.003	K185Q: 167.5 (2.10, 13354.1); 0.019
8	>2 residues: 24.79 (5.66, 108.61); <0.0005	>2 residues: 18.62 (3.43, 101.13); 0.0005	>2 residues: 46.03 (0.86, 2476.12); 0.054

*Adjusted for age, gender, waist-to-hip ratio and BMI, [#]adjusted age, gender, waist-to-hip ratio, BMI, ICAM-1, vWF and thrombomodulin.

CHAPTER FIVE – DISCUSSION AND CONCLUSIONS

5.1 Summary of results

The current study sought to investigate whether HIV-1 subtype C *nef* polymorphisms in HIV-infected subjects are associated with CAD and with biomarkers of endothelial dysfunction. This was accomplished by comparing the plasma levels of VCAM-1, ICAM-1, vWF and thrombomodulin between HIV-infected and non-infected subjects with and without CAD and sequencing the HIV-1 *nef* gene from the HIV-infected subjects. The levels of VCAM-1 and ICAM-1 were found to be higher in subjects with CAD, particularly those without HIV whilst vWF was higher and thrombomodulin was lower in those with CAD irrespective of HIV status. It was observed in the HIV-infected groups that particular amino acid residue variants in Nef were found at different frequencies between subjects with and without CAD and were also associated with the levels of the plasma markers of endothelial dysfunction. Thus, a total of seven variants (N51T, E65G, A84G, F86V, R152K, E175D and K185Q) were identified to be associated with CAD of which F86V was significantly associated with elevated levels of ICAM-1, while N51T, E65G, F86V and K185Q, and N51T, E175D and K185Q were significantly associated with vWF and thrombomodulin, respectively. No association was seen between any of the variants with VCAM-1. In addition, the number of variants associated with CAD were counted in the *nef* gene of each clinical isolate and the prevalence of CAD in the HIV-infected subjects was found to increase in a stepwise manner as the number of CAD-associated amino acid residues rose.

5.2 Discussion

The HIV-infected subjects without CAD had significantly higher BMI and CD4 counts when compared to HIV-infected subjects with CAD. Lower CD4 count levels represent poorer immune function which may be due to poor adherence to HAART or infection with HAART-resistant virus

(Gunda *et al*., 2017; Lailulo *et al*., 2020). This suggest that HIV-infected subjects with CAD have more severe disease and this may explain their lower BMI compared to HIV-infected subjects without CAD. Weight loss is a well-recognized complication of poorly controlled HIV infection (Mangili *et al*., 2006). It was also interesting to note that the HIV subjects with CAD had lower levels of all cholesterol-related lipids than did those without CAD. It is known that HIV infection is associated with lower total cholesterol levels in HAART-naïve subjects and initiation of HAART administration is associated with increased total cholesterol (Riddler *et al*., 2003). The HIV-infected subjects with CAD when compared to those without CAD, have more severe disease, as demonstrated by lower CD4 counts, which would suggest higher viraemia, and this may explain their lower cholesterol levels. Looking at the HIV-uninfected groups, the HIV–/CVD+ group had higher total cholesterol levels, slightly higher BMI and higher waist circumference when compared to HIV–/CVD–. These differences are typical of CVD in HIV-uninfected subjects and may suggest a difference in the aetiology of CAD in the HIV-infected subjects where the virus plays a more prominent role in the development of CAD than do the classical CVD risk factors.

Infection with HIV has been linked to endothelial dysfunction through mechanisms that trigger the expression of adhesion molecules and inflammatory and prothrombotic cytokines (Kearns *et al*., 2017). In the current study HIV-infected subjects, irrespective of their CAD status, had lower plasma levels of ICAM-1 and VCAM-1 compared to HIV-uninfected subjects however, several cross-sectional studies from Africa have reported elevated plasma levels of ICAM-1 and VCAM-1 in HIV-infected subjects compared to HIV-uninfected subjects (Fourie *et al*. 2011; Fourie *et al*. 2015; Mosepele *et al*., 2018). A longitudinal study conducted in South Africa by Mezoh *et al*. (2021) showed plasma levels of ICAM-1 and VCAM-1 to decrease below levels observed in HIV-uninfected subjects following 18 months of HAART administration and other longitudinal studies have also demonstrated that ART causes a decrease in ICAM-1 and VCAM-1 levels in treated patients (Francisci

et al., 2009; van Vonderen *et al.*, 2009; Kristoffersen *et al.*, 2009). Therefore, the current observation of lower levels of ICAM-1 and VCAM-1 in HIV-infected compared to non-infected subjects may be due to the use of HAART in the HIV-infected subjects.

There was no significant difference between the HIV-infected and the non-infected subjects, irrespective of CAD status, with regard to levels of vWF. This does not agree with data from previous studies which show higher vWF levels in those with HIV infection (Constans *et a*l., 1998; de Larrañaga *et al.*, 2003; Mezoh *et al.* 2021) and that vWF plasma levels are reduced by ART (Francisci *et al.*, 2009; van Vonderen *et al.*, 2009). Once again, it is therefore possible that vWF levels are similar between those with and without HIV infection due to use of HAART in the HIV-positive subjects in the current study.

The HIV-infected subjects without CAD had significantly lower thrombomodulin levels than the non-infected healthy subjects whilst those with CAD had thrombomodulin levels that were similar to the HIV-negative subjects with CAD. Data from the literature on thrombomodulin levels and HIV are sparse and conflicting with some studies showing levels are higher in subjects with HIV infection when compared to healthy control subjects (Seigneur *et al.* 1997; Leucker *et al.*, 2018) whilst other studies show no differences in thrombomodulin levels between HIV-positive and negative subjects (Rönsholt *et al.*, 2015; Kamtchum-Tatuene *et al.*, 2020). No longitudinal studies on the effect of HIV or ART on thrombomodulin levels are available. Further studies are therefore required to determine the effect of both HIV infection and its therapy on thrombomodulin levels.

Biomarkers of endothelial dysfunction such as ICAM-1, VCAM-1 and vWF-1 have been studied extensively within HIV-infected participants but there are few studies measuring these factors in HIV-infected subjects with CAD. Two studies were performed in South Africa measuring VCAM-1 levels

in HIV-positive and HIV-negative subjects with acute coronary syndrome (Becker *et al.*, 2010; Vachiat *et al.*, 2020) and demonstrated higher VCAM-1 levels in the HIV-positive group. However, there is no data on the plasma level of vWF and thrombomodulin in HIV-infected subjects with CAD. Studies investigating ICAM-1 levels in HIV-infected subjects with CAD are more frequent and show that levels are higher in those with evidence of CAD (Bahrami *et al.*, 2016; Chandra *et al.*, 2019; Masiá *et al.*, 2019). Our study did show higher levels of both VCAM-1 and ICAM-1 in HIV+/CVD+ compared to HIV+/CVD– but these differences were not significant. In the HIV-uninfected groups, those with CAD did have higher levels of both these biomarkers than subjects without CAD. It is possible that the insignificant differences in VCAM-1 and ICAM- in the HIV-infected groups may be due to suppression of their levels by ART and also poor power due to the low sample sizes. The current study did demonstrate that HIV-infected subjects with CAD had statistically significantly higher levels of vWF and lower thrombomodulin levels compared to HIV-infected subjects without CAD and similar differences were noted in the HIV-uninfected groups. These are novel findings and suggest that these markers may be more sensitive indicators of CAD in the HIV-infected population than ICAM-1 and VCAM-1. The reason for this is not known but may be related to lower suppressive effects of ART on these molecules, but this hypothesis requires further investigation.

Clinical studies generally show that VCAM-1, ICAM-1 and vWF levels are higher in subjects with CVD, but data for thrombomodulin is less clear (Constans & Conri, 2006). Thrombomodulin is an anti-coagulant that binds to thrombin and reduces its procoagulant activity. Studies have shown that low thrombomodulin levels are associated with a higher risk of CAD in healthy subjects whilst higher levels are associated with disease recurrence in those with a previous myocardial infarction (Blann *et al.*, 1997; Constans & Conri, 2006). The current study demonstrates lower levels of thrombomodulin in subjects with recently diagnosed CAD, irrespective of HIV status suggesting that low thrombomodulin levels are associated with a greater risk of CAD.

The HIV-1 Nef peptide, a multifunctional accessory protein, has been described to cause endothelial dysfunction by altering vascular homeostasis via effects on macrophages and endothelial cells through discrete pathways (Duffy *et al*., 2009; Rosa *et al*., 2015; Walker *et al.,* 2007). Several mechanisms by which HIV-1 Nef may cause endothelial dysfunction, such as activation of the nuclear factor kappa-B signaling pathway and apoptosis of endothelial cells through an NADPH oxidase-dependent mechanism, have been studied, however, these mechanisms are not clearly described (Wang *et al*., 2014). The current study found the consensus sequence amino acids N51T, E65G, and F86V, and the non-consensus sequence amino acid K185Q (*i.e.* Q) are associated with higher levels of vWF. Additionally, the non-consensus sequence amino acids N51T, E175D and K185Q were each associated with lower levels of thrombomodulin whereas a higher ICAM-1 level was associated with non-consensus V residue of F86V. It is noteworthy that these variants were located in the N- and C-terminal domains of the HIV-1 *nef* gene. Likewise, a study conducted by Mezoh *et al*. (2020) found HIV-1 *nef* polymorphisms at the N- and C-terminal domains to be associated with biomarkers of endothelial dysfunction. Interestingly, a study by Walker *et al*. (2007) outlined polymorphisms at the N- and C-terminal domains of HIV-1 *nef* that were linked to a loss of MHC-1 downregulation and other functions involved in cell signaling.

Downregulation of MHC-1 and CD4 requires amino acid residues located at the N-terminal domain of HIV-1 Nef (Mandic *et al*., 2001; Stove *et al*., 2003). Moreover, MHC downregulation may also involve a cluster of acidic amino acid residues of the Nef protein (Stove *et al*., 2003). The C-terminal domain contains amino acid residues involved in the internalization of the Nef protein (Mandic *et al*., 2001). A study by Mann *et al*., (2020) showed N-terminal polymorphisms of Nef at residues 199Y, 108D, 105R and 102H to be individually associated with HLA-I downregulation. Mezoh *et al*. (2020) identified five HIV-1 *nef* polymorphisms (V16I, H40Y, T50H,A, and S169N, H188Q,S located at the

N- and C terminal of the Nef protein, respectively) to be associated with higher ICAM-1 levels, and one C-terminal mutation (Y202F) associated with higher VCAM-1 levels. The current study found no overlap of mutations associated with VCAM-1 or ICAM-1 levels that were identified in this study with those mutations identified by Mezoh *et al.* (2020). It is noteworthy that the HIV-infected subjects from the study conducted by Mezoh *et al.* (2020) were HAART-naïve, whereas HIV-infected subjects in the current study were on HAART. Administration of HAART has been demonstrated to significantly decrease plasma levels of ICAM-1 and VCAM-1 (Mezoh *et al.*, 2021). In addition, the current study involved HIV-infected subjects with and without confirmed CAD, whilst the study by Mezoh *et al.* (2020) used subjects that were assumed to be CAD-free.

The combined effect of the HIV-1 *nef* vWF- and thrombomodulin-associated residues was analysed by counting the number of amino acid risk residues in each subject, grouping subjects into 3 groups (0, 1 and ≥ 2 risk residues) and comparing levels of vWF and thrombomodulin between the groups using one-way ANOVA and a Tukey *post-hoc* test. Plasma levels of vWF increased, while thrombomodulin decreased with an increase in the number of CAD-associated risk residues, although the vWF association did not reach statistical significance. These observations therefore suggest that plasma levels of thrombomodulin are dependent on the additive effect of HIV-1 *nef* polymorphisms.

A further analysis demonstrated that Nef variants N51T, E65G, A84G, F86V and E175D had a significantly higher frequency of the consensus sequence amino acid in the HIV+/CVD– group, whilst variants R152K and K185Q had a significantly higher frequency of the consensus sequence in the HIV+/CVD+ group. The combined effect of these variants was analysed by grouping subjects into 2 groups based on the number of CAD-associated residues observed in the Nef peptides sequenced in their clinical isolates. The 2 groups were ≤ 1 and ≥ 2 risk residues, and the results showed that CAD prevalence increased significantly with increasing numbers of CAD-associated amino acid residues.

This observation and the association of the individual variants with CAD was further analysed in multiple logistic regression models adjusted for possible confounding variables. The model which included the combined variants showed that adjusting for age, gender, waist-to-hip ratio and BMI had little effect on the odds ratio or the p-value. However, additional adjustment for ICAM-1, vWF and thrombomodulin caused the p-value to increase from 0.0005 to 0.054, whilst the odds ratio also increased but the 95% CIs broadened considerably (this is a reflection of the small sample size). These data suggest that the major effect of these variants on CVD risk maybe mediated by endothelial dysfunction. The models for the individual variants showed similar trends with the exception of the K185Q variant which remained significantly associated with CVD risk after adjustment, although with a higher p-value.

5.3 Conclusions

In conclusion, this study demonstrates that HIV-1 *nef* polymorphisms are associated with endothelial dysfunction and with CAD. This therefore provides preliminary evidence of HIV Nef as a possible causative factor in the development of CAD in HIV-infected subjects receiving HAART. It is possible that even in the presence of HAART that HIV reservoirs are still active and produce viral peptides, including Nef (Raymond $et\ al.$, 2011). The study further suggests that endothelial dysfunction caused by Nef may lead to CAD. Therefore, biomarkers of endothelial dysfunction and HIV Nef polymorphisms may be used to monitor and predict severe CAD within the HIV-infected individuals on HAART for early intervention. Additionally, HIV Nef can be targeted to develop anti-CAD strategies aiming to ameliorate CAD among HIV-infected individuals receiving HAART. These findings may be included in an awareness campaign to educate the community about how HIV infection can lead to complications such as CAD despite use of HAART. Such awareness campaigns should target clinical staff and patients as well as the general population.

5.4 Study limitations and strengths and future studies

A limitation of this study is that HIV-1 *nef* was sequenced from host genomic DNA and not circulating viral RNA. The administration of HAART limits viral replication and further infection of CD4+ T cells (Reeves *et al.*, 2018). In the current study, 80% of the HIV-infected subjects had viral load levels below 1000 cells/mm^3. Thus, viral RNA could not be extracted from plasma due to the low number of circulating virions. The early phase of the HIV-1 replication cycle starts with attachment of the virion to the host's cell surface and ends with integration of the proviral DNA into the host's genome (Kirchhoff, 2013). The HIV genetic information may therefore be obtained from the host's genomic DNA. Integration of the proviral DNA can occur at any location of the host's genome (Craigie & Bushman, 2012), thus, specific integration sites are unknown. The HIV DNA may therefore not be a true reflection of an intact provirus. This can result in difficulties of obtaining viral PCR products, mainly due to mismatches between the target viral sequence and primers (Diallo *et al.*, 2012, Kirchhoff *et al.*, 1999). Thus, we were able to obtain 25 HIV-1 *nef* sequences from 33 samples. A similar observation was reported in a previous study by Mezoh *et al.* (2020) in which 60 viral sequences were obtained from 123 samples. A further limitation to this study was the small sample size, aggravated by the problem of not quantifying biomarkers of endothelial dysfunction from all study subjects and not obtaining *nef* sequences from all HIV-infected subjects. Despite the small sample size, the current study identified *nef* polymorphisms associated with biomarkers of endothelial dysfunction and CAD. However, the number of identified polymorphisms may be reduced by lack of statistical power. An additional limitation of this study is the discrepancies between the HIV-sequenced and non-sequenced population and between subjects in whom endothelial biomarkers were or were not measured. These differences suggest some level of bias in the subjects from whom full data was obtained. Therefore, further studies with a larger sample size, which will limit bias and improve the power of the study, must be conducted to validate the results of the current study. The present study was cross sectional and we were only able to investigate associations between variables

and not causative relationships. These need to be studied using both longitudinal analyses and *in vitro* functional analyses.

The strengths of this study are that endothelial dysfunction was compared between HIV-infected subjects with and without CAD. This is one of very few studies conducted in sub-Saharan Africa that has looked at endothelial function in HIV-positive subjects with CAD and the only study to analyse the association of Nef variants with CAD risk. Additionally, results obtained in this study were supported by findings from a previous study (Mezoh, 2021). Despite the small sample size, significant associations were observed however, future studies with a larger sample size are essential to confirm the findings of the current study. Another future study could involve investigating the association of biomarkers of endothelial dysfunction in HIV-infected HAART-naïve subjects with and without CAD compared to HIV-infected subjects on-HAART with and without CAD. This would enable us to gauge if the administration of HAART has a role to play in the observed association of HIV-1 *nef* polymorphisms with biomarkers of endothelial dysfunction and CAD. Additionally, an *in vitro* functional study should be carried out to investigate the effect of the HIV-1 *nef* variants reported in this study on expression levels of vWF and thrombomodulin in endothelial cell lines. The link between Nef and endothelial dysfunction should be investigated in clinical studies in which Nef plasma levels are measured in HIV-positive subjects alongside plasma biomarkers of endothelial dysfunction and direct measures of endothelial function such as flow-mediated dilatation. Currently, assays for the measurement of Nef in plasma are of poor sensitivity and specificity and therefore need to be refined. This may be achievable using liquid chromatography mass spectrometric (LCMS) techniques, and we are presently developing such assays.

CHAPTER SIX – REFERENCES

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APPENDICES

Appendix A: Human research ethical clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Mr O Mannafela

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M190714

NAME: (Principal Investigator)	Mr O Mannafela
DEPARTMENT:	School of Pathology Department of Chemical Pathology Medical School University
PROJECT TITLE:	Identification of HIV-1 nef Polymorphims in HIV-positive cardiac patients
DATE CONSIDERED:	2019/07/26
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Ms G Mezoh and Professor NJ Crowther
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2020/01/28

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **July** and will therefore reports and re-certification will be due early in the month of **July** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 19/02/2020

PLEASE QUOTE THE CLEARANCE CERTIFICATE NUMBER IN ALL ENQUIRIES

Appendix B: Permission to recruit study participants from the Cardiology Unit and Vascular Surgery Unit of Charlotte Maxeke Johannesburg Academic Hospital

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

DIVISION OF CARDIOLOGY

19 June 2019

Onkemetse Mannafela

Department of Chemical Pathology
Wits Medical School
7 York Road, Parktown
Johannesburg, 2193
South Africa

Mr Onkemetse Mannafela

Approval of study collaborator

Dear Mr Mannafela

This letter serves to confirm my support and approval for Mr Onkemetse Mannafela's study: **Identification of HIV-1 Type C Nef Polymorphisms in HIV Positive Cardiac Patients.**

Yours sincerely,

Dr Ngoba Tsabedze
Academic & Clinical Head - Division of Cardiology
Charlotte Maxeke Johannesburg Academic Hospital
University of the Witwatersrand
Email: Ngoba.Tsabedze@wits.ac.za
Tel: +2711 488 3611
CMJAH Speed Dial: #6462

Charlotte Maxeke Johannesburg Academic Hospital | Department of Internal Medicine | Division of Cardiology
York Road, Parktown 2193 | T +27 488 3611 | F +27 11 642 9041 | E info.cardiology@wits.ac.za | www.health.gov.za
IYUNIVESITHI YASEWITWATERSRAND | YUNIVESITHI YA WITWATERSRAND

02 November 2020

Genevieve Mezoh
Department of Chemical Pathology
Wits Medical School
7 York Road, Parktown
Johannesburg, 2193
South Africa

Approval of Study Collaborator

Dear Dr Genevieve Mezoh

This letter serves to confirm my support and approval as a collaborator for your project entitled "**Investigation into the effect of HIV viral proteins on endothelial function in the HIV-infected population**" and Mr Onkemetse Mannafela's study entitled "**Identification of HIV-1 Nef polymorphisms in HIV positive cardiac patients**". You are therefore granted permission to recruit study participants from our unit.

Yours Sincerely,

Dr. AJ ALAin
Head Vascular Surgery
CHBH.

Appendix C: Ethical clearance granted to Dr Genevieve Mezoh to use samples previously collected by Dr Alinda G. Vos and Dr Nereshni Lutchman

R14/49 Ms Genevieve Mezoh

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150979

NAME: (Principal Investigator)	Ms Genevieve Mezoh
DEPARTMENT:	Chemical Pathology
PROJECT TITLE:	Investigation into the Effect of HIV Viral Proteins on Endothelial Function in the HIV Infected Population (Previously M10408 Dr Nereshni Lutchman)
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	Renewal and New Principal Investigator
SUPERVISOR:	Prof Nigel Crowther

APPROVED BY: Professor P Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 23/09/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature Date 23/09/2015

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

07 September 2018

Ms Genevieve Mezoh
Chemical Pathology

Sent by email to: Genevieve.mezoh@wits.ac.za

Dear Ms Genevieve Mezoh

Re: **Protocol Ref no: M150979**
Protocol Title: Investigation into the effect of HIV Viral proteins on endothelial function in the HIV-Infected Population
Principal Investigator: Ms Genevieve Mezoh
Protocol Amendment: addition of samples

This letter serves to confirm that the Chairperson of the Human Research Ethics Committee (Medical) has approved the amendment for the above mentioned protocol, as detailed in your letter, dated 26 June 2018.

The following documents were received:

- Summary Letter
- Original ethics application
- Protocol
- Ethics clearance
- Information sheet and consent form
- Gatekeeper permission, protocol and information document

UNIVERSITY OF THE WITWATERSRAND
OFFICE OF THE DEPUTY VICE CHANCELLOR RESEARCH
07 SEP 2018
RESEARCH OFFICE HREC (Medical)
JOHANNESBURG

Thank you for keeping us informed and updated.

Yours Sincerely,

.....
Mr Joshua Ndlangamandla
Administrative Officer
Human Research Ethics Committee (Medical)

Research Office Secretariat: Faculty of Health Sciences, Phillip Tobias Building, 3rd Floor, Office 304, Corner York Road and 29 Princess of Wales Terrace, Parktown, 2193 Private Bag 3, Wits 2050 I **T**+27 (0)11-717-1234/2656/2700/1252 **E**: Rhulani.Mkansi@wits.ac.za I **Office E** HREC-Medical.ResearchOffice@wits.ac.za IWebsite: www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Appendix D: Patient information sheet

PATIENT INFORMATION SHEET

Study Title: **Identification of HIV-1 *nef* polymorphisms in HIV-positive cardiac patients**

Hello sir/madam

Introduction:
My name is Onkemetse Mannafela. I am a Masters student within the Department of Chemical Pathology at the University of Witwatersrand. The research project I am carrying out for my Masters degree seeks to understand how the human immunodeficiency virus (HIV) and treatment affects the blood vessels and heart of the body. Individuals infected with HIV may be at an increased risk of cardiovascular diseases (CVD), such as heart conditions and stroke, which result from the body's blood vessels becoming blocked or not functioning adequately. These effects have been shown in studies carried out on HIV-positive individuals in other countries. In South Africa, there are insufficient studies that associate HIV infection and highly active antiretroviral therapy (HAART) with increased risk of CVD. I would therefore like to look at these effects on South African urban HIV-positive adults who have experienced a cardiovascular event.
Research is a way to answer questions one has about a particular illness and to understand how the illness and its treatment affects the body. Accepting to take part in this research would entail you undergoing tests and processes which you were probably not previously familiar with in the clinic.

Invitation to participate:

I would like to invite you to take part in my study. Your participation in this study is voluntary and should you choose not to participate or leave the study at any time, this will not change your treatment at the clinic.

What is involved in this study:

1. I will ask and record your personal information (age, gender, treatment) according to a questionnaire. The questionnaire will require me to ask you questions about changes in body fat that you may have noticed at different regions on your body (face, arms, legs, back, breasts, belly, buttocks/hips). This process will take approximately 30 minutes and will be held at the Cardiology Unit of the Charlotte Maxeke Johannesburg Academic Hospital or Chris Hani Baragwanath Academic Hospital.

2. To do this study I must also carry out blood tests and body measurements where we will measure your height, weight, waist circumference and hip circumference, and your blood pressure. I will also ask you questions about your illness and treatment. I will ask you to come to the clinic early in the morning without eating breakfast (your last meal will be supper the night before) to do these tests. 5 ml of Bloods will be drawn by placing a needle into your arm. In your blood there are proteins and genetic material known as DNA. Changes in proteins and DNA are involved in disease processes. The tests done on your blood sample will include: measurements of (1) cholesterol; (2) glucose; (3) insulin; (4) a sample to measure cells involved in repair of the lining of blood vessels (5) a sample for protein and DNA analysis for future studies (2 - 10 years); (6) a sample to measure proteins involved in the functioning and inflammation of blood vessels; and, (7) a sample to measure proteins from the virus. The blood samples will be drawn by a qualified nursing sister assisting me and will be transported to the laboratory for storage by myself. The blood samples will be stored in a safe place with your study number as identification. The blood samples will be used only for the purposes explained to you and agreed to by you.

3. The imaging studies will involve using a probe that takes images/pictures of your blood vessels and heart to determine whether you have any cardiovascular diseases (angina/myocardial infarction/peripheral artery disease). This will be done by a qualified doctor in the Cardiology Unit or Vascular Surgery Unit of the Charlotte Maxeke Johannesburg Academic Hospital or Chris Hani Baragwanath Academic Hospital.

The sample of blood for protein and DNA / genetic studies will be used to look for changes in proteins and genes that are involved in endothelial dysfunction and development of CVD. Please note that all blood samples, including the DNA sample, will be stored in a safe place in my department where my supervisor and I will be the only people to have access to them. After completion of the study, the samples will be stored by my supervisor, Mrs Genevieve Mezoh, to be used for further analysis for the identification of potential novel markers that can be used to assess endothelial dysfunction in HIV-infected individuals. I will ask that you sign a separate

consent form to give me permission to use your DNA. The samples that you will provide will be stored for use until completion of the project for which it is intended for and up to a maximum period of 10 years for further analysis, thereafter, the samples will be discarded and destroyed. The blood samples will be used for no other purposes other than those I have described to you.

Risks and benefits of participating in the study:

The tests to be done on you are not harmful. The blood tests that will be done will involve using needles and this will be a little painful. The imaging tests are a bit uncomfortable as in when you have your blood pressure taken.

The potential benefit to you in participating in this study is that I will be able to determine if you have sugar diabetes, cholesterol problems, body fat changes or heart problems that were not previously picked up. In this way your treatment can include treating you for these conditions, or your HIV medication may be changed to a different combination of drugs. You may be referred to a different clinic or department for further treatment if any of the above-mentioned conditions are found. Also, as the study will give information about how the HIV virus and its treatment affects the heart and blood vessels, it may be possible that treatment can be given to all HIV-positive patients early to reduce the risk for these conditions.
The cardiologist (co-investigator) following you up for treatment will also receive the results and have access to the data identifier link. Therefore, your results will be given back to you via your cardiologist.

There is neither cost nor payment in taking part in the study.

Confidentiality:

Your name will be known to me only. I will give you a number and this number will be used at all times as your identification on forms, tests, and during blood sampling. Your name with the corresponding number that will be given to you and all other information recorded from you will remain confidential (please see separate patient data sheet identified with number only). The data sheets with your name and identifying number will be kept by me i.e. the researcher, in a secure place (under lock and key) or my supervisor with the number allocated to you as per data sheet. Your information will be given with your permission to your doctor who is treating you if this will benefit your treatment. Information and results that will be published from this study will not result in you being identified as an individual. This will be done anonymously using the test results as a group. Please be advised that every effort will be made to keep your information confidential. However, absolute confidentiality cannot be guaranteed as your personal information may be disclosed if required by law.

Contact details of researcher:

If you have any questions or queries at any time during the study, please contact me on telephone number 076 564 1036, or by e-mail on sirmannafela@gmail.com, or my supervisor, who is Mrs G Mezoh on telephone number 011 489 8559, or by e-mail on genevieve.mezoh@wits.ac.za

This study has been approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg ("Committee"). A principal function of this Committee is to safeguard the rights and dignity of all human subjects who agree to participate in a research project and the integrity of the research.

If you have any concern over the way the study is being conducted, please contact the Chairperson of this Committee who is Dr Clement Penny, who may be contacted on telephone number 011 717 2301, or by e-mail on Clement.Penny@wits.ac.za. The telephone numbers for the Committee secretariat are 011 717 2700/1234 and the e-mail addresses are Zanele.Ndlovu@wits.ac.za and Rhulani.Mukansi@wits.ac.za

Thank you for reading this information sheet.

January 2020

Appendix E: Consent form

Study title: ***Identification of HIV-1 nef Polymorphisms in HIV-1 cardiac patients***

I have read the Information Sheet provided, or it has been read to me. Any questions I had were answered to my satisfaction. I agree to take part in the above clinical study. The procedures to be carried out have been explained to me. The possible discomforts, risks and benefits involved in taking part in the test have also been described to me. I understand that I can leave the study at any point, without any consequence. I also understand that if I have any questions concerning the test then the investigator will explain these to me.

Contact details:

Mr O Mannafela, Principal Investigator, telephone number 076 564 1036, or by e-mail on sirmannafela@gmail.com,

Mrs G Mezoh, Supervisor, telephone no. 011 489 8559, or by e-mail at genevieve.mezoh@wits.ac.za.
Prof N Crowther, Co-supervisor, telephone no. 011 489 8525, or by email at nigel.crowther@nhls.ac.za

Dr CB Penny, Chairperson of the Human Research Ethics Committee (Medical) at the University of Witwatersrand, on telephone no. 011 717 2301, or by e-mail at Clement.Penny@wits.ac.za.
Ms. Z Ndlovu or Mr Rhulani Mkansi, Committee Secretariat, telephone nos.: 011 717 2700 or 1234, or by e-mail at: Zanele.Ndlovu@wits.ac.za or Rhulani.Mkansi@wits.ac.za

Name of Participant: _____

Date: _____

Place: _____

Signature or mark _____

Witnessed by:

Name of Witness: _____

Signature: _____

Date: _____

Appendix F: Informed consent for DNA sampling and storage

INFORMED CONSENT FOR DNA SAMPLING AND STORAGE

Study title: ***Identification of HIV-1 nef Polymorphisms in HIV-1 cardiac patients***

I have read the Information Sheet provided on blood storage for DNA testing, or it has been read to me. Any questions I had were answered to my satisfaction. I understand that:

1. Only the investigator, Mr Mannafela and his supervisor will have access to the blood sample.
2. The sample of blood for protein and DNA/ genetic studies will be used to look for genes and proteins that are involved in endothelial dysfunction and development of CVD. The blood samples will be used for no other purposes.
3. I will be identified by study number and not by name.
4. My blood sample will be stored until completion of the study and for a maximum period of 10 years for further analysis, thereafter, the samples will be discarded and destroyed. However, should I choose not to take part in further studies, my samples will be discarded and destroyed immediately upon completion of this study.
5. I may withdraw from the study at any time, without having to give a reason and without any consequence, following which my samples will be discarded and destroyed immediately.

Contact details:

Mr O Mannafela, Principal Investigator, telephone number 076 564 1036, or by e-mail on sirmannafela@gmail.com,

Mrs G Mezoh, Supervisor, telephone no. 011 489 8559, or by e-mail at genevieve.mezoh@wits.ac.za.
Prof N Crowther, Co-supervisor, telephone no. 011 489 8525, or by email at nigel.crowther@nhls.ac.za

Dr CB Penny, Chairperson of the Human Research Ethics Committee (Medical) at the University of Witwatersrand, on telephone no. 011 717 2301, or by e-mail at Clement.Penny@wits.ac.za.
Ms. Z Ndlovu or Mr Rhulani Mkansi, Committee Secretariat, telephone nos.: 011 717 2700 or 1234, or by e-mail at: Zanele.Ndlovu@wits.ac.za or Rhulani.Mkansi@wits.ac.za

Name of Participant: _____

Date: _____

Place: _____

Signature or mark _____

Witnessed by:

Name of Witness: _____

Signature: _____

Date: _____

Appendix G: Data collection sheet

DATA COLLECTION SHEET

Participant's Number: _____ **Date:** _____

Patient's age: _____ HIV status: _____

Anti-retroviral therapy: YES ☐ NO ☐

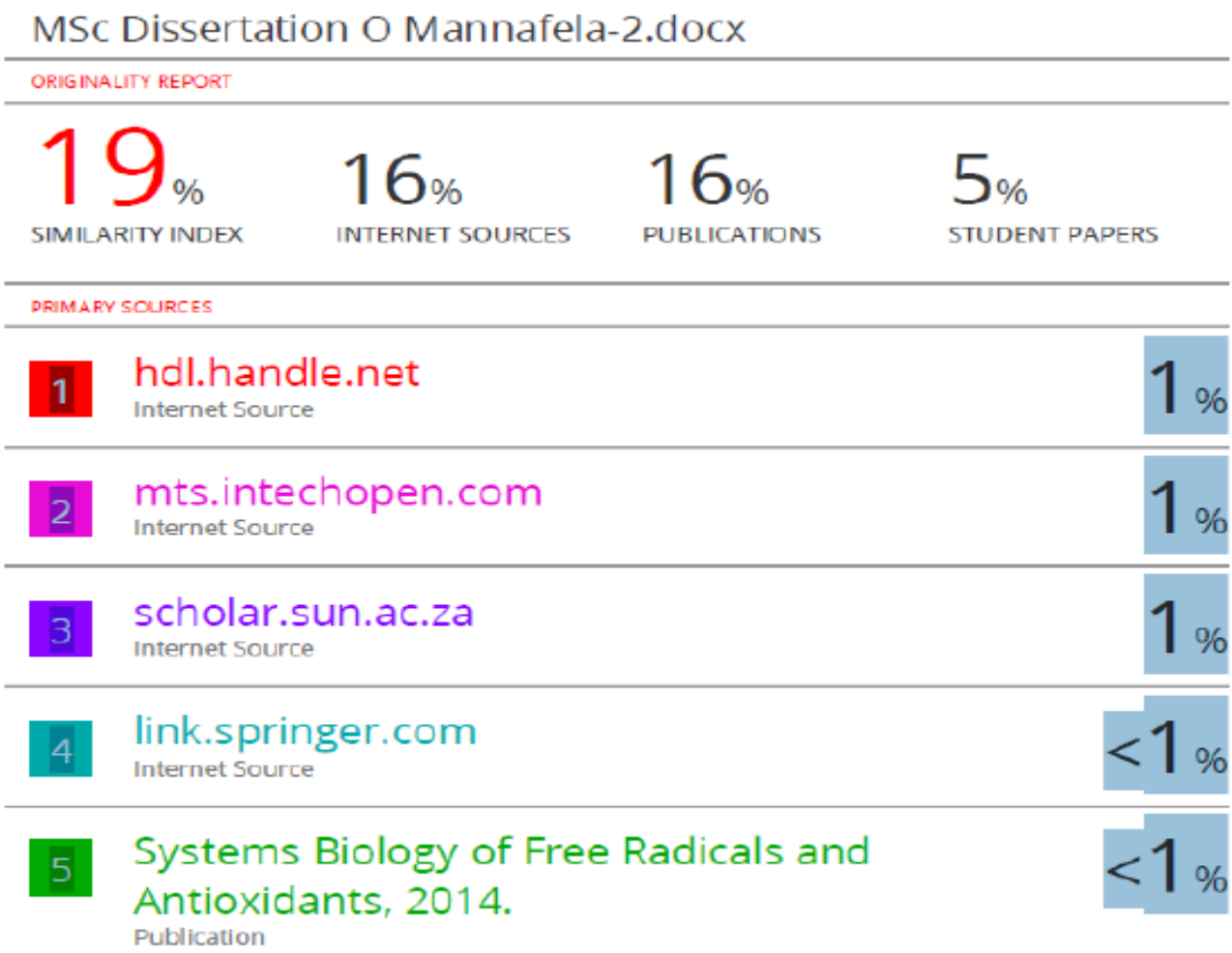
Anti-retroviral treatment regimen:

- Current regimen:
- Previous regimen (if any):

List of any current medications:

CLINICAL FEATURES	PERSONAL HISTORY	FAMILY HISTORY
Coronary artery disease		
Diabetes mellitus		
Hypertension		
Cerebrovascular events		
Recent infections		
Malignancies		
TRADITIONAL RISK FACTORS OF CVDs		
Medication		
Alcohol		
Smoking		
Recreational drugs		
BIOCHEMICAL MEASUREMENTS		
Viral load		
CD4 COUNT		
Glucose		
high-density lipoprotein cholesterol		
Total cholesterol		
Low-density lipoprotein cholesterol		
Triglycerides		
Insulin		
ANTHROPOMETRIC MEASUREMENTS		
Height		
Weight		
Waist circumference		
Hip circumference		
BP Dia/Sys		

Appendix H: Turn-it-in Report



MSc Dissertation O Mannafela-2.docx

ORIGINALITY REPORT

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