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Investigation into the Effect of HIV Viral Proteins on Endothelial Function in the HIV-Infected Population

Genevieve Mezoh



A Thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy in Medicine (Chemical Pathology)

Johannesburg, 2020.

Supervisor: Prof Nigel J Crowther

Co-supervisor: Prof Penny L Moore

Declaration

I, Genevieve Mezoh declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

Student Name	Signature	Date
Ms Genevieve Mezoh	.....	09th September 2020

Dedication

I hereby dedicate this thesis to my husband, the king of Muwah - Pinyin, HRM King Taka IV (Muluh Taka Winston), for his unwavering love and support, and my children, Princess Tema, Chloe Mahwungum, and Nigel Pennji, for their patience and emotional support.

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Mezoh G., Lutchman N., Prigge K., Cave E., Worsley C., Mayne E., Mabvakure B., Lambson B., Moore P.L. and Crowther N.J. (2018) HIV-1 *nef* Polymorphisms Associated with Endothelial Dysfunction (poster)

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Mezoh G. and Crowther N.J. (2019) Deciphering Endothelial Dysfunction in the HIV-Infected Population. *Advances in Experimental Medicine and Biology*, 1134**,** 193-215.

ABSTRACT

Background: Infection with HIV is associated with an increased risk of cardiovascular disease (CVD). This may be due to the negative effect of viral proteins (Nef and Tat) on endothelial function. This has been proven in *in vitro* experiments but not in clinical studies. Currently, the measurement of Nef and Tat in human serum is hampered by the lack of availability of validated assays. Furthermore, the viral *nef* and *tat* genes contain numerous sequence variants, which may be associated with different levels of endothelial dysfunction. Therefore, the aims of this study were to investigate whether endothelial dysfunction is present in African subjects with HIV infection, identify its possible determinants in this population and assess if it is related to variants in the viral *nef* and *tat* genes. Lastly, to develop an efficient method for the quantification of HIV viral proteins in human serum.

Materials and Methods: Eighty HIV-infected treatment-naïve Black South Africans were recruited and compared against 60 healthy age-matched HIV-uninfected individuals. The HIV-infected participants were followed up for 18 months (n=13) following initiation of anti-retroviral therapy (ART). Blood pressure, height, weight, body mass index (BMI), waist and hip circumference, triglycerides, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol, glucose, viral load and CD4 counts were measured. Serum biomarkers of endothelial function *i.e.* intercellular adhesion molecule-1 (ICAM-1), endothelial leukocyte adhesion molecule-1 (E-selectin), vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1), as well as markers of inflammation *i.e* tumor necrosis factor-α (TNF-α), interleukin 6 (IL6) and interleukin 8 (IL8) were quantified using the Luminex platform, while von Willebrand Factor (vWF) and HIV-1 Nef were quantified by ELISA. HIV-1 *nef* and *tat* genes were sequenced from 30 and 34 patients respectively, and sequence analysis performed to identify potential mutations

associated with high levels of biomarkers of endothelial function and inflammation. A second cohort consisting of 140 HIV-infected individuals on ART was recruited for whom carotid-intima medial thickness (CIMT) measurements were available. This cohort was divided into 2 groups: CIMT < 0.50 and CIMT > 0.58 mm. The HIV-1 *nef* gene was successfully sequenced from 60 patients, and variant frequencies compared between the two groups. An *in silico* tryptic digest was performed with HIV-1 *nef* sequences to identify conserved, endogenous target Nef peptides for quantification by tandem mass spectrometry (LC-MS/MS) using the Sciex QTrap 5500 with MicroLC.

Results: The HIV-infected cohort had higher levels of ICAM-1, VCAM-1 and vWF compared to the HIV-uninfected cohort of which ART initiation decreased plasma levels of ICAM-1 and VCAM-1 despite increases in serum levels of TNF-α, IL-6 and IL-8 post-treatment. Serum levels of VCAM-1 and vWF correlated positively with low CD4 counts and high viral load, respectively. HIV-1 polymorphisms that were significantly associated with markers of endothelial dysfunction and inflammation were seen with the following HIV-1 Nef peptide sequence variants: V16I, H40Y, T50H,A, S169N and H188Q,S with ICAM-1; Y202F with VCAM-1; K182M with MCP-1; and D205N with TNF-α. In the HIV-infected cohort on ART, the following HIV-1 Nef peptide sequence variants were found to be significantly associated with higher CIMT: K39R, H40Y, D177E, F143Y and V180T. The Nef assay method developed on the LCMS identified the target Nef peptide standards and endogenous Nef in human serum.

Conclusions: Collectively, our data shows evidence of endothelial dysfunction in the South African HIV-infected population, despite ART administration, and several variants in HIV Nef associated with elevated serum levels of markers of endothelial dysfunction and CIMT, confirming a possible role for this viral protein in impacting endothelial function. Thus, targeting HIV Nef is a potential therapeutic strategy to circumvent the development of CVD in

HIV-infected patients. A LC-MS/MS method for the quantification of Nef in human serum was developed but requires further refinement.

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List of Abbreviations

AIDS - Acquired immunodeficiency syndrome

ART - Antiretroviral therapy

ARV - antiretroviral drug

bFGF - Basic fibroblast growth factor

BMI - Body mass index (BMI)

CA - Capsid

CACs - Circulating angiogenic cells

CAD - Coronary artery disease

CCA - Common carotid artery

CECs - Circulating endothelial cells

CIMT - carotid-intima media thickness

CMD - Cardiometabolic disease

CRP - C-reactive protein

crPWV - Carotid radialis pulse wave velocity

CVD - Cardiovascular disease

ELISA - Enzyme-linked immunosorbent assay

EMPs - Endothelial micro-particles

eNOS - Endothelial nitric oxide synthetase

Env - Envelope

EPCs - Endothelial progenitor cells

E-selectin - Endothelial leukocyte cell adhesion molecule-1

ESI - Electrospray ionization

FMD - Flow-mediated dilation

Gag - Group-specific antigen

GMCSF - Granulocyte-macrophage colony-stimulating factor

HAART - Highly active antiretroviral therapy

HCAEC - Human coronary arterial endothelial cells

HDL-C - High-density lipoprotein cholesterol

HIV - Human immunodeficiency virus

HIV-1 - Human immunodeficiency virus type-1

HIV-2 - Human immunodeficiency virus type-2

ICAM-1 - Intercellular adhesion molecule-l

IL-1β - Interleukin-1β

IL-6 - Interleukin-6

IL-8 - Interleukin-8

IL-17 - Interleukin-17

IN - Integrase

INSTIs - Integrase strand transfer inhibitors

IRIS - Immune reconstitution inflammatory syndrome

LC - Liquid chromatography

LCMS - liquid chromatography mass spectrometry

LC-MS/MS - Liquid chromatography tandem mass spectrometry

LTR - Long terminal repeats

MA - Matrix

MCP-1 - Monocyte chemoattractant protein-1

MCSF - Macrophage colony-stimulating factor

MMP - Matrix metalloproteinase

MS - Mass spectrometer

NC - Nucleocapsid

Nef - Negative regulator factor

Nef - Viral negative effector

NF-kB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NNRTIs - Non-nucleoside reverse transcriptase inhibitors

NO - Nitric oxide

NRTIs - Nucleoside reverse transcriptase inhibitors

PAK2 - p21 activated protein kinase

PCRs - Polymerase chain reactions

PDGF - Platelet derived growth factor

PIGF - Placental growth factor

PIs - Protease inhibitors

Pol - Polymerase

PR - Protease

Rev - Regulator of virion gene expression

ROS - Reactive oxygen species

RT - Reverse transcriptase

SAX - Strong anion exchange

SCX - Strong cation exchange

SISPROT - Simple and integrated spintip-based technology

STATs - Signal transducers and activators of transcription

SU - Surface glycoprotein

Tat - Transcriptional transactivator protein

TM - Transmembrane glycoprotein

TNF-α - Tumour necrosis factor α

UHPLC - Ultra-high-performance liquid chromatography

VCAM-1 - Vascular cell adhesion molecule-1

VEGF - Vascular endothelial growth factor

Vif - Viral infectivity factor

Vpr - Viral protein r

Vpu - Viral protein u

vWF - von-Willebrand factor

WHO - World health organization

Chapter 1: Literature Review

1.1 General introduction

There are more than 37 million people infected with the human immunodeficiency virus (HIV), with sub-Saharan Africa being the most severely affected region (UNAIDS, 2017). The introduction of highly active antiretroviral therapy (HAART) has increased the life expectancy of HIV-infected patients however, cardiovascular disease (CVD) has emerged as an important cause of mortality among infected patients. Despite effective antiretroviral therapy, HIV-infected patients have a higher risk of myocardial infarction and cardiovascular death than age-matched uninfected controls (Freiberg et al., 2013, Chow et al., 2012). Although an increased prevalence of traditional risk factors, such as dyslipidaemia and smoking, among HIV-infected patients likely contributes to this increased cardiovascular morbidity, growing evidence suggests that HIV infection and treatment are more directly linked to endothelial dysfunction, likely through inflammation and immune dysregulation (Wang et al., 2015b, Ronsholt et al., 2013). This thesis seeks to investigate the potential role of HIV viral proteins, such as HIV-1 Nef, in endothelial dysfunction,

1.2 HIV

1.2.1 Epidemiology of HIV/AIDS

The human immunodeficiency virus (HIV) is a lentivirus that causes the progressive deterioration of the human immune system over time. This condition, referred to as acquired immunodeficiency syndrome (AIDS), renders infected persons more susceptible to life-threatening opportunistic infections.

The global pandemic of HIV infection currently stands at about 5000 new HIV infections per day (UNAIDS, 2019). A total of 32.7 – 44.0 million people are infected with HIV of which 50 % are women

above the age of 15 years and 5 % are children below the age of 15 years (UNAIDS, 2019, UNAIDS, 2017). The development of antiretroviral drugs (ARVs) for HIV treatment and increased accessibility to ARVs has decreased the number of reported HIV cases from 3.7 million new infections in 1997 to 1.7 million new infections in 2018 (UNAIDS, 2019). In addition, AIDS-related deaths has decreased by 48 % from 1.7 million reported cases in 2004 to 770, 000 in 2018 (UNAIDS, 2019). Sub-Saharan Africa accounts for up to 61 % of the global HIV-infected population (UNAIDS, 2019). However, a decrease in new HIV infections has been observed in this region, due to the effective rollout of ARVs in sub-Saharan Africa (UNAIDS, 2019). In the Eastern and Southern Africa region, out of the 85 % of HIV-infected people who knew their status, 67 % were on treatment with 58 % of them attaining viral suppression (UNAIDS, 2019). Despite an increase in ARV coverage in Eastern Europe and Central Asia, the number of AIDS-related deaths has increased by 5 % from 36,000 in 2010 to 38,000 in 2018 (UNAIDS, 2019). Of the 71 % of people living with HIV in the Eastern Europe and Central Asia regions who knew their status, only 38 % were on treatment with only 29 % being virally supressed (UNAIDS, 2019).

Figure 1.1 gives an overview of the HIV pandemic across the African continent in 2016. Out of the 36.7 million people living with HIV globally, 71 % were in Africa of which 53 % were in Eastern and Southern Africa, 17 % in Western and Central Africa, while only 1 % were in the Middle East and North Africa (UNAIDS, 2017). In Africa, 11 countries have an HIV prevalence of greater than 5 % (percentage of population 15 - 49 years of age). At the top of the list is Swaziland with an adult HIV prevalence of 28.8 %, followed by Lesotho, Botswana, South Africa, Zimbabwe, Namibia, Zambia, Mozambique, Malawi, Uganda and Kenya. Eastern and Southern Africa accounted for the majority of new infections compared to the rest of Africa in 2017 (UNAIDS, 2017). Several hypotheses have been proposed to explain the disparity in the global distribution of HIV, with sub-Saharan Africa carrying the highest burden of HIV

cases. The driving factors behind this disparity are complex and include a mixture of socioeconomic, sociocultural and epidemiological factors (Temah, 2009).

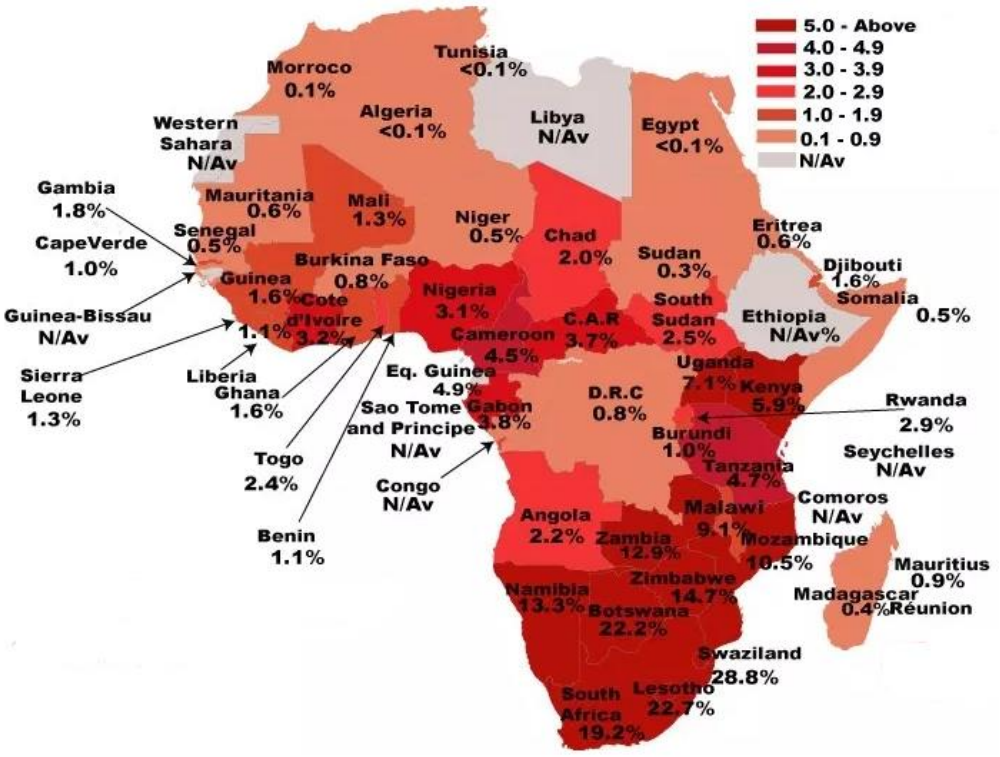


Figure 1.1: Map of Africa showing HIV prevalence in adults. Adult HIV prevalence, as defined by the % of population 15 - 49 years of age, is represented by the different colour codes indicated in the top right corner of the map. Taken from Afri-dev.info, licensed under a creative commons attribution 2.0 generic license (Afri-Dev.Info, 2016).

South Africa has one of the largest HIV-infected populations, and is home to about 7.5 million people living with HIV, with the number of HIV-infected women being twice that of men, as shown in Table 1.1 (UNAIDS, 2019). However, the rate of new HIV infections has decreased between 2005 to 2018 (see Table 1.1). New infections appear to be more common in young females and older males (Zuma et al.,

2016, Shisana et al., 2014). Although South Africa has the largest HIV treatment programme in the world (UNAIDS, 2017), a large proportion of infected individuals do not receive treatment (Table 1.1).

Table 1.1: HIV epidemiologic data of South Africa from UNAIDS, 2019

	Population	Range
HIV-Infected Population According to Age Group	Adults (≥ 15yrs)	7,500,000 [6,800,000 – 8,100,000]
	Children (≤ 14yrs)	260,000 [200,000 - 360,000]
HIV-Infected Adults According to Gender	Female	4,700,000 [4,300,000 – 5,000,000]
	Male	2,800,000 [2,500,000 - 3,100,000]
HIV Prevalence	Female (15 - 49yrs)	23.7 [20.9 - 26.7]
	Male (15 - 49yrs)	14.0 [10.9 - 15.8]
New HIV Infections for Both Adults and Children	2005	500,000 [470,000 - 530,000]
	2010	380,000 [350,000 - 410,000]
	2018	240,000 [210,000 - 270,000]
New HIV Infections According to Gender	Female (≥ 15yrs)	140,000 [120,000 - 160,000]
	Male (≥ 15yrs)	86,000 [75,000 -100,000]
	Children (≤ 14yrs)	14,000 [11,000 - 35,000]
ART coverage	Female (≥ 15yrs)	66% [61% - 72%]
	Male (≥ 15yrs)	53% [48% - 58%]
	Children (≤ 14yrs)	58% [45% - 77%]

1.2.2 Molecular epidemiology of HIV

The HIV displays a high degree of genetic variability and has been categorised into groups and sub-types, as shown in Table 1.2. The HIV is divided into HIV-1 and HIV-2, however, HIV-1 is more predominant worldwide compared to HIV-2 (Robertson et al., 1995). The HIV-1 is further classified into four different groups; M, N, O and P. Group M is the most common, accounting for up to 90% of HIV/AIDS cases and is further divided into subtypes (or clades) A, B, C, D, E, F, G, H, J and K (Robertson et al., 1995). The

HIV-1 group M is geographically distributed across the globe with subtype C being the most prevalent subtype in South and East Africa (Hussein et al., 2000, Harmelen et al., 1999), and India (Neogi et al., 2012), whereas subtype B is the most predominant version in North and South America, Europe and Australia (Paraskevis et al., 2009). The HIV-1 groups N, O and P have only been reported thus far in Cameroon (Vallari et al., 2011, Ayouba et al., 2000). However, with constant migration and mixing of populations the geographical distribution of the various HIV clades is not static. In addition to this, continuous evolution of the virus has resulted in new versions, classified as circulating recombinant forms (CRFs) and unique recombinant forms (URFs), increasing the complexity of the molecular epidemiology of HIV (Barin et al., 1997, Sönnerborg et al., 1997). The CRF01_AE is more common in South-East Asia, while CRF02_AG is more prevalent in West Africa and CRF03_AB in Eastern Europe and Central Asia (Hemelaar et al., 2006). The HIV-2 is divided into eight groups; A, B, C, D, E, F, G and H, with group A and B being the most common forms (Santiago et al., 2005). The virulence of HIV-1 is high whereas that of HIV-2 is low (Campbell-Yesufu and Gandhi, 2011).

Table 1.2: Molecular classification of HIV and geographical distribution

Specie	Virulence	Group	Subtype	Prevalence
HIV-1	High	M	A	Eastern Europe and Central Asia
			B	Australia, Western Europe, the Americas and the Caribbean
			C	Southern Africa, Ethiopia and India
			D	North Africa and Middle East
			E	Southeast Asia
			F	Central Africa and Latin America
			G	West and Central Africa
			H	Central Africa
			J	Central Africa
			K	Central Africa
		N	-	Cameroon
		O	-	Cameroon
		P	-	Cameroon
HIV-2	Low	A	-	West Africa, Angola, Mozambique, Brazil, India, Europe and U.S.
		B	-	West Africa
		C	-	Liberia
		D	-	Liberia
		E	-	Sierra Leone
		F	-	Sierra Leone
		G	-	Ivory Coast
		H	-	Ivory Coast

1.2.3 HIV morphology and genomic organisation

The HIV-1 virion is a spherical retrovirus composed of two single strands of RNA, that codes for the HIV-1 genome, enclosed in a capsid (Figure 1.2). The genome of HIV comprises 9 genes; *gag* (group-specific antigen), *pol* (polymerase), *vif* (viral infectivity factor), *vpr* (viral protein r), *tat* (transcriptional transactivator protein), *rev* (regulator of virion gene expression), *vpu* (viral protein u), *env* (envelope) and *nef* (viral negative effector), that encode for 16 different proteins. The structural proteins that make up the virion and outer membrane envelope i.e. matrix (MA), capsid (CA), nucleocapsid (NC) and p6, are encoded for by *gag*, while the viral enzymes protease (PR), reverse transcriptase (RT), and integrase (IN), responsible for viral replication are encoded for by the Pol gene (Frankel and Young, 1998). The HIV-1 virion uses the host cell membrane to form the viral envelope. This envelope contains complex proteins made up of a coat protein, gp120, which is a surface glycoprotein (SU) attached to a transmembrane glycoprotein (TM), gp41 (Frankel and Young, 1998). The virus also has four accessory proteins; Vpr, Vif, Vpu and Nef, and two regulatory proteins, Tat and Rev, that control its ability to infect target host cells, produce new virions and cause disease (Frankel and Young, 1998).

The HIV Nef protein is a myristoylated accessory protein known to play a major role in HIV replication and pathogenesis (Foster et al., 2011). It is made up of 206 amino acids and has a molecular weight of 27-35 kD. Myristoylation of the Nef protein is a prerequisite for its biological activity and is determined by the first 6 amino acids (MGXXXS) at the N-terminal domain (Peng and Robert-Guroff, 2001). Some of the known functions of HIV Nef include the downregulation of CD4 and MHC class I molecules, activation of p21 activated protein kinase (PAK2), enhancement of virion infectivity via a CD4-independent mechanism and inhibition of immunoglobulin class switching (Foster et al., 2011). However,

there are several unknown Nef functions that are yet to be elucidated, some of which are suspected to be contributors to the pathogenic activity of HIV (Watkins et al., 2013).

The HIV-1 Tat protein is made up of 86 to 101 amino acid residues, depending on the HIV subtype, encoded for in two exons. Thus, Tat exon 1 consists of 72 amino acids while the second coding exon of HIV-1 Tat is made up of 28 amino acid residues. Tat Exon 1 is responsible for activating transcription from the 5' LTR (Kuppuswamy et al., 1989). The main function of Tat exon 2 has not yet been elucidated, however, it is postulated to contribute towards the transactivation activity of Tat (Jeang et al., 1993), and may also play a role in trans-repression (Howcroft et al., 1993) and virus replication (Neuveut and Jeang, 1996). The main role of gp120 is that of viral entry into the host cell. The gp120 protein is extremely polymorphic, compared to Nef and Tat, with a divergence rate of 1 % - 2 % per year (Novitsky et al., 2009).

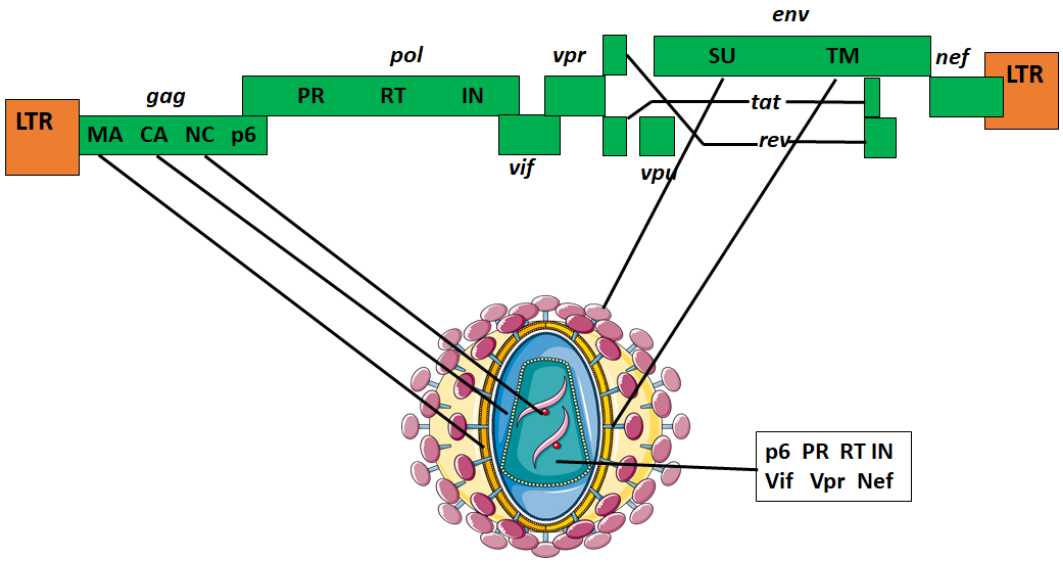


Figure 1.2: Genome organisation of HIV-1. The genome of HIV consists of 9 genes; *gag*, *pol*, *vif*, *vpr*, *tat*, *rev*, *vpu*, *env* and *nef*, and two long terminal repeats (LTR) at both ends (unpublished G Mezoh, 2019).

1.2.4 Infection and replication of HIV

The virus gains entry into the body by the transfer of vaginal fluids, semen or blood, following which it infects T helper cells and replicates to produce copies of the virus. The infection and replication cycle of HIV (Figure 1.3) comprises several steps. The first step involves binding of the HIV gp120 surface protein to the CD4 molecules on the surface of the target host cell. In addition, there are two chemokine co-receptors on the host cell; CCR5 and CXCR4, that facilitate entry of the HIV depending on the viral strain. Binding of gp120 to the CD4 molecule initiates a conformational change in gp120, exposing the chemokine binding domains on gp120, and enabling gp41 to penetrate the cell membrane. During this process, the viral and cellular membranes fuse together permitting virus entry into the host cell, following which the viral coat is removed, thereby releasing its RNA genome into the cell.

The viral RNA strands are reverse transcribed into a double stranded linear DNA helix, catalysed by the viral RT (Zheng et al., 2005). Given that this process is prone to errors, several mutations can be introduced into the viral DNA. The linear viral DNA is then transported into the nucleus of the host cell as a pre-integration complex where it integrates into the host's genome, facilitated by the viral IN. The viral DNA is flanked at both ends by long terminal repeats (LTR) of which the 5' LTR acts like a eukaryotic promoter, initiating the transcription process, while the 3' LTR acts as the polyadenylation and termination site. The viral DNA is then transcribed into mRNA, some of which is spliced into mRNA transcripts. The spliced mRNAs encode for the accessory and regulatory proteins while the unspliced mRNAs encode for the structural proteins. The spliced mRNA transcripts are exported from the nucleus to the cytoplasm where they are translated into their corresponding proteins: Vpr, Vif, Vpu, Nef, Tat and Rev. The spliced mRNAs can move from the nucleus to the cytoplasm without any support, however, the unspliced mRNAs require

Rev for their nuclear export to the cytoplasm where they are translated to Gag and Env (Pollard and Malim, 1998). Transcription of the provirus is largely upregulated by Tat following cellular activation.

The final step of the HIV replication cycle is the assembly of the newly formed virions which commences at the plasma membrane of the infected host cell. Furin facilitates cleavage of the Env protein into the gp120 and gp41 envelope glycoproteins in the Golgi complex. The glycoproteins are transported to the host cell's plasma membrane where gp120 holds gp41 to the infected cell's membrane. As the newly formed virion begins to bud from the infected cell, the Gag and Gag-Pol proteins, along with the HIV genomic RNA, associate with the inner surface of the plasma membrane. The viral PR catalyses cleavage of the Gag polyproteins into the structural proteins MA, CA and NC, which then assemble to yield the mature HIV virions that can move on to infect other cells (Sundquist and Kräusslich, 2012) .

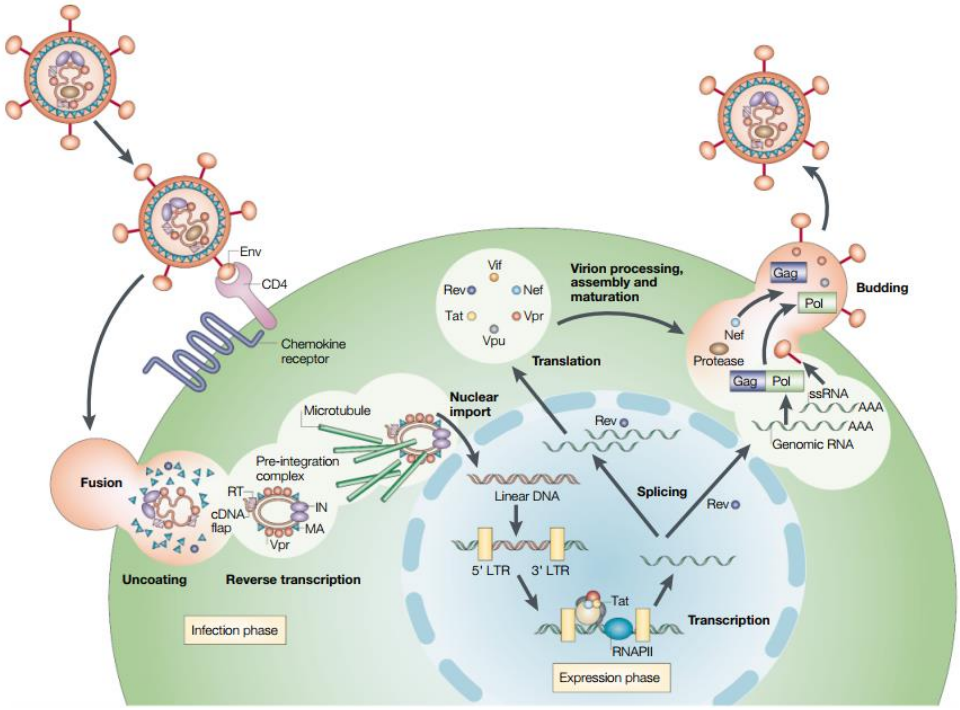


Figure 1.3: HIV-1 replication cycle. The HIV-1 replication process consists of nine main steps: 1) Fusion of the viral particle to the target cell to permit its entry; 2) Uncoating of the viral particle thereby releasing the RNA strands; 3) Reverse transcription of the viral RNA to DNA; 4) Nuclear import of the viral DNA; 5) Transcription of the viral DNA in the nucleus of the infected cell; 6) Splicing to yield the HIV mRNA transcripts which are exported out of the nucleus; 7) Translation of the viral RNA transcripts into biologically active proteins; 8) Assembly of the new HIV; 9) Budding at the host cell membrane to release the new mature HIV. Reproduced with permission from Springer Nature: Nature Publishing Group, Nature Reviews Immunology (Peterlin and Trono, 2003), Copyright 2003.

1.3 HIV infection and treatment

1.3.1 Antiretroviral therapy

To date, there is no eradication cure for HIV. Therefore, treatment is a life-time commitment to antiretroviral therapy (ART). There are six classes of ARTs, designed to inhibit viral proteins required for HIV entry into the host cell and replication. These include nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors such as the integrase strand transfer inhibitors (INSTIs), and fusion and entry inhibitors such as the CCR5 antagonists (Cihlar and Fordyce, 2016). Currently, to achieve virologic suppression, the recommended ART regime is a combination of at least three drugs from two different classes (Cihlar and Fordyce, 2016). This proposed ART regimen, regarded as highly active antiretroviral therapy (HAART), has proven to be effective in supressing viral load, as it targets more than one process required for HIV replication (Barton et al., 2013). Table 1.3 shows the different drugs currently approved by the U.S. Food and Drug Administration (FDA) for HIV treatment (Services, 2018).

The first group of ARVs to be synthesised were the NRTIs. They act as competitive inhibitors, competing for the active binding site of reverse transcriptase, thereby prohibiting the reverse transcription of viral RNA into DNA (Weller and Williams, 2001). However, due to the high level of toxicity of NRTIs, NNRTIs were developed (Weller and Williams, 2001). The NNRTIs act as non-competitive inhibitors in which they bind to sites other than the active site of reverse transcriptase, resulting in a conformational change of the active site of the enzyme, and as a consequence, inhibiting enzyme activity (Weller and Williams, 2001). Although toxicity with the NNRTIs is low, inhibition is specific to the reverse transcriptase of only HIV-1 (Weller and Williams, 2001). Given that the viral RT is subject to point mutations, its activity is not effectively inhibited by NRTIs and NNRTIs when administered as

monotherapy, thus, a combination of drugs is required. The third major class of ARVs are the PIs, which compete for the protease active binding site, resulting in enzyme inactivity. Inhibition of viral protease prevents formation of the viral structural proteins; hence, immature viral particles are produced.

Challenges such as high toxicity with long term usage, inadequate antiviral potency and low degree of tolerability, has led to the development of new classes of ARTs, including fusion and entry inhibitors (Meanwell and Kadow, 2007). These new classes block the viral receptors and viral entry. Another recently developed class of ARTs is the INSTIs, which inhibit viral integrase, prohibiting integration of the viral DNA into the host's genome (Evering and Markowitz, 2007).

Table 1.3: List of FDA-approved drugs for the treatment of HIV

Drug Class	Generic Name	Brand Name	Release Date
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	Lamivudine and zidovudine	Combivir	27-Sep-97
	Emtricitabine, FTC	Emtriva	02-Jul-03
	Lamivudine, 3TC	Epivir	17-Nov-95
	Abacavir and lamivudine	Epzicom	02-Aug-04
	Zalcitabine, dideoxycytidine, ddC (withdrawn)	Hivid	19-Jun-92
	Zidovudine, azidothymidine, AZT, ZDV	Retrovir	19-Mar-87
	Abacavir, zidovudine, and lamivudine	Trizivir	14-Nov-00
	Tenofovir disoproxil fumarate and emtricitabine	Truvada	02-Aug-04
	Enteric coated didanosine, ddI EC	Videx EC	31-Oct-00
	Didanosine, dideoxyinosine, ddI	Videx	9-Oct-91
	Tenofovir disoproxil fumarate, TDF	Viread	26-Oct-01
	Stavudine, d4T	Zerit	24-Jun-94
	Abacavir sulfate, ABC	Ziagen	17-Dec-98
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Rilpivirine	Edurant	20-May-11
	Etravirine	Intelence	18-Jan-08
	Delavirdine, DLV	Rescriptor	4-Apr-97
	Efavirenz, EFV	Sustiva	17-Sep-98
	Nevirapine, NVP	Viramune (Immediate Release)	21-Jun-96
	Nevirapine, NVP	Viramune XR (Extended Release)	25-Mar-11
Protease Inhibitors (PIs)	Amprenavir, APV (withdrawn)	Agenerase	15-Apr-99
	Tipranavir, TPV	Aptivus	22-Jun-05
	Indinavir, IDV,	Crixivan	13-Mar-96
	Saquinavir (withdrawn)	Fortovase	7-Nov-97
	Saquinavir mesylate, SQV	Invirase	6-Dec-95
	Lopinavir and ritonavir, LPV/RTV	Kaletra	15-Sep-00
	Fosamprenavir Calcium, FOS-APV	Lexiva	20-Oct-03
	Ritonavir, RTV	Norvir	1-Mar-96
	Darunavir	Prezista	23-Jun-06
	Atazanavir sulfate, ATV	Reyataz	20-Jun-03
	Nelfinavir mesylate, NFV	Viracept	14-Mar-97
Fusion and entry inhibitors - CCR5 co-receptor antagonist	Enfuvirtide, T-20	Fuzeon	13-Mar-03
	Maraviroc	Selzentry	06-Aug-07
HIV integrase strand transfer inhibitors	Raltegravir	Isentress	12-Oct-07
	Dolutegravir	Tivicay	13-Aug-13
	Elvitegravir	Vitekta	24-Sep-14

Despite success with the new anti-HIV drugs approved by the FDA, complete eradication of the virus remains a problem owing to the existence of HIV reservoirs, thus inflammation persists, possibly due to the continued release of viral peptides. Non-communicable diseases such as diabetes mellitus, liver, renal and cardiovascular diseases (CVDs), are more prevalent in the HIV-infected population compared to uninfected individuals, with CVDs now being the leading cause of death in the HIV-infected population (Lorenc et al., 2014).

1.3.2 HIV infection and therapy as cardiovascular disease risk factors

The role of HAART in the increase in CVD incidence in the HIV-infected population is controversial. In a population-based cohort study conducted in Denmark, HIV-infected patients receiving HAART had an increased risk of ischemic heart disease compared with the general population (Obel et al., 2007). A similar study carried out in France showed an increased risk of myocardial infarction in HIV-infected patients receiving HAART compared to the general population (Lang et al., 2010). In a nested case-control study conducted in Québec involving 7,053 HIV-infected subjects on ARVs, compared to 27,681 HIV-uninfected subjects, the risk of acute myocardial infarction (AMI) increased with usage of ARVs such as abacavir, efavirenz, lopinavir and ritonavir (Durand et al., 2011). Acute myocardial infarction risk was highest with the protease inhibitor, ritonavir compared to the nucleoside reverse transcriptase inhibitor, abacavir and the non-nucleoside reverse transcriptase inhibitor, efavirenz (Durand et al., 2011).

However, a retrospective study conducted in Northern California showed no association between ARVs and CVDs such as AMI and coronary heart disease (CHD) (Klein et al., 2002). The study consisted of 4,159 HIV-infected males, 35 to 64 years of age, compared with 39,877 gender and age-matched HIV-uninfected controls. Risk of AMI and CHD did not increase with exposure to protease inhibitors or other

ARVs however, the risk of these diseases was higher in the HIV-infected cohort compared to the uninfected control group (Klein et al., 2002). This suggests that the virus, rather than ARVs, itself could be responsible for the increased CVD risk in the HIV-infected population. This hypothesis is supported by the Strategies for Management of Antiretroviral Therapy (SMART) Study which involved 5,472 adult HIV-infected participants on treatment, with a CD4+ cell count of greater than 350 cells/mm^3 at the time of recruitment (El-Sadr et al., 2006). In this study, 50 % stayed on treatment while 50 % had their treatment interrupted based on levels of their CD4+ cell count (El-Sadr et al., 2006). Participants who had episodic HIV treatment were more predisposed to renal, hepatic and cardiovascular disease (El-Sadr et al., 2006).

Some studies suggest increased levels of lipids in HIV-infected subjects on ARVs (Viale et al., 2016, Zephy et al., 2015). Dyslipidaemia was proposed as a mechanism by which ARVs contribute to the increased CVD risk. A study carried out with 8,033 Swiss participants showed that individuals receiving ART had higher levels of triglycerides and cholesterol (Glass et al., 2006). In a cross-sectional study in Denmark involving 17,852 HIV-infected participants, of whom 13 % were ART-naïve, lipodystrophy and long-term exposure to ARVs were associated with increased cholesterol levels (Friis-Møller et al., 2003). However, a longitudinal study has shown that HIV infection is associated with a fall in serum cholesterol levels, which increase after the initiation of ART (Riddler et al., 2003). This data therefore suggests that the rise in lipid levels observed with ART is due to a reduced viral load and a resulting increase in serum lipids back to pre-ART levels.

1.4 Cardiovascular disease in the HIV-infected African population

The incidence of CVD in the HIV-infected population of high-income countries has been extensively studied. Although sub-Saharan Africa is the most affected region in terms of the HIV epidemic, the

incidence of CVD in the HIV-infected population is unclear. In a study from Congo, out of 157 HIV-infected ARV-naïve patients, 25 to 51 years of age, 55 % of the participants experienced a cardiac event within a follow-up period of 7 years (Longo-Mbenza et al., 1998). In Malawi, 36 % of the 1,703 HIV-infected patients receiving ARVs had heart disease while 43 % had stroke (SanJoaquin et al., 2013). In a more recent study in Malawi, 379 HIV-infected patients on ART did not have a higher risk of developing CVD compared to 356 uninfected individuals (Rücker et al., 2018), contrary to findings reported from European and American studies (Chow et al., 2012, Bergersen et al., 2004). The authors suggested that the conflicting observations could be as a result of the different ART regimes used as well as different HIV-infected sub-populations. In a cross-sectional study carried out in Cameroon on 44 HIV-infected patients, 35 – 60 years of age, of which 70.5 % were on ART, HIV infection was positively associated with heart disease (Menanga et al., 2015). In South Africa, 30 ART-naïve HIV-infected patients, 35 – 50 years of age, compared against HIV-uninfected individuals with acute coronary syndrome revealed the HIV-infected group to be much younger and have a higher degree of thrombus formation, despite fewer traditional CVD risk factors compared to the non-infected group (Becker et al., 2010). This finding is supported by a cross-sectional study carried out in Botswana involving 179 participants in which HIV-infected patients had an increased risk of cardiomyopathy and pericarditis (Schwartz et al., 2012). On average, 54 % of the study population were receiving ART. In the African studies discussed above, only half (3 out of 6) included an HIV-uninfected control group.

In a meta-analysis carried out with 12,757 HIV-infected participants, 50 % of which were on HIV treatment, compared with 16,998 HIV-uninfected participants from sub-Saharan Africa, HIV infection was associated with elevated levels of triglycerides and lower levels of high-density lipoprotein cholesterol (HDL-C), body mass index (BMI), systolic and diastolic blood pressure, whereas HIV

treatment was associated with higher levels of low-density lipoprotein cholesterol (LDL-C) and HDL-C (Dillon et al., 2013).

Studies from outside of Africa tend to suggest a direct role of HIV in CVD risk, while the results from Africa are less consistent. The mechanisms by which viral infection may modulate risk for CVD are currently not well understood, however some investigations have shown that HIV effects on endothelial function may be involved.

1.5 The endothelium

1.5.1 Endothelial site and function

The endothelium is a single layer of cells that runs throughout the body internally lining the walls (intima) of the blood vessels. It is therefore the principal barrier separating blood from tissues. Endothelial cells secrete a number of substances to regulate numerous blood vessel functions including vascular tone, which involves vasodilatory substances such as endothelium derived hyperpolarizing factor, nitric oxide and prostacyclin, or vasoconstrictive substances such as endothelin-1 and thromboxane (Sandoo et al., 2010); cell adhesiveness, which involves adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and activated endothelial leukocyte cell adhesion molecule-1 (E-Selectin) (Reglero-Real et al., 2016); and coagulation involving substances such as von Willebrand factor (vWF) and thrombomodulin (Wang et al., 2018, Brevetti et al., 2008). An imbalance in the levels of these mediators can result in endothelial dysfunction (Ludmer et al., 1986). This systemic pathological state of the endothelium is the earliest clinically detectable stage of cardiovascular disease (Verma S, 2002).

1.5.2 Endothelial cell activation

Endothelial cells express transmembrane adhesion proteins such as endothelial leukocyte adhesion molecule-1 (E-selectin), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). The expression of these adhesion molecules by the endothelium is increased in response to proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Gimbrone et al., 1997) and in response to turbulent blood flow. These factors increase the expression of endothelial adhesion molecules by activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-*k*B) pathway (Bruyndonckx et al., 2013). This phenomenon, referred to as endothelial cell activation, enables the transmigration of monocytes across the endothelium, which is a primary factor in the initiation of atherosclerotic plaque formation (Liao, 2013, Gimbrone et al., 1997). During the course of infection, migration of monocytes from their site of origin (progenitor cells in the bone marrow) across the endothelium to infected areas plays a vital role in the control and clearance of viruses, bacteria, fungi and protozoa (Shi and Pamer, 2011).

1.5.3 Endothelial dysfunction

Endothelial dysfunction is defined as the decreased bioavailability of nitric oxide (NO) derived from the endothelium, and is caused by a number of factors including hypercholesterolaemia, smoking and oxidative stress (Liao, 2013). The synthesis of NO in endothelial cells is catalysed by the enzyme endothelial nitric oxide synthetase (eNOS), which is activated by shear stress brought about by the continuous rhythmical flow of blood (Dimmeler et al., 1999). Endogenous NO has several functions which include inhibition of white cell and platelet activation, inhibition of vascular smooth muscle cell proliferation, smooth muscle relaxation and vasodilation (Furchgott and Zawadzki, 1980). Levels of NO are reduced by its interaction with superoxide, which is produced from oxygen by the action of NADPH

oxidase (Virdis et al., 2010). Failure of the endothelium to respond to shear stress, due to endothelial dysfunction, would therefore lead to a decrease in the endothelium-derived NO with a concomitant build-up of superoxide which further decreases levels of NO. Given the vital role NO plays in smooth muscle relaxation, a prerequisite for vasodilation, a decrease in NO also leads to vasoconstriction (Flammer and Luscher, 2010, Virdis et al., 2010). A decrease in the bioavailability of NO also leads to greater endothelial activation which results in increased platelet aggregation, vascular smooth muscle cell proliferation, greater monocyte adhesion and transmigration and oxidative stress, which promote atherosclerosis (Davignon and Ganz, 2004).

1.5.4 Atherosclerotic plaque formation

Atherosclerosis is characterised by the narrowing of blood vessels, due to plaque formation, which obstructs blood flow to the heart (coronary artery disease), brain (ischemic stroke) or lower extremities (peripheral vascular disease). The mechanism of plaque formation in atherosclerosis involves several complex processes which take place in the intima of arteries, and includes the retention of lipoproteins, recruitment of inflammatory cells, formation of foam cells, apoptosis and necrosis, smooth muscle cell proliferation and matrix synthesis, calcification, angiogenesis, arterial remodelling, rupturing of the fibrous cap and thrombosis (Bentzon et al., 2014). The process begins with the accumulation of LDL-C in the arterial intima. The lipid portions of LDL-C are oxidised and the modified LDL-C molecules aggregate. Modification of LDL-C by oxidation and aggregation enable them to stimulate innate and adaptive immune responses, initiated by their binding to the proteoglycans of the intima. This results in endothelial cell activation, characterised by the expression of adhesion molecules such as ICAM-1 and VCAM-1; chemoattractants such as monocyte chemoattractant protein-1 (MCP-1); and growth factors such as granulocyte-macrophage colony-stimulating factor (GMCSF) and macrophage colony-stimulating

factor (MCSF) (Bentzon et al., 2014). Monocytes are subsequently recruited and differentiate into dendritic cells and macrophages which ingest the retained lipoproteins to form foam cells (Bentzon et al., 2014). The mechanisms enabling this process involves scavenger-like receptors that mediate the uptake of oxidized LDL-C, hydrolysis of free cholesterol from aggregated LDL-C and direct uptake of remnant lipoproteins or native LDL-C (Bentzon et al., 2014). Over time, the foam cells accumulate within the proteoglycan layer of the intima to form xanthomas or fatty streaks. Further progression of the latter results in the development of atherosclerotic lesions which may further develop into necrotic cores (referred to as fibroatheromas). Collagen-rich fibrous connective tissue expands within the lesions to replace the loose fibrocellular tissue. With time, the necrotic core and surrounding tissue undergoes calcification resulting in the formation of fibrocalcific plaques (Bentzon et al., 2014).

1.5.5 Risk factors for atherosclerosis

Post-mortem studies show that the atherosclerotic process starts in individuals as early as childhood, and by the early third decade of life, 20% of adults will have advanced lesions (Hong, 2010, Stary et al., 1995). Core factors known to influence endothelial function and to therefore modify risk for atherosclerosis include age (Skaug et al., 2013), male sex (Skaug et al., 2013), family history of diabetes and CVD (Goldfine et al., 2006), physical activity (Siasos et al., 2013), insulin resistance (Steinberg et al., 1996), hyperglycaemia (Makimattila et al., 1996), active and passive smoking (Celermajer et al., 1993), visceral fat (Romero-Corral et al., 2010), obesity (Steinberg et al., 1996), hypertension (Virdis et al., 2001) and dyslipidaemia (Masia et al., 2010). Infection with HIV has been proposed as a further risk factor for endothelial dysfunction (Grunfeld et al., 2009, Solages et al., 2006) (Figure 1.4).

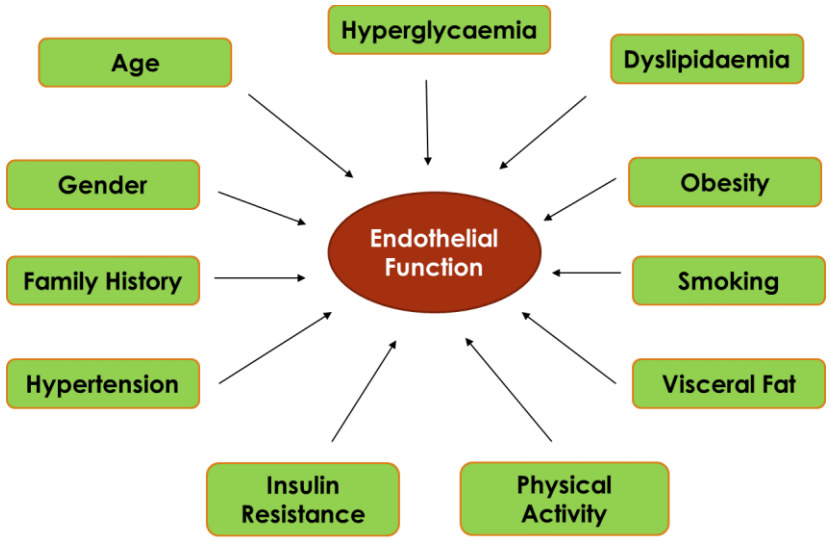


Figure 1.4: Factors that affect endothelial function. Several factors are known to have an impact on the endothelial cell state. Some of these include hyperglycaemia, dyslipidaemia, obesity, smoking, visceral fat, physical activity, insulin resistance, hypertension, family history of endothelial dysfunction, gender and age.

1.5.6 Endothelial repair

The endothelium is exposed to shear stress induced by turbulent blood flow, which is common in areas where the vasculature branches and are sites at which atherosclerotic plaques are often formed (McLenachan et al., 1990). Endothelial damage at such sites initiates a repair process which involves the localization of circulating cells originating from the bone-marrow. These cells include circulating angiogenic cells (CACs) and endothelial progenitor cells (EPCs). Angiogenic cytokines, such as the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), placental growth factor (PlGF) and platelet derived growth factor (PDGF) are produced from the site of endothelial damage and induce movement of EPCs to the damaged endothelium where they are integrated into the sites of active angiogenesis (Bruyndonckx et al., 2013, Asahara et al., 1997). A recent study has shown that in an animal

model of atherosclerosis, treatment with EPCs reduces the level of atherosclerosis (Georgescu et al., 2016). Endothelial damage also results in the release of circulating endothelial cells (CECs) and endothelial micro-particles (EMPs) into the blood circulation (Blann et al., 2005). As a consequence, CACs are recruited to the site of endothelial damage for vascular repair (Wassmann et al., 2006).

The formation of an atherosclerotic plaque can therefore be seen as a complex process, involving both endothelial activation and dysfunction. Reduced production and/or activity of endothelial-derived NO appear to be central to this process, but the molecular pathways involved are only partially understood. In addition, recruitment of EPCs to the site of endothelial damage seems to be essential to the attenuation of plaque formation. Many factors can initiate endothelial dysfunction and activation, and HIV infection may be one such agent.

1.5.7 Endothelial dysfunction in HIV-infected patients

1.5.7.1 ARVs and endothelial dysfunction

Several studies report endothelial dysfunction in the HIV-infected population despite controlled viremia (Hsue et al., 2009, Blanco et al., 2006). Some studies suggest that HAART has no negative impact on the endothelium; rather, it alleviates endothelial dysfunction (Francisci et al., 2009, Arildsen et al., 2013). However, a study conducted by Hsue *et al.* (2009) showed endothelial dysfunction to be associated with abacavir. In this study, HIV-infected patients receiving the NRTI, abacavir, as first line therapy, had significantly lower flow-mediated dilation (FMD) measurements compared to HIV-infected patients receiving the NRTI, tenofovir, as treatment (Hsue et al., 2009). On the contrary, other studies report ARVs to ameliorate endothelial function. In a longitudinal study involving 56 HIV-infected subjects, of which 28 were either on PIs or NNRTIs, and 10 were ART-naïve, compared with 28 gender and age-matched

HIV-uninfected controls, levels of VCAM-1, MCP-1 and vWF in the HIV-infected cohort on treatment decreased over a period of 12 months, with no significant difference observed between the two classes of ARVs (Francisci et al., 2009). However, for subjects that were ART-naïve, levels of VCAM-1, MCP-1 and vWF were higher than HIV-uninfected controls, which remained unchanged after the 12 months follow-up period. In a retrospective cohort study conducted in Italy on HIV-infected patients before and after initiating HAART it was observed that HIV infection, and not HIV treatment, influenced endothelial function (Francisci et al., 2009). Short-term treatment with HAART rather improved endothelial dysfunction. In addition, a more recent study conducted in Denmark confirmed this observation, with administration of HAART improving endothelial dysfunction (Arildsen et al., 2013).

Long-term virological suppression of HIV by the administration of ARVs appears not to fully restore endothelial function, as seen by reports from studies conducted on HIV-infected patients of African descent (Mosepele et al., 2018, Fourie et al., 2015). Thus, in a study conducted in South Africa involving 66 HIV-infected patients on ART for a duration of 2 years, compared with 78 HIV-infected ART-naïve and 166 HIV-uninfected Black African subjects, the odds ratio for elevated levels of ICAM-1 and VCAM-1 was lower in the HIV-infected cohort on ART compared to the ART-naïve group (Fourie et al., 2015). In a similar study carried out in Botswana with 112 HIV-infected adults on long-term ART (10 years), compared with 84 HIV-uninfected controls, multivariate analysis showed HIV infection to be associated with higher levels of ICAM-1 and VCAM-1 (Mosepele et al., 2018). However, it is important to note that the studies discussed above were cross sectional and not longitudinal studies.

1.5.7.2 Viral effects on endothelial function

Several studies show that the virus itself may affect endothelial function. Thus, a cross-sectional study demonstrated that a high viral load was associated with lower FMD (Solages et al., 2006). In addition, HIV-infected ART-naïve patients are reported to have higher levels of circulating markers of endothelial dysfunction such as ICAM-1, VCAM-1, vWF and E-selectin compared to healthy individuals (Fourie et al., 2011). This therefore suggests that the HIV potentially affects endothelial cells, either by a direct or indirect mechanism, ultimately resulting in endothelial impairment (Bussolino et al., 2001). The mechanism by which the virus may affect the endothelium is not known, however, there is some evidence to suggest that it may be related to the effects of viral proteins.

Despite the control of viral replication and viremia by ART, it is suggested that HIV stays hidden in reservoirs where they continue to replicate (Lorenzo-Redondo et al., 2016, Hellmuth et al., 2015) and to exert actions through the production and release of viral proteins (Wang et al., 2015a, Wang et al., 2014, Matzen et al., 2004). These viral proteins could potentially be a causative factor contributing towards the development of CVD within the ARV-treated, HIV-infected population by their effects on endothelial function (see section 1.5.1).

1.5.8 Assessment of endothelial function

There are several methods by which endothelial function can be assessed, which can be either invasive or non-invasive (Table 1.4) (Flammer et al., 2012). Non-invasive methods are preferred due to low cost, more patient compliance and ease of use when compared to invasive strategies (Flammer et al., 2012,

Verma et al., 2003). Invasive strategies include techniques such as quantitative coronary angiography, intracoronary Doppler and strain-gauge venous impedance plethysmography (Newby and Fox, 2002).

The quantification of cells involved in the process of endothelial repair, such as the endothelial micro-particles (EMPs) and endothelial progenitor cells (EPCs), by flow cytometry, has been used as a strategy to investigate endothelial dysfunction (da Silva et al., 2011). However, this technique is limited by the fact that the circulating EPCs are present in low numbers, and there is no specific marker currently available that can be used for their identification (Yoder, 2009). To address this problem, a combination of markers has been utilised to measure EPC numbers (da Silva et al., 2011, Masouleh et al., 2010). However, there are several drawbacks to the use of these flow cytometric methods for the assessment of endothelial dysfunction such as the requirement of highly specialised equipment, trained personnel, and low turn-around times.

Another non-invasive strategy is the measurement of flow-mediated dilation (FMD). This involves the ultrasound measurement of the diameter of the brachial arteries following a brief period of occlusion and reflow in which reactive hyperaemia is induced. Reduction of endothelial function as demonstrated by a decrease in flow-mediated vasodilation of the brachial artery has been observed in HIV-infected subjects (Sun et al., 2015, Grunfeld et al., 2009). However, this procedure is not subject to automation, is time consuming and requires the availability of specialised equipment and skilled personnel. In addition, significant variation has been observed in day-to-day measurements, most likely due to changes in the biological circadian rhythms (Verma et al., 2003).

A more recent non-invasive approach is the plethysmographic recordings obtained from the fingertip using the EndoPAT (endothelial peripheral arterial tonometry) device (Kuvin et al., 2003, Lavie et al., 2000).

The finger arterial pulse wave amplitude is recorded at rest, following which the forearm is cuffed for 5 minutes to induce reactive hyperaemia, and plethysmographic readings taken in 30 seconds intervals for 4-5 minutes following occlusion. Advantages of the EndoPAT over FMD include the fact that it is easier to perform, less variability in day-to-day readings, and higher sensitivity (Flammer et al., 2012). However, this technique is more expensive compared to FMD as it requires the use of disposable finger probes. Therefore, as with FMD, this approach may not be easily adopted in resource-limited countries. Moreover, EndoPAT readings have been associated with several traditional and metabolic cardiovascular risk factors, thus non-endothelial factors can influence the signal (Hamburg et al., 2011, Schnabel et al., 2011).

Ultrasound measurement of the thickness of the two inner layers of the carotid artery (the intima and the media), referred to as carotid-intima media thickness (CIMT), has been proposed as a non-invasive strategy to assess endothelial dysfunction as it provides a quantitative measure of sub-clinical atherosclerosis. Advancements in ultrasound technology has led to the creation of smaller, user-friendly and less-costly instruments, with automated edge detector programs, thereby making measurements faster and less variable (Darabian et al., 2013). Evaluation of the carotid arteries is achieved using linear phased array probes with a frequency of 7 to 15 MHz. The distance between two echogenic lines, separated by echo lucent space in the wall of the artery, provides a measure of CIMT. Measurements of the intima and media are each taken thrice in the anterior, lateral and posterior planes. CIMT measurements are affected by age, gender and ethnicity.

The thickness of the arterial wall increases as lipids and inflammatory cells accumulate in the vessel wall. An increase in the thickness of the arterial wall is associated with an increase in the risk of myocardial infarction and stroke. Several studies report a positive correlation to exist between CIMT and CVD risk

(Owolabi et al., 2015, Den Ruijter et al., 2012). Measurement of CIMT can therefore me used as a tool to screen for individuals at high risk of cardiovascular events. In a meta-analysis conducted with eight population-based cohort studies, a 0.1 mm increase in the CIMT was associated with a 10–15% increase in the risk of myocardial infarction and a 13–18 % increase in stroke events (O'Leary and Bots, 2010). However, a lack of correlation between CIMT and markers of endothelial function has been observed in some studies (Hileman et al., 2014, Falcao Mda et al., 2012).

The use of circulating plasma markers for the assessment of endothelial function would be a more practical and sustainable approach in resource-limited environments. Such immunoassays could easily be automated enabling shorter turn-around times. However, this technique may lack specificity in that some of these markers are elevated under conditions not associated with endothelial dysfunction (Worm and Hsue, 2010, Witkowska and Borawska, 2004), and do not necessarily originate from endothelial cells (Fonsatti et al., 1997). This problem may be partially overcome by measuring multiple blood markers. The following section of this thesis will discuss each of these blood markers, including circulating cells, as markers of endothelial function in more detail.

Table 1.4: Methods used for the assessment of endothelial function

	Technique	Principle	References
Invasive Strategies	Quantitative coronary angiography	Infusion of a vasodilator such as acetylcholine, bradykinin or serotonin, followed by measurement of changes in the vascular diameter. Patients with good endothelial function show a vasodilatory response whereas either vasoconstriction or no change in vascular diameter is observed in those with endothelial dysfunction.	Aymong (2002), Ludmer et al. (1986)
	Intracoronary Doppler	Infusion of an endothelium-dependent vasodilator (such as acetylcholine) and direct dilator of vascular smooth muscle (such as papaverin) by means of a Doppler catheter followed by measurement of intracoronary blood flow velocity and cross-sectional arterial area.	Zeiher et al. (1991)
	Strain-gauge venous impedance plethysmography	Direct brachial artery administration of agonists of vasodilation in the forearm, followed by measurement of change in blood flow.	Verma et al. (2001), Mather et al. (2001)
Non-invasive Strategies	Brachial artery ultrasound (flow mediated vasodilation)	Brachial artery diameter measured using a high-resolution ultrasound transducer before and after a brief period of occlusion. Reflow after occlusion results in raised shear stress that causes endothelial dependent dilation in patients with healthy endothelial function. This function is impaired in patients with endothelial dysfunction.	Skaug et al. (2013), Thijssen et al. (2011)
	Peripheral arterial tonometry (EndoPAT)	Arterial blood volume measured in the fingertip using a plethysmograph before and after a brief period of occlusion. Reflow results in arterial volume changes which increases the measured signal.	Kuvin et al. (2003), Costa-Hong et al. (2018)
	Immunoassays including enzyme-linked immunosorbent assays (ELISAs)	Formation of an antibody-antigen complex, with antigen concentration determined by the quantification of light emitted by a conjugated fluorescent protein (antibody or antigen).	De Pablo-Bernal et al. (2014), Fourie et al. (2011)
	Flow cytometry	Quantification of forward and scattered-light emitted by labelled cells.	Sinha et al. (2016), da Silva et al. (2011)
	Immuno-magnetic separation	Antibody-antigen binding of CECs (similar to ELISA) coupled with fluorimetry for quantification of the selected autofluorescent CECs.	Lopez et al. (2012), Makin et al. (2004)

1.5.9 Blood-based markers of endothelial function

1.5.9.1 Adhesion molecules

The ICAM-1 and VCAM-1 molecules are trans-membrane proteins belonging to the immunoglobulin superfamily and found on the surface of leukocytes and endothelial cells (Garton et al., 2003, Fonsatti et al., 1997). Though attached to the membrane, they also exist in a soluble form in biological fluids (Teppo et al., 2001, Fonsatti et al., 1997). These adhesion proteins are similar in structure and function. They play a major role in cell-cell adhesion by stabilizing cell-cell interactions (Huo et al., 2000), facilitate the migration of leukocytes across the endothelium and are expressed upon cytokine stimulation (Garton et al., 2003). Thus, cytokines such as TNF-α and IL-6 activate the signal transducers and activators of transcription (STATs) signalling pathway, and the STATs bind to NF-*k*B in the ICAM-1 promotor leading to upregulation of expression (Wung et al., 2005). Reactive oxygen species (ROS) are required for the binding of STATs to NF-*k*B (Roy et al., 2001). Therefore, increases in the levels of ROS as a result of endothelial dysfunction would further increase levels of ICAM-1 and VCAM-1. Increased expression of the adhesion molecules on endothelial cells results in the recruitment of inflammatory immune cells (Newton and Dixit, 2012, Muller, 2011).

The ICAM-1 and VCAM-1 molecules are not simultaneously expressed in all diseases involving an inflammatory process (Cybulsky et al., 2001). Thus, a study conducted by Mason et al showed the levels of sICAM-1 and sVCAM-1 to be significantly higher in patients with rheumatoid arthritis compared to healthy controls, however, in patients with lupus erythematosus, only sVCAM-1 was elevated and not sICAM-1 (Mason et al., 1993). Therefore, different inflammatory diseases may be associated with specific adhesion or inflammatory molecules.

Elevated levels of ICAM-1 and VCAM-1 in blood indicates an increase in their expression on the cell surface, and thus an increase in the transmigration of leukocytes across the endothelial cells. This increase in movement across the endothelium results in the build-up of pressure at the surface of the endothelial cells (Nagel et al., 1994). Eventually, endothelial shedding occurs resulting in the detachment of the endothelial trans-membrane proteins and their shedding into the blood circulation (Bruyndonckx et al., 2013).

Blood flow through the blood vessels is generally laminar. However, obstruction to blood flow as a result of atherosclerotic plaques generates rhythmic patterns. *In vitro* studies show that laminar and oscillatory shear stress upregulates ICAM-1 expression (Chiu et al., 2003, Chappell et al., 1998). However, up-regulation of VCAM-1 is either to a much lesser extent compared to ICAM-1 or non-existent (Walpola et al., 1995, Nagel et al., 1994), and prolonged exposure to shear stress down-regulates both adhesion molecules (Sampath et al., 1995, Chiu et al., 2003).

Endothelial leukocyte adhesion molecule-1, often referred to as E-selectin or ELAM-1, is similar to VCAM-1 and ICAM-1 in that it is also a trans-membrane adhesion molecule that facilitates cell-cell interaction and the transmigration of leukocytes into tissues following cytokine activation. However, E-selectin differs from VCAM-1 and ICAM-1 in that it is solely expressed on endothelial cells. In addition, it promotes the adhesion of resting CD4+ memory cells to endothelial cells (Shimizu et al., 1991, Chiu et al., 2003). This adhesion molecule could therefore be regarded as a plasma marker of choice for the assessment of endothelial function given that it is endothelium specific. However, continuous blood flow and influx of leukocytes to inflamed tissues results in shear stress which inhibits E-selectin expression (Chiu et al., 2007, Chiu et al., 2003). Levels of sICAM-1, sVCAM-1 and E-selectin are equally elevated

in other conditions not associated to endothelial dysfunction such as multiple sclerosis (Tsukada et al., 1993) and cancer (Liu et al., 1999, Pizzolo et al., 1993). In addition, other factors such as ethnicity (Miller et al., 2003) and high-density lipoprotein cholesterol (HDL-C) (Toth, 2010, Nofer et al., 2002) also influence blood levels of sICAM-1 and sVCAM-1. High-density lipoprotein cholesterol down regulates expression of the adhesion molecules and upregulates endothelial cell migration and proliferation (Nofer et al., 2002).

A number of studies has observed higher serum levels of adhesion molecules in HIV-infected compared to healthy controls, and these are described in Table 1.5 below.

Table 1.5: Studies comparing serum markers of endothelial dysfunction in HIV-infected and non-infected subjects

Authors	Study populations	Effectors and markers of endothelial function analysed	Results of comparison of cases versus controls	Associations
Miller et al. (2010)	106 HIV-infected children, of which 86% were on HAART, against 55 normal control children aged 12-15 years	C-reactive protein (CRP) IL-6 MCP-1 sICAM-1 sVCAM-1 E-Selectin	Increase Increase Increase Increase Increase Increase	Correlation between all endothelial markers with higher waist:hip ratio, low CD4 counts and high viral load.
Giuliano Ide et al. (2008)	83 HIV-infected children on HAART against 83 uninfected healthy children aged 7-13 years	CIMT CRP	Increase Increase	Positive association between HIV infection with CIMT and elevated CVD risk factors. Negative association between CIMT and HDL-C
De Pablo-Bernal et al. (2014)	A cross-sectional study of 39 HIV-infected ARV-naïve patients aged 29-45 years compared with three different groups; (i) 26 age-matched (27-37 years) and (ii) 26 elderly (64-81 years) uninfected healthy controls; and (iii) 26 non-survivor elderly subjects (74-87 years).	CRP TNF-α INF-γ IL-1β IL-6 IL-8 IL-17	Increase Increase Increase Below detection Increase Increase Below detection	No association between any markers of inflammation and HAART.
	A longitudinal study of 39 HIV-infected ARV-naïve patients at baseline, and after 48 and 96 weeks of ARV administration.	CRP TNF-α INF-γ IL-1β IL-6 IL-8	No change Decrease Decrease Below detection Decrease Decrease	

		IL-17	Below detection	
Fourie et al. (2011)	300 ARV-naïve HIV-infected participants compared with 300 uninfected controls aged 36-52 years.	IL-6 CRP sICAM-1 sVCAM-1 Carotid radialis pulse wave velocity (crPWV)	Increase Increase Increase Increase No difference	Positive association between crPWV and age in the HIV-infected group. Positive association of HIV-infection with levels of ICAM-1 and VCAM-1.
Fourie et al. (2015)	66 HIV-infected subjects on HAART compared with 165 HIV-uninfected controls.	IL-6 CRP sICAM-1 sVCAM-1 crPWV	No difference No difference Increase Increase No difference	Positive association between HIV-infection with ICAM-1 and VCAM-1. Inverse association between VCAM-1 with CD4 count.
Seigneur et al. (1997)	90 HIV-infected subjects on HAART versus 90 uninfected controls	vWF sTM E-selectin TNF-α IFN-α	Increase Increase Increase Increase Increase	Negative correlation between sTM and CD4+ cell count. Positive correlation of vWF with TNF-α and IFN-α Higher level of markers of endothelial dysfunction in HIV-infected subjects.
Mosepele et al. (2018)	112 HIV-infected adults on long-term HAART, 35-45 years of age, compared with 84 HIV-uninfected controls aged 33-43 years.	IL-6 sICAM-1 sVCAM-1 E–selectin CIMT	No difference No difference Increase No difference No difference	Positive correlation between HIV infection with increased levels of VCAM–1 but not ICAM–1 or E–selectin. No association of IL-6 with markers of endothelial dysfunction.

1.5.9.2 von Willebrand factor (vWF)

von Willebrand factor (vWF) is a glycoprotein present in blood plasma that plays a role in haemostasis (Sadler, 1998). It is synthesized by endothelial cells (Jaffe et al., 1974) as well as megakaryocytes (Nachman et al., 1977). It is thought to be an excellent blood-based marker for the assessment of

endothelial function, with several studies demonstrating a strong inverse correlation between vWF and FMD (Freestone et al., 2008, Felmeden et al., 2003). Thus, a study conducted by Felmeden et al (Felmeden et al., 2003) in which 89 hypertensive subjects were compared against 43 healthy subjects before and after 6 months of cardiovascular risk management, revealed a significant ($r = -0.517$, $P < 0.001$) inverse correlation between vWF and FMD. Following 6 months of antihypertensive treatment and intensive cardiovascular risk factor management, vWF levels decreased with a simultaneous increase in FMD. Moreover, both vWF and FMD correlated significantly with 10-year cardiovascular risk. In contrast, another study in which 32 obese children were compared against 20 healthy controls with regards to measurements of E-selectin, vWF, thrombomodulin, FMD and carotid intima-media thickness (CIMT), no association was found to exist between FMD and plasma endothelial markers (Meyer et al., 2006). Within the context of HIV infection, a study performed by Seigneur et al involving 90 HIV-infected subjects versus controls revealed the HIV-infected cohort to have higher levels of vWF, sTM (soluble thrombomodulin), E-selectin, TNF-α and IFN-α compared to the HIV-uninfected cohort (Seigneur et al., 1997). Levels of vWF correlated positively with TNF-α and IFN-α while sTM correlated negatively with CD4+ cell count (see Table 1.5).

1.5.9.3 Thrombomodulin

Similar to E-selectin, thrombomodulin (TM) is a transmembrane protein specifically expressed on the surface of endothelial cells (Sadler, 1997). However, it differs in its function in that it acts as a protein C co-factor and possesses anticoagulant activity (Sadler, 1997). Thrombomodulin has been found to exist in a soluble form within the blood circulation, thus, easily quantified by ELISA (Boffa et al., 1991, Ishii et al., 1991). Clinical studies revealed a positive association between levels of sTM and endothelial damage, thereby warranting its use as a plasma marker of endothelial damage (Ware et al., 2003, Ileri et al., 2001,

Boffa et al., 1991). A positive correlation between plasma levels of sTM with CECs, but not with E-selectin, was observed by Strijbos et al (Strijbos et al., 2008), while a strong negative correlation has been reported to exist between levels of sTM with CD4+ cell count (Seigneur et al., 1997).

1.5.9.4 Markers of inflammation

C-reactive protein (CRP) is a pentameric protein found in plasma and largely synthesised by hepatocytes in response to acute inflammation (Black et al., 2004, Pepys and Hirschfield, 2003). However, expression of CRP in other cells such as neurons, monocytes, lymphocytes and foam cells have been reported, though, most likely at insignificant plasma levels (Jialal et al., 2004, Burke et al., 2002). The C-reactive protein stimulates phagocytosis by activating the complement pathway and binds to immunoglobulin receptors thereby playing a role in innate immunity as a defence mechanism against infections (Black et al., 2004). Expression of CRP is principally induced by the inflammatory cytokines interleukin-6 (IL-6), which can be enhanced by interleukin-1 (IL-1) (Kushner et al., 1995). C-reactive protein has gained acknowledgement as an independent marker of inflammation and can be used in the clinical evaluation of endothelial function and CVD risk (Fichtlscherer et al., 2000, Ridker et al., 2000). C-reactive protein has been observed to be high in HIV-infected patients, despite long periods of HAART administration (Neuhaus et al., 2010, Shikuma C, 2009). C-reactive protein can decrease endothelial nitric oxide synthase mRNA (Verma S, 2002), and upregulate the adhesion molecules ICAM-1 and VCAM-1 and chemoattractant chemokines such as MCP-1 (Deshmane et al., 2009, Verma et al., 2003). In addition, *in vivo* and *in vitro* studies have shown that CRP upregulates angiotensin type 1 receptor in vascular smooth muscle cells, thereby increasing proliferation and migration of these cells, as well as increasing restenosis and the production of reactive oxygen species (Wang et al., 2003). These mechanisms would ultimately lead to a decrease in NO levels, and as consequence, endothelial dysfunction.

Interleukin-6 is a soluble protein secreted by a variety of cells such as monocytes, macrophages, fibroblasts, vascular smooth muscle cells, stromal cells and endothelial cells (Sprague and Khalil, 2009, Febbraio and Pedersen, 2005). It acts not only as a pro-inflammatory cytokine, but also has anti-inflammatory effects, mediated by its ability to inhibit TNF-α and IL-1, and activate IL-1 receptor antagonist (IL-1Ra) and IL-10 (Febbraio and Pedersen, 2005). Interleukin-6 activates the NF-*k*B pathway, resulting in the expression of ICAM-1, VCAM-1 and E-selectin, and inhibits NO production by downregulating eNOS expression (Mathew et al., 2010). Interleukin-6 also plays a role in the development of atherosclerotic plaques. Thus, after migration of monocytes across the endothelium these cells develop into foam cells as they absorb modified lipoproteins, and secrete IL-6 and TNF-α Both these cytokines mobilize further immune cells leading to the progressive development of the atherosclerotic plaque (Baker et al., 2011).

Interleukin-8 (IL-8) is a cytokine produced not only by macrophages, but also by other types of cells such as epithelial, smooth muscle and endothelial cells (Sprague and Khalil, 2009, Hedges et al., 2000). Interleukin-8 also plays a role in atherogenesis in that it induces the adhesion of CXCR2-expressing monocytes to the endothelium under physiological flow conditions (Mehra et al., 2005), however, to a much lesser extent when compared to IL-6 (Sprague and Khalil, 2009).

Tumour necrosis factor alpha (TNF-α), is a transmembrane cell signalling protein primarily expressed by activated macrophages in response to acute systemic inflammation (Chu, 2013, Carswell et al., 1975). During inflammation, TNF-α and IL-1 are released by the macrophages in the inflamed tissue which in turn induces overexpression of the adhesion molecules, VCAM-1, ICAM-1 and E-selectin (Wang et al.,

2004, Signorelli et al., 2003). In a study performed in HIV-infected subjects, TNF-α was found to correlate strongly with levels of vWF (Seigneur et al., 1997).

Monocyte chemoattractant protein-1, also known as CCL2, is a small proinflammatory cytokine mainly produced by bone and brain cells upon neuroinflammation, where it displays chemotactic activity by attracting monocytes, memory T lymphocytes and dendritic cells to the site of MCP-1 expression (Gerard and Rollins, 2001). Increase in serum levels of MCP-1 is thus primarily associated with neurodegenerative disorders and diseases of the central nervous system. However, MCP-1 equally plays a role in diseases characterised by the infiltration of monocytes, such as atherosclerosis (Xia and Sui, 2009). The MCP-1 binds to the CC-Chemokine receptors, CCR2 and CCR4, located in the plasma membrane (Craig and Loberg, 2006).

1.5.9.5 Circulating cells

Studies conducted by da Silva et al. (2011) demonstrated that FMD and the systemic levels of EPCs to be lower and EMPs to be higher in HIV-infected ARV-naïve individuals when compared to healthy controls. A report from López et al. revealed the numbers of EPCs to be significantly lower and CACs to be significantly higher in HIV-infected ARV-naïve subjects versus controls (Lopez et al., 2012). The authors hypothesized that direct infection of EPCs by HIV could account for the reduced levels of EPCs in HIV-infected patients as these cells may possess the chemokine receptors CCR5 and CXCR4, that are used by HIV to infiltrate host cells (Teofili et al., 2010). This is evident by the observation that administration of HAART to HIV-infected patients fully restores the level of EPCs (Papasavvas et al., 2012). This observation was confirmed by Costiniuk et al. (2012) who observed reduced levels of EPCs in HIV-infected versus HIV-uninfected controls in a study conducted with 30 ARV-naïve HIV-infected men against 36 HIV-uninfected men. However, no correlation was found to exist between EPC levels and

CD4+ cell count or viral load (Costiniuk et al., 2012). In contrast, a cross-sectional and a longitudinal study conducted with 50 and 66 chronic HIV-infected subjects respectively, in which both cohorts were receiving ARVs, showed EPC levels in HIV-infected subjects to be significantly higher compared to negative controls (Papasavvas et al., 2012). Moreover, a correlation was found to exist between EPC numbers and CD4+ cell count, but no association could be seen between EPC numbers or change in EPC numbers over time with CIMT measurements.

Circulating angiogenic cell (CAC) levels and their migration to the site of endothelial damage have been shown to correlate with endothelial function (Van Craenenbroeck et al., 2010, Heiss et al., 2005). However, levels of CACs can be influenced by factors such as age (Heiss et al., 2005) and dyslipidemia (Fabbri-Arrigoni et al., 2012). To date, no study has been conducted to assess levels of CACs in HIV-infected patients under conditions of continuous viral replication or suppressed viremia.

Studies have revealed a significant positive correlation between levels of CECs with vWF levels (Makin et al., 2004, Chong et al., 2004) and a significant negative correlation (r=−0.423, P=0.002) between levels of CECs and FMD measurements (Chong et al., 2004). Also, HIV-infected ARV-naïve patients are reported to possess higher EMP levels (da Silva et al., 2011) and CEC levels (Lopez et al., 2012) compared to healthy individuals. These data therefore suggest that HIV infection results in significant endothelial injury. However, to date, there are no studies to show if controlled viremia with administration of HAART reduces levels of EMPs and CECs.

1.5.9.6 Metabolites of endothelial nitric oxide synthetase

As previously discussed, the enzyme, eNOS, plays a vital role in maintaining the body's vasculature in a healthy state. Endothelial dysfunction is characterised by a decrease in eNOS with the concomitant decrease in NO and increase in reactive oxygen species (ROS). An increase in ROS results in the increase oxidation of NO to NO_2, which is further oxidised to NO_3^-. This increase in NO_3^- and NO_2^- can be measured in human biological fluids (Helmke and Duncan, 2007). Given that NO has a short half-life in blood of about 0.1s (Kelm and Schrader, 1990), the measurement of the NO metabolites, NO_3^- and NO_2^-, as biomarkers for the assessment of endothelial dysfunction, has been proposed by Lomelí and colleagues (Lomelí et al., 2018, Helmke and Duncan, 2007). A study conducted on 32 subjects with Marfan syndrome, a connective tissue disorder, with 35 healthy controls, revealed an inverse correlation to exist between NO_3^- / NO_2^- and NO_2^- with FMD (Lomelí et al., 2018). However, although a significant difference was observed between the two groups with regards to NO_3^- / NO_2^- ($p=0.002$), NO_2^- ($p=0.03$) and VCAM-1 ($p=0.03$), no significant difference was observed between the groups with regards to markers of inflammation (IL-6, IL-8 and TNF-α), and a marker of endothelial function (ICAM-1) (Lomelí et al., 2018). In addition, no studies on these molecules have been performed in HIV-infected subjects.

1.6 Potential aetiology of CVD in the HIV-infected population

1.6.1 Role of HIV viral proteins, Nef and Tat, in endothelial dysfunction

The development of CVD in HIV-infected individuals may occur via the induction of endothelial dysfunction by the HIV proteins Nef (Wang et al., 2014), Tat (Matzen et al., 2004) and gp120 (Jiang et al., 2010) (Figure 1.5). This hypothesis is supported by *in vitro* and *in vivo* studies conducted by Matzen and colleagues (2004) using human microvascular endothelial cells and C57BL/6 rats respectively, in which they showed that adhesion of leukocytes to the endothelium was selectively activated by the

synergistic action of HIV-1 Tat and TNF-α. The development of vascular diseases has been associated with an increase in the adhesion of leukocytes to the endothelial cells (Zietz et al., 1996). In a study conducted by Dhawan and colleagues (Dhawan et al., 1997) using human umbilical vein endothelial cells, HIV-1 Tat was found to induce expression of E-selectin, ICAM-1 and VCAM-1. Research conducted by Wang and co-workers (2014) using human coronary arterial endothelial cells (HCAEC), and Indian rhesus macaque heart tissue, showed that HIV-1 Nef contributes to endothelial dysfunction via two distinct pathways; an NADPH oxidase-dependent mechanism resulting in the apoptosis of endothelial cells, and by the expression of MCP-1 through the NF-*k*B signalling pathway. In addition, gp120 with TNF-α pre-treatment was found to significantly reduce endothelial function by downregulating the expression of endothelial nitric oxide synthase and upregulating ICAM-1 expression in HCAEC (Jiang et al., 2010). Hence, research conducted thus far on the effect of HIV proteins on endothelial function, shows that the circulating viral proteins act individually via different mechanisms to induce endothelial dysfunction, with HIV tat and gp120 acting in synergy with TNF-α.

The *in vitro* studies described above show that HIV viral proteins can affect endothelial function, while *in vivo* studies have shown that HIV-infected subjects display some level of endothelial dysfunction. However, no data exists to show that endothelial dysfunction in HIV-infected subjects is related to the serum levels of viral proteins. In-house HIV-1 Nef (Fujii et al., 1996) and Tat (Poggi et al., 2004) antigen assays have been developed by research groups for the quantification of HIV-1 viral proteins from human sera. There are commercially available enzyme-linked immunosorbent assay (ELISA) kits for the measurement of HIV-1 Nef and Tat proteins in serum (Raymond et al., 2011, Santosuosso et al., 2009), however, these kits have not been validated. Moreover, sequence diversity within the *nef* (Bredell et al., 2007) and *tat* (Li et al., 2012) genes, among HIV-infected individuals, could lead to inaccurate

quantification of the viral proteins by ELISA. Mass spectrometric techniques, such as liquid chromatography mass spectrometry (LCMS), provide a possible alternative to immunoassays for measuring serum levels of HIV proteins, which will be discussed further in Chapter 5.

1.6.2 The domino effect linked to CVD in HIV-infected individuals

Figure 1.5 seeks to illustrate potential mechanisms by which HIV-infected individuals could develop CVD. Invasion of cells of the immune system by HIV initiates immune responses that involve an upregulation of cytokine production (Deeks et al., 2013). In addition, studies have shown immune reconstitution inflammatory syndrome (IRIS) to occur upon ARV administration, characterised by excessive proinflammatory cytokine activity (Walker et al., 2015). Cytokines, as well as age and turbulent blood flow are factors known to activate the NF-*k*B pathway resulting in endothelial cell activation, which subsequently leads to endothelial dysfunction (Liao, 2013). The HIV viral protein, Nef, is suggested to cause endothelial dysfunction via activation of the NF-*k*B pathway within endothelial cells (Wang et al., 2014). It is proposed that Nef is released into the circulation within vesicles derived from the infected cell and enters the endothelial cells by cell-to-cell adhesion (Wang et al., 2014). A recent study conducted by Serena *et a*l. showed that HIV Nef could evade proteolysis by its interaction with human peroxisomal thioesterase 8 (ACOT8), hence, stay in circulation for much longer (Serena et al., 2016). Endothelial activation and dysfunction put into motion a series of events which include vasoconstriction, smooth muscle cell proliferation, platelet aggregation, leukocyte adhesion, LDL oxidation and matrix metalloproteinase (MMP) activation, which initiates atherogenesis, and as consequence, the development of atherosclerotic diseases.

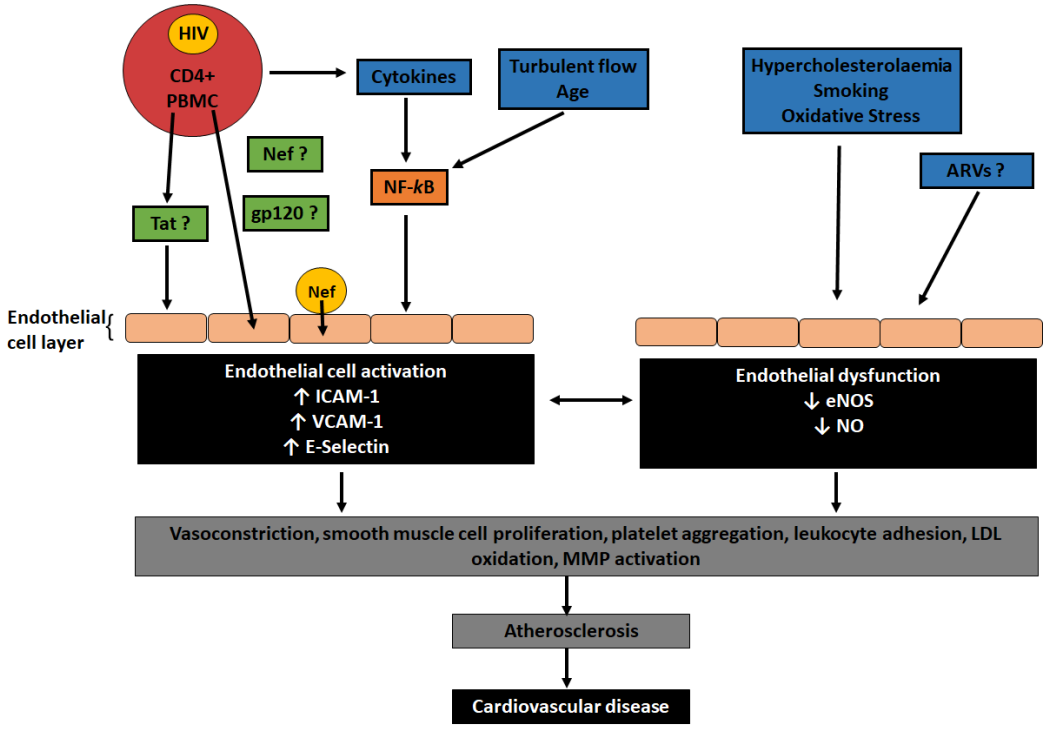


Figure 1.5: Schematic illustration of the potential mechanisms behind CVD in the HIV-infected population. HIV-infected individuals are more predisposed to endothelial dysfunction. This occurs not only as a result of inducers such as hypocholesterolaemia, smoking, oxidative stress, turbulent blood flow and age, but also as a result of immune activation upon HIV infection and direct interaction between HIV viral proteins such as Nef, Tat and gp120 with endothelial cells (adapted from Liao, 2013).

1.7 Genetic and functional heterogeneity of HIV-1 *nef* and *tat* alleles

Given the highly polymorphic nature of HIV, it is conceivable that sequence variation in HIV viral proteins could possibly influence its pathogenic activity (Robertson et al., 1995). Polymorphisms in the HIV-1 *nef* gene have been associated with disease progression in HIV-1 patients (Walker et al., 2007). Unique mutations specific to slow progressors were identified at the N-terminal and C-terminal domains of HIV-1 *nef* genes isolated from a cohort of HIV-infected infants and children at different stages of disease progression (Walker et al., 2007). In addition, HIV-1 *nef* mutants have been positively correlated

with pulmonary hypertension in HIV-infected patients of which the identified *nef* polymorphisms were not influenced by age, treatment or duration of HIV infection (Almodovar et al., 2012).

Mutations in *tat* exon 1 have been shown to inhibit the transactivation activity of Tat (Jeang, 1996). Genetic variations in the HIV-1 *tat* gene have also been associated with neurological disorders (Li et al., 2012, Cowley et al., 2011, Mayne et al., 1998). In a comparative study involving HIV-infected patients with and without dementia, HIV-1 *tat* sequence analysis revealed a positive association between a cluster of *tat* mutations and neurological injury (Bratanich et al., 1998). In addition, the ability of HIV-1 *tat* to stimulate TNF-α production is greatly influenced by sequence variation (Campbell et al., 2007, Siddappa et al., 2006).

These studies suggest that sequence variation in viral genes, particularly *nef* and *tat,* alter the progression of not only HIV infection but also of diseases associated with viral infection. It is therefore possible that the ability of these viral proteins to interact with the endothelium may also be influenced by viral gene variation. However, as of date, no studies have been performed to test this hypothesis. In addition, the mechanism by which HIV infection increases cardiovascular risk is not fully understood and therefore evidence that viral protein sequence variation associates with markers of endothelial dysfunction would strongly implicate these viral peptides in CVD aetiology. This would allow the development of focussed therapies in subjects carrying these viral gene variants. Also, the development of assays for the accurate quantification of serum Nef levels would allow investigation of the association of Nef with CVD risk factors and endpoints, further cementing the link between HIV and endothelial dysfunction.

1.8 Project aims and objectives

The overall aim of this research project was therefore to determine whether endothelial dysfunction is present in subjects with HIV infection and is related to variants in the viral *nef* and *tat* genes.

The objectives of this research project were to:

1. Compare endothelial function in Black South African HIV-infected and non-infected subjects by measuring serum levels of vWF, ICAM-1, VCAM-1, MCP-1 and E-selectin, and compare inflammation by quantifying serum levels of IL-6, IL-8 and TNF-α.

2. Measure the association of serum markers of endothelial function and inflammation with CVD risk factors in HIV-infected and HIV-uninfected subjects.

3. Sequence the genes coding for the HIV-1 Nef and Tat viral proteins from subjects with HIV infection and compare endothelial function and inflammation across the different viral gene sequence variants.

4. Determine whether *nef* or *tat* gene variants are associated with CIMT.

5. Develop a method for the accurate quantification of HIV viral proteins from human serum using liquid chromatography-mass spectrometry (LCMS) and compare with an available ELISA method.

1.9 References

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Chapter 2: HIV-1 Infection is Associated with Endothelial Dysfunction in Black South Africans

2.1 Introduction

The prevalence of HIV continues to rise with just over 37 million people currently living with HIV (UNAIDS, 2019). The rate of new HIV infections is highest in developing countries with the sub-Saharan Africa region accounting for up to 70% of the total number of reported cases (UNAIDS, 2019). The high prevalence of HIV infection in sub-Saharan Africa has led to the large scale roll-out of ART in this region, especially in South Africa (Johnson, 2012). The development and improvement of highly active anti-retroviral therapy (HAART) has resulted in a dramatic increase in the lifespan of HIV patients (Grinsztejn et al., 2013). However, there are growing concerns in the public health sector regarding the adverse metabolic effects of both HIV infection and long-term HAART, in an aging population, which is also exposed to increased levels of classic cardiovascular disease (CVD) risk factors (Chow et al., 2012, Jerico et al., 2005).

Systemic inflammation enhances atherosclerosis, and biomarkers indicative of both of these processes are found at higher levels in HIV-infected individuals (van Leuven et al., 2007). Reports from developed countries show the prevalence of CVD to be higher in the HIV-infected population versus uninfected subjects (Freiberg et al., 2013, Chow et al., 2012, van Leuven et al., 2007). However, little is known about the prevalence of CVD in the sub-Saharan HIV-infected population. Although an increased prevalence of traditional risk factors, such as dyslipidaemia, smoking, hypertension, hypercholesterolaemia and diabetes, among HIV-infected patients likely contributes to this increased cardiovascular morbidity,

growing evidence suggests that HIV infection itself is directly linked to endothelial dysfunction, likely through inflammation and immune dysregulation (Freiberg et al., 2013).

Endothelial dysfunction is regarded as the earliest clinically detectable stage of CVD. It is characterised by activation of the endothelial cells, induced by reactive oxygen species, and augmented by exposure to CVD risk factors (Deanfield et al., 2007). This leads to the up-regulation of endothelial adhesion molecules, such as VCAM-1 and ICAM-1, which promote adherence of leukocytes to the endothelium and the progression of atherosclerotic plaque formation. Increased adhesion of leukocytes to the aortic endothelium, in subjects with HIV infection, has been observed in conjunction with increased expression of VCAM-1 and E-selectin (Zietz et al., 1996). Furthermore, serum markers of endothelial dysfunction (ICAM-1, VCAM-1, MCP-1 and vWF) and serum markers of inflammation (CRP and IL-6), are known to be higher in HIV-infected subjects (Mosepele et al., 2018, Borges et al., 2015, De Pablo-Bernal et al., 2014, Fourie et al., 2011, Neuhaus et al., 2010).

The majority of studies investigating endothelial dysfunction in HIV-infected subjects have been conducted outside of the African continent, despite the very high prevalence of infection in sub-Saharan Africa. Therefore, the main aim of this study was to compare endothelial dysfunction and systemic inflammation between indigenous African HIV-infected, treatment naïve and HIV-uninfected populations. Secondary aims were to characterise the main determinants of endothelial dysfunction in HIV-infected subjects, and to assess the effect of anti-retroviral therapy (ART) on both endothelial function and inflammation. These aims were achieved by measuring a broad array of appropriate serum biomarkers in HIV-infected and control subjects. The HIV-infected patients included two groups of subjects: those with a CD4 count $\leq$350 and those with CD4 count >350 cells/mm^3. These sub-groups were

used to determine if subjects who were more immune-compromised had a greater severity of endothelial dysfunction. Classical CVD risk factors were measured in all subjects. A small longitudinal sub-study measured serum biomarkers of endothelial dysfunction and systemic inflammation after initiation of ART.

2.2 Materials and Method

2.2.1 Ethics

Ethical clearance for patient recruitment and blood sampling was granted to Dr Nereshni Lutchman by the Human Research Ethics Committee of the University of the Witwatersrand under the ethical clearance number M10408 (Appendix 1). Participants were recruited and blood samples collected by Dr Lutchman. Ms Genevieve Mezoh was granted ethical clearance by the same ethics committee under the clearance number M150979 (Appendix 2) to carry out all further analyses on these blood samples.

2.2.2 Patient population

The study consisted of a population of Black South African urban adults between the ages of 30 to 50 years. Eighty (80) HIV-infected, ART-naïve patients including 38 subjects with CD4 count ≤ 350 (low CD4 group) and 42 subjects with CD4 count >350 (high CD4 group), were recruited from the Nthabiseng and Zazi Clinics, Chris Hani Baragwanath Hospital. These CD4 cut points were chosen because of their clinical significance. Thus, at the time of recruitment of subjects into this study, only those with a CD4 count below 350 cells/mm^3 were eligible for ART (Moorhouse et al., 2016, Govender et al., 2014). A group of 60 HIV-uninfected individuals were recruited from the Zazi Clinic. Participants of the HIV-uninfected group were screened for HIV at the Perinatal HIV Research Unit of Zazi Clinic using the Bioline HIV 1/2 3.0 rapid test kit (Standard Diagnostics Inc, Gyeonggi-do, Republic of Korea) (Govender et al., 2014). The groups were matched for age and gender. Informed consent was obtained from all

participants. Following initiation of ART, the HIV-infected participants from both the low and high CD4 groups were followed up for 18 months. Serum markers of inflammation and endothelial dysfunction were measured in these subjects at baseline, i.e. pre-ART, and at 18 months after initiation of either a first-line tenofovir-containing ART regimen (Tenofovir/Lamivudine/Efavirenz) or second-line ART treatment (Darunavir/Ritonavir and Lopinavir/Ritonavir).

2.2.3 Exclusion criteria

The exclusion criteria for this study were designed in an effort to eliminate subjects who may have potentially received drugs that influence their glucose, lipid or blood pressure levels. Subjects with clinical conditions known to influence endothelial function were also excluded. The exclusion criteria were therefore as follows:

- Personal history of diabetes mellitus, coronary artery disease, cerebrovascular accident, peripheral vascular disease
- Current AIDS-defining illness or opportunistic infection or neoplasm
- Acute illness in the last three months
- Active drug abuse
- Patient drug history e.g. statin or anti-hypertensive therapy
- Alcohol abuse as defined by: a woman who has more than seven drinks per week or more than three drinks per occasion. A man who has more than 14 drinks per week or more than four drinks per occasion. One drink is defined as one bottle of beer (340 mL) or 150 mL glass of wine or 40 mL of distilled spirits
- Smoking defined as $\geq$10 cigarettes/day in the past year
- Pregnant females

2.2.4 Data and sample collection

Blood was collected from all participants into EDTA plasma and serum tubes. The plasma and serum samples were divided into 1 mL and 500 µL aliquots and stored at -80°C until further analysis. Anthropometric data such as height, weight, body mass index (BMI), waist and hip circumference were measured at the time of recruitment as described by Crowther and Norris (2012). Briefly, weight and height measurements were taken using an electronic scale and wall mounted stadiometer (Holtain, UK), respectively. Prior to taking these measurements, participants removed their shoes and any heavy clothing. To measure waist circumference, a measuring tape was put around the waist above the umbilicus in the standing position, and the smallest girth recorded to the nearest 0.5 cm. Hip circumference was taken by measuring the length over the widest part of the gluteal region using a soft measuring tape. For blood pressure measurements, participants were asked to sit and rest their right arm for about 5 minutes following which readings were taken thrice from the right arm using an automated sphygmomanometer (Omron M6; Omron, Kyoto, Japan) and the final reading recorded.

A fasting morning blood sample was collected from all participants and glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglycerides quantified using the Siemens ADVIA 1800 Chemistry Immunoassay System following standard protocols within the NHLS Chemical Pathology routine laboratory unit of the Charlotte Maxeke Johannesburg Academic Hospital. Viral loads were measured from EDTA plasma samples using the Cobas® HIV-1 quantitative nucleic acid test on the Cobas® automated 6800 system, while CD4 count was measured from EDTA plasma samples by flow cytometry, following standard protocols within the HIV Molecular Routine Laboratory of the Charlotte Maxeke Johannesburg Academic Hospital.

Other relevant information was obtained from patient files and by direct questioning, using a structured questionnaire, in relation to personal history of coronary artery disease, diabetes mellitus, hypertension, cerebrovascular events, recent infections or diagnosed malignancies, drug history, alcohol intake, smoking and recreational drug use.

2.2.5 Measurement of biomarkers of endothelial function

2.2.5.1 Quantification of biomarkers of endothelial dysfunction by a multiplex assay

Serum levels of ICAM-1, VCAM-1, E-Selectin, MCP-1, TNF-α, IL6 and IL8 were measured using a customised Human Magnetic Luminex Screening Assay kit (R&D Systems, Minneapolis, MN, U.S.A) on the Bio-Plex® Multiplex System (Bio-Rad, Hercules, CA, U.S.A) with Luminex xMAP technology, according to the manufacturer's instructions. The Luminex assay is based on multi-analyte profiling bead technology enabling the simultaneous detection and quantitation of up to 100 analytes in a single test sample. Therefore, human ICAM-1, VCAM-1, E-Selectin, MCP-1, TNF-α, IL6 and IL8 were multiplexed using the R&D Systems Luminex Assay Customization Tool.

The beads were conjugated to protein-specific capture antibodies and dyed with fluorophores of differing intensities. The Luminex xMAP technology enables multiplexing as it binds different ligands to different colour beads. The target proteins in the plasma sample were captured by the protein-specific capture antibodies. After washing to remove unbound proteins, protein-specific biotinylated detector antibodies were added and incubated with the beads for 1 hr to allow binding to the appropriate immobilized proteins. The excess biotinylated detector antibodies were removed by washing and conjugated streptavidin R-phycoerythrin (streptavidin-RPE) added. During incubation, the streptavidin-RPE binds to the biotinylated detector antibodies associated with the immune complexes on the beads, forming a four-

member sandwich. The fluorescent protein, R-phycoerythrin, emits light which was then detected by the Bio-Plex® Multiplex System. Protein concentration was determined by monitoring the spectral properties of the beads and the amount of associated R-phycoerythrin fluorescence.

2.2.5.2 Quantification of vWF by enzyme-linked immunosorbent assay (ELISA)

The vWF was measured by ELISA using a human vWF ELISA kit (Merck, Darmstadt, Germany) as described by the manufacturer. The assay is based on the formation of a double antibody sandwich complex. This can be used to accurately quantify a single target protein in clinical samples. Briefly, plasma samples were diluted 8,000 fold with the provided diluent, and 100 µL of each sample, standard and blank added in duplicates to a 96 well plate pre-coated with the vWF capture antibody. After incubating and washing to remove unbound substances, a second horseradish peroxidase (HRP)-conjugated target-analyte antibody was added to bind the captured human vWF, followed by another round of incubation and washing procedures to remove unbound antibody-HRP. Incubation with the horseradish peroxidase substrate yielded a blue coloured complex. The reaction was stopped using an acidic solution supplied with the kit, changing the colour of the reaction mixture from blue to yellow. Thereafter, samples were quantified by spectrophotometry using an ELISA plate reader set at 450nm and 540nm, and wavelength reading corrected by subtracting readings at 540 nm from the readings at 450 nm.

2.2.6 Statistical analysis

Statistical analysis was performed using the Statistica software v13.3 (Statsoft Inc., Tulsa, OK, U.S.A.). Data that was not normally distributed was either log-transformed and/or square rooted to normalize their distribution. Data was compared across the 3 groups (HIV-uninfected, HIV-infected high CD4 and HIV-infected low CD4) using one-way ANOVA and a Tukey *post-hoc* test. This was followed by multivariable linear regression analyses in which endothelial dysfunction markers that were found to differ between the 3 groups were included as dependent variables, the 2 CD4 groups included as independent variables, and the HIV-uninfected subjects acting as the reference group. Other variables that differed across the 3 groups were then added to the models to determine whether they attenuated the relationship of the endothelial dysfunction markers with HIV status. Data was compared between baseline and follow-up time points using a Students paired t test.

Multivariable linear regression analysis was used to find the main determinants of serum biomarkers of endothelial dysfunction (dependent variables) in HIV-infected subjects, particularly noting the input of CD4 counts and viral load. Firstly, appropriate study variables (independent variables) were included in univariate regression analyses with each of the dependent variables (univariate results are shown in appendix 3). These independent variables were gender (male vs female), age, waist and hip circumferences, BMI, systolic and diastolic blood pressure, glucose, total cholesterol, LDL-C, HDL-C, triglycerides, TNF-α, IL6, IL8, CD4 count and viral load. Those variables that correlated with the dependent variable at $p<0.20$ were included in the multivariable regression model. This p-value was used because previous studies have shown that when choosing only variables that correlate with the dependent variable in a univariate analysis at $p<0.05$ for inclusion in a multivariable model, one can leave out variables that will have significant effects when included in the model. It has therefore been suggested

that a $p<0.20$, or even higher, should be used for selecting variables for inclusion in multivariable models (Greenland, 1989). Variables were assessed for collinearity using the variance inflation factor (VIF), and any variables with a VIF>5.0 were excluded from the model. Backward, stepwise, linear regression analyses were then performed with the stepwise removal of non-significant variables until a final model was reached where all independent variables correlated with the dependent variable at $p<0.05$.

The sample size for this study was determined by infrastructural constraints and data from the literature. At the time of this study, no data was available from South Africa on serum levels of biomarkers of endothelial dysfunction in HIV-infected subjects. Hence, a study conducted in Italy by Francisci et al. (2009) was used to estimate the appropriate sample size. This study reported a significant difference in serum markers of endothelial dysfunction between HIV-uninfected and HIV-infected subjects using 28 subjects per group. We therefore ensured that the sample size for each of our study groups was greater than 28.

2.3 Results

2.3.1 Comparison of demographic, anthropometric and cardiometabolic variables between study groups

No significant differences were observed between the three groups for age, anthropometry, blood pressure, glucose or triglyceride levels (Table 2.1). However, there were significantly fewer male participants in the HIV-infected high CD4 group compared to the HIV-uninfected group. No difference in total cholesterol was seen between the HIV-uninfected and high CD4 group, however levels of total cholesterol were significantly lower in the low CD4 group compared to the HIV-uninfected and high CD4 group. Serum levels of LDL-C were significantly higher in the high CD4 group compared to both the HIV-uninfected

group and the low CD4 group. In addition, levels of HDL-C were lower in both the low and high CD4 group compared to the HIV-uninfected group.

Table 2.1: Demographic, anthropometric, cardiometabolic and immunological characteristics of study groups

Variables	HIV-uninfected (n = 60)	HIV-infected CD4 > 350 (n = 42)	HIV-infected CD4 ≤ 350 (n = 38)
Age (years)	36.48 ± 4.80	36.19 ± 6.59	38.89 ± 6.07
Gender, male n (%)	25 (41.67)	5 (11.90) ^{aa}	10 (26.32)
BMI (kg/m^2)	26.02 ± 5.27	27.52 ± 8.31	25.71 ± 5.31
Waist circumference (cm)	88.20 ± 12.20	87.50 ± 12.34	85.63 ± 10.47
Diastolic BP (mmHg)	80.37 ± 11.25	78.38 ± 12.17	81.92 ± 16.47
Systolic BP (mmHg)	130.22 ± 16.88	130.10 ± 17.84	137.82 ± 23.88
Glucose (mmol/L)	4.62 ± 0.54	4.67 ± 1.94	4.44 ± 0.40
Triglyceride (mmol/L)	0.74 [0.42, 0.99]	0.87 [0.61, 1.07]	0.64 [0.50, 0.86]
Total cholesterol (mmol/L)	3.84 ± 0.85	3.99 ± 0.83	3.21 ± 0.76^{aa,bb}
LDL-C (mmol/L)	1.58 ± 0.65	2.42 ± 0.58^{aaa}	1.88 ± 0.63^{bb}
HDL-C (mmol/L)	1.64 [1.31, 2.38]	1.11 [0.84, 1.35]^{aaa}	0.97 [0.84, 1.20]^{aaa}
CD4+ cell count (cells/mm^3)	-	691.55 ± 232.08	224.18 ± 92.54^{bbb}
Viral load (copies/mL)	-	37109.72 ± 71099.85	81741.74 ± 90049.05^{bb}

Data given either as mean ± standard deviation or median [lower quartile, upper quartile]; n indicates the number of participants; ^{aa}p<0.005, ^{aaa}p<0.0005 vs HIV-uninfected; ^{b}p<0.05, ^{bb}p<0.005, ^{bbb}p<0.0005 vs CD4>350.

2.3.2 Comparison of markers of endothelial dysfunction and inflammation between study groups

No significant difference was observed across the three groups with regards to plasma levels of IL-8, E-selectin and MCP-1 (Table 2.2). In addition, plasma levels of TNF-α, IL-6, ICAM-1 and VCAM-1 did not differ significantly between the HIV-infected high CD4 and HIV-uninfected groups. However, plasma levels of TNF-α, ICAM-1 and VCAM-1 were higher in the HIV-infected low CD4 group compared to the HIV-uninfected group, with levels of vWF being higher in both the low and high CD4 group compared to the HIV-uninfected group. Comparing the HIV-infected, low and high CD4 groups, significant differences between these two groups were observed for plasma levels of TNF-α, IL-6 and VCAM-1, which were significantly higher in the low compared to the high CD4 group.

Table 2.2: Comparison of endothelial dysfunction and inflammation between study groups

Variables	HIV uninfected (n = 60)	HIV-infected CD4 > 350 (n = 42)	HIV-infected CD4 ≤ 350 (n = 38)
TNF-α (pg/mL)	4.6 [3.1, 7.4]	5.4 [3.6, 7.2]	8.2 [6.2, 10.4]^{aa,b}
IL-6 (pg/mL)	3.4 [2.0, 4.6]	2.2 [1.0, 3.4]	4.6 [3.8, 5.3]^{bbb}
IL-8 (pg/mL)	7.6 [4.3, 20.9]	7.9 [3.1, 15.5]	6.3 [4.7, 9.3]
ICAM-1 (ng/mL)	335.6 [158.5, 575.4]	462.1 [331.9, 919.3]	839.4 [419.7, 1042.5]^{a}
VCAM-1 (ng/mL)	464.3 [266.5, 609.6]	526.1 [434.9, 657.9]	1163.1 [701.5, 1606.4]^{aaa,bbb}
E-selectin (ng/mL)	30.0 [20.9, 44.1]	27.9 [19.9, 33.9]	30.3 [22.7, 40.0]
vWF (µg/mL)	14.1 [9.2, 20.7]	22.2 [15.8, 36.5]^{aa}	21.3 [13.4, 43.9]^{aa}
MCP-1 (pg/mL)	120.8 [99.3, 171.5]	131.2 [102.3, 178.6]	139.3 [115.0, 208.1]

Data given as median [lower quartile, upper quartile]; n indicates the number of participants; $^{a}p<0.05$, $^{aa}p<0.005$, $^{aaa}p<0.0005$ vs HIV-uninfected; $^{b}p<0.05$, $^{bbb}p<0.0005$ vs CD4>350.

2.3.3 Determinants of systemic inflammation and endothelial dysfunction

It is possible that significant differences noted in ICAM-1, VCAM-1 and vWF between the HIV-infected groups and the HIV-uninfected group are due to confounding by other variables that also differ across these groups, i.e. gender, total cholesterol, LDL-C, HDL-C (see Table 2.1), TNF-α and IL-6 (see Table 2.2). Therefore, multivariable regression models were set up for each of the 3 markers of endothelial dysfunction with the 2 CD4 groups as independent variables and with the HIV-uninfected group as the reference. The models were run with and without adjustment for gender, LDL-C, HDL-C, TNF-α and IL-6. Total cholesterol was not included due to high collinearity with LDL-C. The models are shown in Table 2.3. In the unadjusted models, the differences noted in Table 2.2 for the CD4 groups with the HIV-uninfected group were repeated, as would be expected. After adjustment for the possible confounding variables, there was some attenuation of the β-coefficients noted in the unadjusted models, but all p-values remained significant except that for the ICAM-1 levels in the CD4≤350 group which changed from p=0.019 in the unadjusted model to p=0.059 in the adjusted model.

Table 2.3: Comparison of endothelial dysfunction between HIV-infected and HIV-uninfected groups using regression analysis without and with adjustment for possible confounding variables

Model number	Dependent variables	Independent variables with standardised β-coefficient (p-value) [a]	
		Without adjustment	With adjustment [b]
1	ICAM-1[c]	CD4>350 0.123 (0.206) CD4≤350 0.229 (0.019)	CD4>350 0.110 (0.374) CD4≤350 0.223 (0.059)
2	VCAM-1[c]	CD4>350 0.012 (0.890) CD4≤350 0.429 (<0.0005)	CD4>350 0.006 (0.955) CD4≤350 0.376 (0.0006)
3	vWF [d]	CD4>350 0.324 (0.002) CD4≤350 0.342 (0.001)	CD4>350 0.325 (0.012) CD4≤350 0.278 (0.026)

[a] The β-coefficients and p-values for the CD4>350 and the CD4≤350 groups are shown in reference to HIV-uninfected subjects; [b] adjusted for gender, LDL-C, HDL-C, TNF-α and IL-6; [c] square root and [d] logged values used.

Table 2.4 shows the results of regression models for each of the serum biomarkers of endothelial dysfunction in the HIV-infected population (both CD4 groups combined). These models were generated to isolate the main determinants of endothelial dysfunction in this population, and in particular to observe the effects of CD4 counts and viral load.

Table 2.4: Multivariable backward stepwise linear regression models for serum biomarkers of endothelial dysfunction in HIV-infected subjects

Model number	Dependent variable	Independent variable with standardised β (p-value)		Adjusted R^2 (p-value) for full model
1	VCAM-1[a]	CD4 counts	-0.474 (<0.0005)	0.214 (<0.0005)
2	E-selectin	Male [c] Diastolic BP	0.367 (0.002) 0.247 (0.036)	0.172 (0.001)
3	vWF [b]	Viral load	0.344 (0.007)	0.103 (0.007)
4	MCP-1[a]	Age	0.357 (0.002)	0.115 (<0.002)

[a] Square root and [b] logged values used; [c] compared to females; models were built by performing univariate analyses of all appropriate study variables (see section 2.2.6 and appendix 3) against each of the 5 dependent variables shown in Table 2.4. Those variables that correlated at $p<0.20$ were included in a multivariable regression model in which backward, stepwise removal of non-significant variables was performed until all remaining variables were significant at $p<0.05$ – these final models are shown in Table 2.4.

Regression analyses show that the only significant determinant of VCAM-1 serum levels was CD4 count (inverse relationship; Table 2.4, model 1) while male gender and diastolic blood pressure were both positively associated with E-selectin levels (model 2). Viral load was a strong positive determinant of vWF levels (model 3) while age correlated positively with MCP-1 (model 4). After backward, regression analysis no significant associations were observed for ICAM-1 serum levels.

2.3.4 Changes in endothelial dysfunction and inflammation following initiation of ART

Subjects who were initiated onto ART and followed-up 18 months later included 74 subjects of who 61 were lost to follow-up leaving only 13 subjects for whom blood samples were available at 18 months. Of note, 61.5% of the 13 subjects had a CD4+ cell count of ≤ 350. Data obtained from these follow-up

participants are reported in Table 2.5. No difference was observed between baseline and follow-up measures with regards to BMI and blood pressure. The waist circumference, total cholesterol and LDL-C levels were significantly higher in the HIV-infected follow-up group compared to baseline; however, glucose level was lower in the follow-up group. Comparing markers of endothelial dysfunction and inflammation, levels of TNF-α, IL-6 and IL-8 were significantly higher after treatment compared with baseline. However, levels of ICAM-1 and VCAM-1 were significantly decreased. No significant difference in plasma levels of E-selectin, vWF and MCP-1 were observed after ART initiation.

Table 2.5: Characteristics of HIV-infected ART-naïve participants at baseline and after 18 months of treatment

Variables	HIV-infected Baseline (n = 13)	HIV-infected 18 months follow-up (n = 13)
Age (years)	38.3 ± 5.6	40.1 ± 5.6 [aa]
BMI (kg/m^2)	25.7 ± 6.3	26.3 ± 5.9
Waist circumference (cm)	84.6 ± 12.2	87.7 ± 11.6 [aa]
Diastolic BP (mmHg)	80.3 ± 16.8	81.7 ± 12.3
Systolic BP (mmHg)	128.7 ± 19.0	132.5 ± 17.2
Glucose (mmol/L)	4.5 ± 0.5	4.3 ± 0.5 [a]
Triglyceride (mmol/L)	0.7 [0.5, 1.0]	0.7 [0.6, 1.0]
Total cholesterol (mmol/L)	3.2 ± 0.8	3.9 ± 0.9 [a]
LDL-C (mmol/L)	1.9 ± 0.5	2.4 ± 0.8 [a]
HDL-C (mmol/L)	1.0 [0.8, 1.0]	1.0 [0.8, 1.0]
CD4+ cell count (cells/mm^3)	433.7 ± 291.6	495.5 ± 157.8
TNF-α (pg/mL)	7.7 [5.9, 9]	19.1 [13.9, 22] [aa]
IL-6 (pg/mL)	3.4 [2.7, 5.0]	11.2 [7.7, 12.0] [aa]
IL-8 (pg/mL)	7.9 [4.9, 13.0]	34.7 [20.1, 43.0] [aa]
ICAM-1 (ng/mL)	425.2 [355.4, 899.8]	149.8 [107.5, 205.0] [a]
VCAM-1 (ng/mL)	676.4 [607.3, 1203.0]	270.7 [151.9, 343.5] [a]
E-selectin (ng/mL)	36.1 [23.4, 37.8]	23.7 [22.2, 28.2]
vWF (µg/mL)	24.4 [19.8, 41.8]	23.4 [20.9, 29.5]
MCP-1 (pg/mL)	175.4 [133.6, 225]	203.6 [153.8, 506]

Data given either as mean ± standard deviation or median [lower quartile, upper quartile]. n indicates the number of participants; [a]$p<0.05$, [aa]$p<0.005$, [aaa]$p<0.0005$ vs baseline.

2.4 Discussion

In this study, HIV-infected participants with CD4 counts >350 cells/mm^3 had more atherogenic lipid profiles than both HIV-infected subjects with CD4 counts ≤350 cells/mm^3 and HIV-uninfected subjects. Serum levels of the inflammatory markers, TNF-α and IL6, were highest in HIV-infected subjects with low CD4 counts. When compared to HIV-uninfected subjects, those who were HIV-infected, most particularly those with the lowest CD4 counts, had the highest serum levels of the endothelial dysfunction markers ICAM-1, VCAM-1 and vWF. These differences, although attenuated, remained significant after adjusting for possible confounding variables. In HIV-infected subjects, CD4 count was a negative determinant of ICAM-1 levels while viral load was positively associated with vWF levels. Following 18 months of ART in a small sub-group of participants, serum levels of ICAM-1 and VCAM-1 were lower, while levels of TNF-α, IL-6 and IL-8 were higher than that observed before treatment. In addition, an increase in waist circumference, total cholesterol and LDL-C with a decrease in glucose levels were observed post treatment.

Serum markers of endothelial dysfunction i.e. VCAM-1, ICAM-1 and vWF, were higher in HIV-infected subjects when compared to HIV-uninfected subjects. This data is supported by a number of other studies (Mosepele et al., 2018, Fourie et al., 2015, van den Dries et al., 2015, De Pablo-Bernal et al., 2014, Fourie et al., 2011). However, serum levels of E-selectin were not elevated in the HIV-infected subjects. A previous study from South Africa also reported no difference in E-selectin levels between HIV-uninfected and HIV-infected, ART-naïve subjects (Hoffman et al., 2018). Serum levels of MCP-1 were higher in both the HIV-infected groups when compared to the controls, but this difference was not statistically significant. Previous studies have shown that MCP-1 levels are higher in HIV-infected subjects (Francisci et al., 2009, Miller et al., 2010), but these studies were conducted in mixed populations (Hispanic, African

American and White) of children (Miller et al., 2010) or in White subjects only (Francisci et al., 2009). A study conducted in Tanzania has also shown that MCP-1 levels were higher in HIV-infected compared to HIV-uninfected subjects before initiation of ART (Haissman et al., 2009). This study had a higher sample size for HIV-infected subjects than the current study and it is therefore possible that our study was not sufficiently powered to detect significant differences in MCP-1 levels within the context of an African population.

Data from the current study demonstrates that the inflammatory cytokines, IL-6 and TNF-α, were elevated in the HIV-infected cohort, particularly in those with low CD4 counts. This persisted even after 18 months of administration of ART. There is thus continuous immune activation in the HIV-infected population despite treatment. Other studies also show that inflammation is maintained in HIV-infected subjects even after ART initiation (Kiefer et al., 2018, Macatangay et al., 2017, Rönsholt et al., 2013). Interleukin-8 levels did not differ between the HIV-infected ART-naïve study groups and the uninfected cohort. This finding is contrary to a report from De Pablo-Bernal et al. (2014) in which serum levels of IL-8 were significantly higher in the HIV-infected ART-naïve group, 27 – 37 years of age, when compared with age-matched uninfected controls, but significantly lower when compared with uninfected subjects, greater than 64 years of age.

The current study demonstrated that biomarkers of endothelial dysfunction that were elevated in HIV-infected, compared to HIV-uninfected subjects, decreased significantly after 18 months of ART. This observation is in agreement with other studies involving individuals on ART for 12 to 24 months (Arildsen et al., 2013, van Vonderen et al., 2009). However, some investigations have shown that endothelial

dysfunction is not alleviated after long-term (8 to 12 years) ART use (Mosepele et al., 2018, Ronsholt et al., 2013).

The serum levels of HDL were found to be lower in the HIV-infected compared to the HIV-uninfected group, irrespective of CD4 counts. Similar data has been reported in previous studies (Fourie et al., 2015, Fourie et al., 2011). Total cholesterol level was lower in subjects with low CD4 counts than those with high CD4 counts and than those who were HIV-uninfected, while LDL-C showed a similar difference but only between the two CD4 groups. It is known that untreated HIV infection is associated with lower total cholesterol levels (Riddler et al., 2003). The current data therefore suggests that this effect is more prominent in those with low CD4 counts and high viral loads.

Multiple regression analyses demonstrated that differences in the level of serum markers of endothelial dysfunction, particularly VCAM-1 and vWF, between the HIV-infected and HIV-uninfected groups were specifically associated with viral infection, and were not due to confounding from other sources such as gender, lipid or cytokine levels. These findings are supported by previous studies conducted at both African sites and elsewhere (Mezoh and Crowther, 2019, Mosepele et al., 2018, Fourie et al., 2011). The current data is novel in that it includes assessment of serum vWF in the panel of endothelial dysfunction biomarkers, and vWF has not previously been studied in the context of HIV infection and CVD risk factors in African populations. Earlier African studies have shown elevated vWF levels in HIV-infected compared to HIV-uninfected subjects, but these studies were performed in the context of stroke (Allie et al., 2015), malaria and sepsis (Huson et al., 2016) and coagulation markers (Jong et al., 2010) and included no adjustment for possible confounding from CVD-related variables.

The present study demonstrates that elevated serum levels of VCAM-1 and vWF in HIV-infected subjects are related to low CD4 counts and high viral load, respectively. This is a novel finding which suggests that endothelial dysfunction in HIV infection is due to direct effects of the virus on the endothelium and to virus-associated immune dysregulation, and previous studies have demonstrated such associations. Thus, it is known from *in vitro* studies that viral proteins such as Nef, Tat and gp120 can induce endothelial dysfunction (Mazzuca et al., 2016) while CD4+ T-cell suppression by the virus may lead to immune activation (Younas et al., 2016), which in turn has been associated with endothelial dysfunction (Beltrán et al., 2015). It is interesting to note that elevated serum levels of VCAM-1 have been associated with activated macrophages in subjects with HIV infection (Grome et al., 2017). However, no studies have investigated the effect of HIV viral proteins on endothelial vWF production.

The main limitation of this study was the relatively small sample size, particularly that of the longitudinal analysis. Despite this, significant differences were observed across the study groups and these findings were supported by data from other studies (Mosepele et al., 2018, Fourie et al., 2015, De Pablo-Bernal et al., 2014, Fourie et al., 2011). Also, regression analyses were performed using cross sectional data, which does not allow us to identify causation, but only to observe associations. In addition, the duration of HIV-infection was not known for those subjects who were HIV-infected. The main advantage of this study was the analysis of a broad range of serum markers of endothelial dysfunction and inflammation in an under-studied population in which HIV-infection is highly prevalent. Furthermore, this is one of the very few studies to measure the levels of such biomarkers before and after the initiation of ART, and to investigate the interaction of these biomarkers with each other and with classical CVD risk factors in an HIV-infected ART-naïve population.

In conclusion, our data shows evidence of endothelial dysfunction in the Black South African ART-naïve HIV-infected population. Commencement of HIV treatment was associated with an improvement in endothelial function, but markers of inflammation continued to increase. This study suggests that endothelial dysfunction may arise from direct effects of the virus on the endothelium and from immune dysregulation. In the following study we attempted to test the hypothesis that the effects of the virus on the endothelium are due to the actions of the Nef and Tat proteins by sequencing the associated viral genes and comparing serum markers of endothelium function between subjects carrying HIV with different *nef* or *tat* variants.

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Chapter 3: Mutations in the HIV-1 *nef* and *tat* Genes are Associated with Endothelial Dysfunction

3.1 Introduction

The effective administration of antiretroviral drugs has resulted in a decrease in the rate of AIDS-related deaths, as well as opportunistic infections, in the HIV-infected population. However, non-communicable diseases such as cardiovascular diseases (CVD) are now an emerging cause of concern ((UNAIDS), 2017), with an increased number of CVD-related deaths in HIV-infected versus HIV-uninfected individuals (Freiberg et al., 2013, Chow et al., 2012). The mechanism for this enhanced CVD in the context of HIV infection has not yet been defined. The direct role of highly active antiretroviral therapy (HAART) as a cause of CVD is controversial with conflicting data (Arildsen et al., 2013, Lang et al., 2010, Obel et al., 2007). Alternatively, HIV infection itself has been proposed to contribute towards the development of CVD in HIV-infected individuals through its effects on the vascular endothelium (Wang et al., 2015).

Endothelial dysfunction is defined as the pathological state of the endothelium in which there is an imbalance between substances produced by or acting on the endothelium (Matzen et al., 2004). It is regarded as a characteristic marker for the development of CVD (Münzel et al., 2008). During endothelial damage, endothelial trans-membrane proteins, which include intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial leukocyte adhesion molecule-1 (E-selectin), are released into the blood circulation (Flammer et al., 2012, Verma et al., 2003). In addition, proteins that act on endothelial cells, such as monocyte chemoattractant protein-1 (MCP-1) and von Willebrand factor (vWF), are upregulated upon oxidative stress brought on by endothelial damage (Miller et al., 2010, Seigneur et al., 1997). Endothelial damage is also accompanied by an inflammatory response

(Bruyndonckx et al., 2013), which involves the upregulation of cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8) (De Pablo-Bernal et al., 2014). Clinical studies conducted on individuals infected with HIV show that some, but not all, display endothelial dysfunction (Fourie et al., 2015, Graham et al., 2013). This differential effect of the virus on endothelial dysfunction may arise from genetic heterogeneity of the virus or heterogeneity of the host response to infection.

The HIV genome exhibits a very high mutation rate and substantial diversity (Sanjuán et al., 2010). Variation in HIV viral protein sequences has been shown to influence the host response to the virus. As an example, *tat* sequence variation has been associated with variation in chemoattractant activity and ability to stimulate TNF-α production (Campbell et al., 2007, Siddappa et al., 2006). Cohort studies have also implicated sequence variation with clinical outcomes. Genetic variation in *tat* has been associated with neurological disorders in HIV-1 patients (Li et al., 2012, Cowley et al., 2011, Mayne et al., 1998). Similarly, sequence analysis of HIV-1 *tat*, isolated from an HIV-infected cohort with and without HIV-associated dementia, revealed an association between signature sequences in this gene and neurological impairment (Bratanich et al., 1998). In addition, *nef* polymorphisms have been associated with disease progression in HIV-1 patients (Walker et al., 2007). Sequence analysis of HIV-1 *nef* genes, isolated from a cohort of HIV-infected, ART-naïve infants and children with varying stages of disease progression revealed unique polymorphisms specific to slow progressors at the N-terminal and C-terminal domains (Walker et al., 2007).

Several studies support the hypothesis of HIV infection being a CVD risk factor in the HIV-infected population, with HIV viral proteins being role players in the development of CVD via their effects on the vascular endothelium. This hypothesis has arisen from data obtained from cell-based and animal studies

(Wang et al., 2014, Matzen et al., 2004, Dhawan et al., 1997). *In vitro* and *in vivo* studies have shown that the HIV viral proteins Nef (Wang et al., 2014), Tat (Matzen et al., 2004, Dhawan et al., 1997) and gp120 (Jiang et al., 2010) upregulate the expression of markers of endothelial function and inflammation. The HIV-1 Nef and Tat amino acid sequences have been extensively studied in relation to HIV disease progression, however limited research has been done on HIV-1 Nef and Tat viral proteins within the context of non-communicable diseases. Research conducted by Almodovar *et al*. revealed HIV-1 Nef polymorphisms to be associated with pulmonary hypertension (Almodovar et al., 2012). Based on these observations, we therefore hypothesized that unique polymorphisms in HIV-1 Nef and Tat may be associated with endothelial dysfunction, as measured by serum levels of ICAM-1, VCAM-1, E-Selectin, MCP-1, vWF, IL-6, IL-8 and TNF-α, in the HIV-infected population.

3.2 Materials and Methods

3.2.1 HIV-1 clinical isolates

Eighty (80) HIV-infected ARV-naïve Black South Africans between the ages of 30 to 50 years were recruited from the Nthabiseng and Zazi Clinics, Chris Hani Baragwanath Hospital as mentioned in Chapter 2, section 2.2.2. Patient data and blood samples were collected as described in Chapter 2, section 2.2.4, and blood samples stored at -80^{o}C until further analysis. Markers of endothelial dysfunction (ICAM-1, VCAM-1, E-selectin and vWF), as well as markers of inflammation (TNF-α, IL-6 and IL-8) were measured as detailed in Chapter 2, section 2.2.5.

3.2.2 Sequencing of HIV-1 *nef* and *tat* from viral RNA

3.2.2.1 Isolation of viral RNA and cDNA synthesis

Viral RNA from EDTA plasma samples of the HIV-infected cohort was extracted using a QIAamp viral RNA minikit (Qiagen, CA, U.S.A) according to the manufacturer's instruction. Thereafter, HIV viral RNA from the clinical isolates was reverse transcribed into cDNA using Super-Script III reverse transcriptase (Invitrogen, CA, U.S.A) and the QuantiTect Reverse Transcription kit (Qiagen, CA, U.S.A) with HIV-1 subtype C specific primers Vif1: 5'-GGGTTTATTACAGGGACAGCAGAG -3' and OFM19: 5'- GGT AGG ATC TCT ACA ATA CTT GGC ACT G -3' (Figure 3.1), following a technique developed by Salazar-Gonzalez *et al*. (2011) which enables amplification of the highest representative population of the HIV-1 genome.

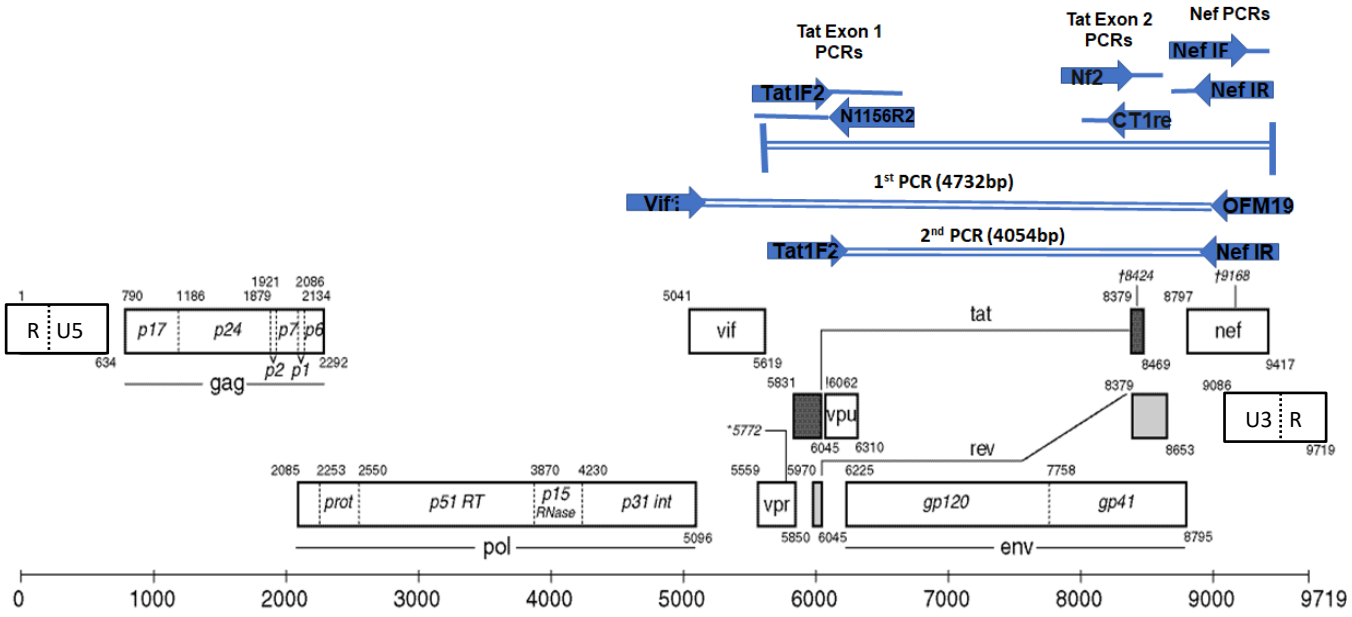


Figure 3.1: Schematic illustration of polymerase chain reactions (PCRs) carried out to obtain HIV-1 *nef* and *tat* from HIV-1 viral RNA. HIV-1 gene map depicting the cDNA and HIV-1 genes obtained following reverse transcription of HIV-1 viral RNA. Outer primer pair used for the first round of PCR, Vif1 and OFM19, are located at position 4886 – 4909 and 9589 – 9617, respectively. The inner primer pair used in the second PCR, TatIF2 and NefIR, are located at position 5449 – 5476 and 9483 – 9502, respectively. Primers used for sequencing HIV-1 *nef*, NefIF and NefIR, are located at position 8735 – 8756 and 9483 – 9502, respectively; while those used for *tat* exon 1 sequencing, Tat IF2 and 1156R2, are respectively found at position 5449 – 5476 and 6196 – 6219, and *tat* exon 2 sequencing primers, Nf2 and CT1re, span the region of 8062 – 8079 and 8572 – 8602, respectively (Adapted from Los Alamos National Laboratory, 2018).

3.2.2.2 Amplification of HIV-1 *nef* and *tat* by nested polymerase chain reaction (PCR)

The amplification of a gene by PCR is governed by the specificity to which the primers bind to the DNA template. Hence, polymorphisms located at the primer binding site of the HIV-1 *nef* and *tat* genes could hinder primer binding. Therefore, the *nef* and *tat* genes were amplified in a nested polymerase chain

reaction (PCR) using Platinum^{TM} *Taq* DNA polymerase high fidelity (Invitrogen, CA, U.S.A) and HIV-1 subtype C Env specific outer primers as described by Salazar-Gonzalez *et al*. (2011), and inner primers as described by Bredell *et al.* (2007) with slight modifications. The HIV-1 encoding region from position 4886 to 9617, which includes *vif, vpr*, *vpu*, *rev*, *nef* and *tat*, was amplified yielding a single amplicon of 4732 bp (Figure 3.1). Outer primers Vif1: 5'-GGGTTTATTACAGGGACAGCAGAG-3' and OFM19: 5'-GGTAGGATCTCTACAATACTTGGCACTG-3' were used in the first PCR reaction, following which the inner primers, Tat1F2: 5'-GGTAGGATCTCTACAATACTTGGCACTG-3' and NefIR: 5'-CTTATATGCAGCATCTGAGG-3', were used in the second PCR reaction. Amplicons were electrophoresed on a 1% agarose gel to confirm successful amplification of the target 4054 bp fragment. Thereafter, PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, CA, U.S.A) according to the manufacturer's instruction, prior to performing HIV-1 *nef* and *tat* sequencing reactions.

3.2.2.3 Sequencing of HIV-1 *nef* and *tat* genes

The *nef* and *tat* encoding regions were sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, CA, U.S.A) according to the manufacturer's instructions. In the sequencing reaction, *tat* exon 1 was sequenced using primers Tat1F2: 5'-GGTAGGATCTCTACAATACTTGGCACTG-3' and N1156R2: 5'-TCATTGCCACTGTCTTCTGCTCT-3', while *tat* exon 2 was sequenced using primers Nf: 5'-TGA CCT GGA TGC AGT GG -3' and CT1re: 5'-GACTTCCCAGATACTTAAGAGCTTCCCACC-3'. The *nef* fragment was sequenced using primers NefIF: 5'-CCTAGAAGAATAAGACAGGGC-3' and NefIR: 5'-CTTATATGCAGCATCTGAGG-3' (Figure 3.1).

Sequencing reactions were purified and DNA concentrated by ethanol precipitation. Briefly, two volumes of a mixture of 0.3M acetate in 100% ethanol was added to the sequencing products and these were centrifuged at 4,000 x g for 30 mins, following which the supernatant was discarded and the pellet washed with cold 70% ethanol and dried by centrifuging at 2,000 x g for 5 mins on an inverted paper towel. Thereafter, products were resolved on an ABI 3500xL automated genetic analyser (Applied Biosystems, CA, U.S.A).

3.2.2.4 Bioinformatic analysis of sequencing data

The nucleotide sequence regions coding for the HIV-1 *nef* and *tat* were identified and assembled using the CLC workbench (CLC Bio) and edited with BioEdit (ver. 5.0.9). Multiple sequence alignments of HIV-1 *nef* and *tat* nucleotide sequences were performed using BioEdit and translated into amino acid sequences. HIV-1 Nef and Tat amino acid sequences were analysed and compared against consensus sequence sets retrieved from the Los Alamos HIV-1 Sequence Database (Los Alamos National Laboratory, 2018). Phylogenetic trees were constructed using MEGA to screen for contamination.

3.2.3 Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc.) and Statistica v13.3 (Statsoft Inc., Tulsa, OK, U.S.A.). Variables that were normally distributed were expressed as mean ± SD while variables that were non-parametric were expressed as median [interquartile range]. Continuous variables were compared between groups using a Students unpaired t test or a Mann-Whitney U test while categorical variables were compared using the χ^2 test. HIV-1 Nef andTat amino acid sequences from the HIV-infected cohort were individually sorted in decreasing order of levels of ICAM-1, VCAM-1, E-selectin, vWF, TNF-α, IL-6 and IL-8. Thereafter, subjects in each of these biomarker groups were divided

into two sub-groups based on the median value of the serum biomarker. The frequency of each Nef or Tat variant was then compared between the 2 sub-groups using a contingency table and a two-tailed Fisher's exact test. To confirm that variants which showed statistically significant differences in frequency between the 2 sub-groups were associated with biomarker levels, a Mann-Whitney U test or Kruskal-Wallis ANOVA was used to compare the biomarker levels between subjects harbouring the wild type and the mutant amino acid Nef sequence.

3.3 Results

3.3.1 Clinical and virologic characteristics of the study group

Remnant plasma samples for use in this study were derived from 65 of the 80 HIV-infected ARV-naïve participants. Viral RNA was extracted from all these samples and the amplicons obtained by nested PCR assessed by agarose gel electrophoresis (appendix 5). However, HIV-1 *nef* and *tat* sequences were successfully amplified from viral RNA extracted from only 35 of these subjects. Thus, HIV-1 *nef* and *tat* sequences were effectively obtained from 30 and 34 patients, respectively. The mean age of this sub-group of 35 subjects was 39 years, with a mean CD4 T cell count of 368 cells/mm^3. Table 3.1 shows the clinical and virologic characteristics of the sub-group.

The low success rate of 54 % (35 / 65) for HIV-1 *nef* and *tat* sequencing, could be because 17 individuals out of the 65 HIV-infected adults had viral load levels below 1,500 cells/mm^3. Attempts to amplify viral RNA from these subjects were unsuccessful. Viral load is known to greatly impact sequencing with a typical threshold for the successful sequencing of HIV viral proteins set at 1,000 cells/mm^3 (Dudley et al., 2012). Therefore, taking the latter into consideration, *i.e.* disregarding individuals that had viral load levels < 1,500 cells/mm^3, our success rate for sequencing HIV-1 *nef* and *tat* was 63 % (30 / 48) and 71 %

(34 / 48), respectively. In addition, given that HIV has a high rate of mutation, it seems likely that amongst other factors, mismatches between the primers and target may also influence amplification efficiency.

Table 3.1: Clinical features of the HIV-infected sub-group from whom HIV-1 *nef* and *tat* sequences were derived

Sample ID	Age (Years)	BMI (kg/m^2)	Viral Load (copies/mL)	CD4 ($cells/mm^3$)
EFH0002	45	21.7	152000	248
EFH0003	40	64.1	36000	406
EFH0006	37	20.1	299000	146
EFH0011	50	28.7	103000	86
EFH0013	34	22.4	81800	59
EFH0014	38	28.8	301000	338
EFH0021	37	32.3	151000	203
EFH0029	35	20.7	150000	222
EFH0030	46	38.9	189000	155
EFH0032	34	22.7	89100	288
EFH0034	31	22.9	12000	228
EFH0037	42	23.5	1500	282
EFH0038	30	18.5	292000	130
EFH0040	37	27.7	102000	24
EFH0050	39	18.4	6900	138
EFH0051	50	30.2	30000	295
EFH0058	30	30.8	208000	320
EFH0060	46	34.2	4250	345
EFH0090	39	21.1	164000	146
EFH0091	48	19.9	63100	416
EFH0096	32	21.8	125000	277
EFH0132	38	18.2	32300	272
EFH0147	41	19.7	158000	342
EFH0001	31	21.6	98100	662
EFH0005	31	31.6	64700	575
EFH0026	40	28.4	1560	501
EFH0027	36	24	34800	771
EFH0033	41	23.5	11000	687
EFH0042	41	20.2	10100	501
EFH0049	32	19.1	199000	1098
EFH0094	37	27.7	155000	823
EFH0128	41	20	211000	649
EFH0144	39	26.6	281000	524

EFH0146	32	50.6	27300	514
EFH0148	46	23.8	230000	478
Mean	39	26.4	116415	376

3.3.2 Comparison of cardiometabolic variables between groups

Available data for the subjects from whom viral DNA sequences were obtained and those from whom no sequence data was obtained were compared to determine whether the sequenced group were different from the latter group. No significant differences were observed between these groups for age, gender, anthropometry, blood pressure, glucose, HDL-C, TNF-α, IL-6, ICAM-1, E-selectin, vWF and MCP-1 levels (Table 3.2). However, a higher frequency of males and higher levels of viral load and VCAM-1 and lower levels of triglycerides, total cholesterol, LDL-C, CD4 counts and IL-8 were observed in the sequenced compared to the non-sequenced group.

Table 3.2: Comparison of HIV sequenced and non-sequenced groups

Variables	HIV non-sequenced (n=45)	HIV sequenced (n=35)
Age (years)	36.71 ± 6.93	38.46 ± 13.31
Gender, male n (%)	5 (11.11)	11 (31.42)*
BMI (kg/m^2)	26.86 ± 4.64	26.41 ± 9.38
Waist circumference (cm)	88.89 ± 9.66	84.46 ± 13.31
Diastolic BP (mmHg)	80.33 ± 15.22	79.71 ± 13.46
Systolic BP (mmHg)	137.07 ± 20.15	129.51 ± 21.93
Glucose (mmol/L)	4.66 ± 1.71	4.38 ± 0.35
Triglyceride (mmol/L)	0.87 [0.61, 1.10]	0.58 [0.38, 0.76]***
Total cholesterol (mmol/L)	3.89 ± 0.84	3.22 ± 0.78**
LDL-C (mmol/L)	2.31 ± 0.62	1.93 ± 0.66*
HDL-C (mmol/L)	1.08 [0.91, 1.35]	0.92 [0.71, 1.20]
CD4+ cell count (cells/mm^3)	542.56 ± 314.68	375.69 ± 241.17*
Viral load (copies/mL)	8981.22 ± 18465.38	116414.57 ± 94695.18***
TNF-α (pg/mL)	6.2 [4.5, 9.6]	7.7 [5.2, 10.5]
IL-6 (pg/mL)	2.9 [1.3, 4.6]	3.9 [2.3, 5.3]
IL-8 (pg/mL)	8.4 [5.2, 24.1]	6.0 [3.2, 7.9]**
ICAM-1 (ng/mL)	464.8 [344.1, 923.1]	848.4 [403.4, 1048.7]
VCAM-1 (ng/mL)	522.9 [426.3, 805.6]	954.3 [669.8, 1477.7]*
E-selectin (ng/mL)	27.9 [19.8, 33.9]	30.0 [23.4, 44.1]
vWF (µg/mL)	20.7 [12.1, 29.9]	26.9 [18.4, 43.9]
MCP-1 (pg/mL)	139.3 [114.9, 181.1]	137.2 [108.5, 209.7]

Data given either as mean ± standard deviation or median [lower quartile, upper quartile]; n indicates the number of participants; *p<0.05, **p<0.005, ***p<0.0005 vs HIV non-sequenced.

3.3.3 Comparison of endothelial dysfunction and inflammation between study groups

No significant difference was observed between the 2 groups with regards to plasma levels of TNF-α, IL-6, ICAM-1, E-selectin and vWF and MCP-1 (Table 3.3). However, plasma levels of IL-8 were significantly lower in the HIV-infected sequenced group compared to the HIV-infected un-sequenced group, while plasma levels of VCAM-1 were higher in the sequenced versus the un-sequenced group.

Table 3.3: Comparison of markers of endothelial dysfunction and inflammation between study groups

Variables	HIV-infected Unsequenced (n = 45)	HIV-infected Successfully sequenced (n = 35)
TNF-α (pg/mL)	6.2 [4.5, 9.6]	7.7 [5.2, 10.5]
IL-6 (pg/mL)	2.9 [1.3, 4.6]	3.9 [2.3, 5.3]
IL-8 (pg/mL)	8.4 [5.2, 24.1]	6.0 [3.2, 7.9]**
ICAM-1 (ng/mL)	464.8 [344.1, 923.1]	848.4 [403.4, 1048.7]
VCAM-1 (ng/mL)	522.9 [426.3, 805.6]	954.3 [669.8, 1477.7]*
E-selectin (ng/mL)	27.9 [19.8, 33.9]	30.0 [23.4, 44.1]
vWF (µg/mL)	20.7 [12.1, 29.9]	26.9 [18.4, 43.9]
MCP-1 (pg/mL)	139.3 [114.9, 181.1]	137.2 [108.5, 209.7]

Data given as median [lower quartile, upper quartile]; n indicates the number of participants; *p<0.05, **p<0.0005 vs HIV-infected un-sequenced.

3.3.4 Sequence analysis of HIV-1 Nef

The HIV-1 subtype C virus is the most dominant subtype in the South African population accounting for up to 98% of HIV infections (Hemelaar et al., 2006). The HIV-1 *nef* and *tat* gene sequences derived from the plasma samples were therefore aligned to the HIV-1 subtype C consensus sequence, retrieved from the Los Alamos sequence database (Los Alamos National Laboratory, 2018) and translated into Nef and

Tat amino acid sequences (Figure 3.2 and 3.4) using the Bio Edit translation tool. The HIV-1 Nef and Tat functional domains were annotated using the HIV-1 proteomics resource (Doherty et al., 2005). The HIV-1 Nef amino acid residues were numbered following HIV-1 HXB2 Nef which is the general sequence used for numbering. The HIV-1 consensus C Nef differs in number from the HXB2 Nef by the addition of an extra E amino acid residue at position 62 resulting in a shift in numbering by 1 residue from position 61.

Looking at the HIV-1 Nef multiple sequence alignments of the clinical isolates from the HIV-infected subset, shown in Figure 3.2, the N-terminal and C-terminal domains spanning position 1 to 79 and 165 to 220 respectively, possess a lot of variations in the HIV-1 Nef consensus C sequence, with the centre region from position 80 to 164 staying relatively conserved. Ten (10) HIV-1 Nef amino acid residue variants were observed that were associated with varying levels of markers of endothelial dysfunction and inflammation and these tended to be located toward the N and C-terminal domains. However, one of these variants (E59Q) that was associated with ICAM-1 serum levels was also observed within the CAWLEAQ functional domain, which is a critical region for the CD4 down-regulation activity of Nef (Mann et al., 2015).

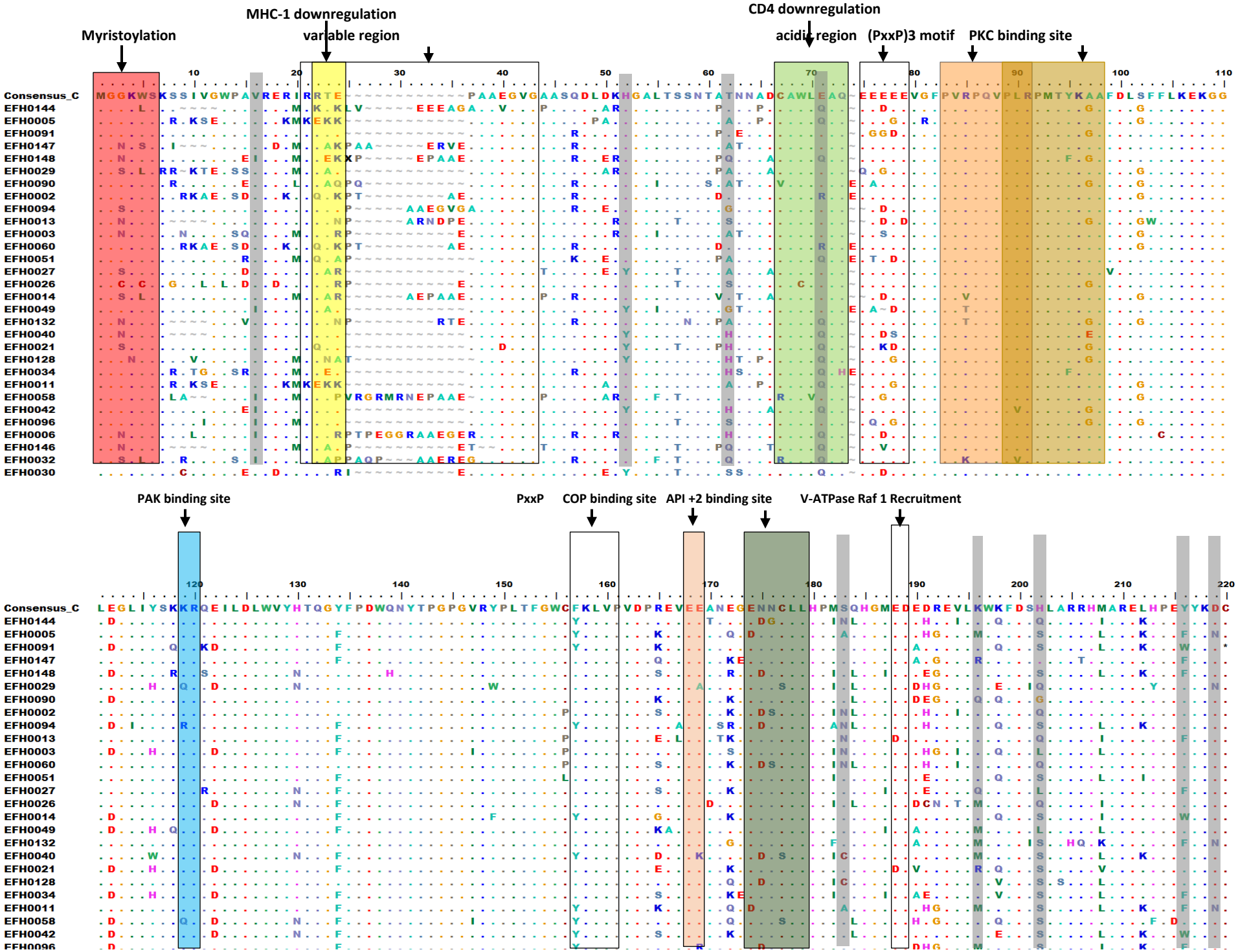

Myristoylation
MHC-1 downregulation
variable region
CD4 downregulation
acidic region
(PxxP)3 motif
PKC binding site
PAK binding site
PxxP
COP binding site
API +2 binding site
V-ATPase Raf 1 Recruitment
Consensus_C
EFH0144
EFH0005
EFH0091
EFH0147
EFH0148
EFH0029
EFH0090
EFH0002
EFH0094
EFH0013
EFH0003
EFH0060
EFH0051
EFH0027
EFH0026
EFH0014
EFH0049
EFH0132
EFH0040
EFH0021
EFH0128
EFH0034
EFH0011
EFH0058
EFH0042
EFH0096
EFH0006
EFH0146
EFH0032
EFH0030

Figure 3.2: Multiple sequence alignment of plasma derived HIV-1 Nef protein sequences. Nucleotide sequences were aligned with the HIV-1 *nef* Clade C consensus sequence (retrieved from Los Alamos) using BioEdit, translated and numbered following HXB2. Sequences have been placed in decreasing order of ICAM-1 levels. Functional domains, which include the sequence regions involved in Nef trafficking and internalization resulting in MHC-1 and CD4 downregulation, are shaded in yellow and green, respectively. Other functional motifs, such as those involved in Nef modification and signalling, which includes the region for myristoylation, the (PxxP)3 motif, the PKC, PAK, COP and API +2 binding sites, are all boxed and shaded in red, orange, purple, blue, pink and brown, respectively. Amino acid residues determined by means of a contingency table and Fisher's Exact two-tailed test to be associated with either a marker of endothelial function or inflammation are shaded in grey. Other annotated indels are boxed. Amino acid residues that are the same as that of Nef Clade C are represented in colour dots (.), while gaps in the alignment are represented with tildes (~).

3.3.5 Sequence analysis of HIV-1 Tat

In contrast to Nef, the HIV-1 Tat multiple sequence alignment of the clinical isolates from the HIV-infected subset, represented in Figure 3.4, revealed variations to the HIV-1 Tat consensus C sequence to be mainly at the C-terminal domain, with the N-terminal domain and central region remaining relatively conserved. The C-terminal domain of Tat is encoded in *tat* exon 2, spanning regions 71 to 101, which is located within the *env* gene (Frankel and Young, 1998). The *env* gene accounts for the high genetic variability displayed by HIV with a substitution rate of 0.0028 per site, per year (Rambaut et al., 2004, Holmes et al., 1992). Tat exon 2 constitutes the RDG domain which is involved in HIV replication (Smith et al., 2003). The HIV-1 Tat amino acid residues associated with varying levels of markers of endothelial

dysfunction were located only in Tat exon 1, at position 12 and 58, within the acidic domain and glutamine-rich motif, respectively.

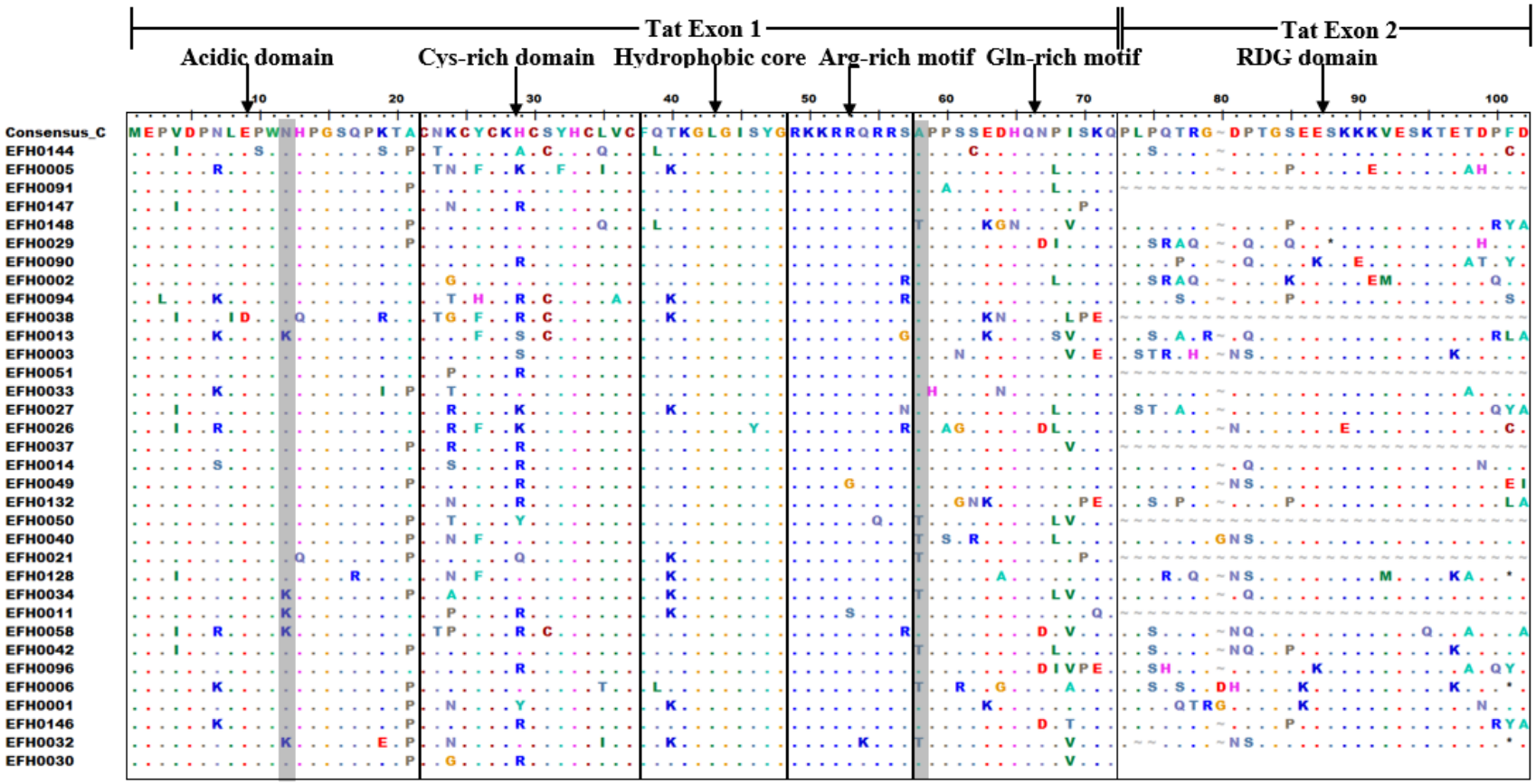


Figure 3.3: Multiple sequence alignment of plasma derived HIV-1 Tat sequences. Sequences were aligned with the HIV-1 *tat* Clade C consensus sequence (retrieved from Los Alamos) using BioEdit, translated and numbered following HXB2. Sequences have been placed in decreasing order of ICAM-1 levels. Amino acid residues determined by means of a contingency table and Fisher's Exact two-tailed test to be associated with a marker of endothelial function are shaded in grey. The functional domains which include the acidic region, cysteine-rich motif, hydrophobic core region, arginine-rich motif, glutamine-rich motif and RDG domain, are all boxed (Mahlknecht et al., 2008). Amino acid residues that are the same as that of HIV-1 Nef Clade C are represented in colour dots (.) while gaps in the alignment are represented with tildes (~).

3.3.6 Determinants of systemic inflammation and endothelial dysfunction

Sequence analysis of amino acid residues of HIV-1 Nef (Figure 3.2) and Tat (Figure 3.3) revealed several associations of statistical significance between sequence variants and markers of endothelial dysfunction (Table 3.4). With regards to Nef, results from the contingency table showed that the consensus amino acids 16V, H40, 50T, and 59E were associated with higher levels of ICAM-1, while the consensus amino acids 182K and 205D were associated with higher levels of MCP-1 and TNF-α, respectively. However, the 59E variant just failed to show a significant effect (p=0.059). A mutation at codon 169 from Ser to Asn, and 188 from His to Gln, was associated with higher levels of ICAM-1. The HIV-1 Nef consensus C amino acid residue at codon 188 (His) was present in only one subject out of the 30 sequenced samples with 20 % (6/30) of the study population having the variant Gln, and 63 % (19/30) having the variant Ser. In addition, a mutation at codon 202 from Tyr to Phe was associated with increased levels of VCAM-1.

Sequence analysis of HIV-1 Tat using a contingency table and two-tailed Fisher's exact test showed only two codons to be associated with markers of endothelial dysfunction. The consensus amino acid, 58A, was associated with higher levels of ICAM-1 while a mutation at codon 12 from Asn to Lys was associated with higher levels of VCAM-1. However, the association of these variants with ICAM-1 and VCAM-1 just missed statistical significance (p=0.059, and p=0.052, respectively).

Table 3.4: HIV-1 Nef and Tat polymorphisms significantly associated with biomarkers of endothelial dysfunction and inflammation

Biomarker	Variant	Amino acid	N	Biomarker level (median [interquartile range] (ng/mL))	p value
Nef sequences:					
ICAM-1	V16I	V	18	913.08 [528.05, 1048.70]	**0.032**
		I	8	379.44 [136.50, 747.48]	
	H40Y	H	21	1007.92 [694.52, 1194.61]	**0.029**
		Y	7	528.05 [355.44, 800.43]	
	T50H,A	H	6	467.10 [355.44, 528.05]	**0.017**
		A	9	1007.92 [899.79, 1194.61]	
		T	6	1064.07 [830.47, 1636.41]	
	E59Q	E	13	1007.92 [830.47, 1148.37]	0.059
		Q	15	509.03 [107.38, 926.38]	
	S169N	S	19	800.43 [355.44, 1148.37]	**0.045**
		N	6	1037.62 [1007.92, 1122.46]	
	H188Q,S	Q	6	1075 [1006, 1195]	**0.017**
		S	19	509 [166, 926]	
VCAM-1	Y202F	Y	17	847.69 [505.69, 1324.55]	**0.020**
		F	9	1293.34 [1173.32, 2337.36]	
MCP-1	K182M	K	18	149.58 [115.49, 217.43]	**0.048**
		M	8	103.46 [93.18, 130.39]	
TNF-α	D205N	D	25	8.27 [6.70, 11.05]	**0.037**
		N	5	5.21 [4.06, 6.64]	
Tat sequences:					
ICAM-1	A58T	A	26	909.52 [509.03, 1122.46]	0.059
		T	8	476.61 [231.41, 659.75]	
VCAM-1	N12K	N	29	932.77 [669.84, 1293.34]	0.052
		K	5	1477.71 [1202.95, 1617.01]	

Data given as median [lower quartile, upper quartile]; N indicates the number of individuals; significant values are in bold characters ($p<0.05$).

3.3.7 Effect of HIV-1 Nef polymorphisms on ICAM-1

Table 3.3 shows that 5 different polymorphic sites in the *nef* gene were significantly ($p<0.05$) associated with serum levels of ICAM-1. These associations were studied in more detail by expressing them as dot plots (see Figure 3.4). These data clearly show the separation in ICAM-1 levels between the different amino acid residues expressed at each of the 5 polymorphic loci. Next, to determine if there was an additive effect of these variants on ICAM-1 levels, the number of ICAM-1-associated Nef amino acid residues (residue count) present in the viral isolates of each patient (range of 0 to 5) were counted and plotted against the serum levels of ICAM-1 (Figure 3.5). The level of ICAM-1 can be seen to rise with an increase in the number of ICAM-1-associated Nef amino acid residues.

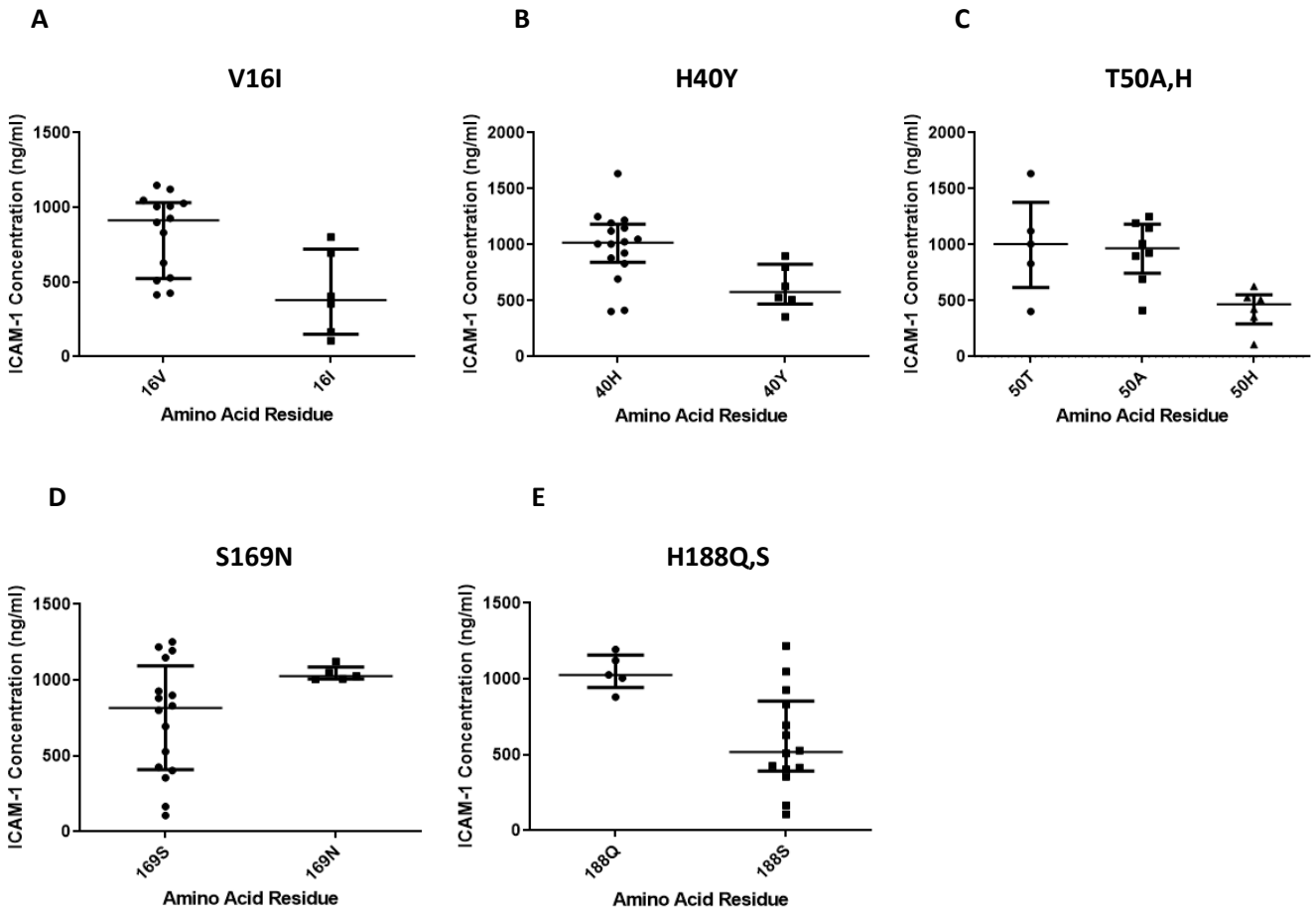


Figure 3.4: Dot plot of ICAM-1 levels versus amino acid residue at each of the 5 polymorphic loci that were associated with ICAM-1 (A-E). Data expressed as median with interquartile range; all graphs show data that are significantly different between groups at $p < 0.05$.

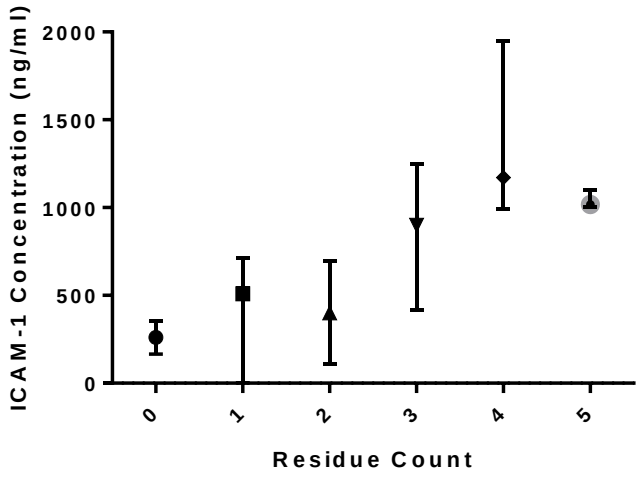


Figure 3.5: Plot of ICAM-1 levels across residue counts. The number of Nef ICAM-1-associated amino acid residues in each patient was counted (residue count) and subjects grouped according to the number of ICAM-1 associated residues and plotted against their ICAM-1 concentrations. Data are expressed as median with interquartile range; p=0.0038

3.4 Discussion

In this study we sought to investigate whether HIV infection was directly linked to the endothelial dysfunction observed in the HIV-infected population via the action of the viral proteins Nef and Tat. This was accomplished by sequencing the *nef* and *tat* genes from clinical isolates and the amino acid residues at polymorphic loci were screened for association with plasma levels of markers of endothelial dysfunction and inflammation. A total of 8 amino acid residues of significant ($p<0.05$) importance were identified in the Nef protein, of which five were linked to increased levels of ICAM-1, and one each with VCAM-1, MCP-1 and TNF-α. No HIV-1 Tat sequence variants of significant importance were linked to increased levels of any of these biomarkers, although 2 variants were identified that showed associations with ICAM-1 and VCAM-1 levels that just missed statistical significance. In addition, our analysis

showed that ICAM-1 levels increased with a rising residue count of the 5 loci that were individually associated with ICAM-1 levels.

The HIV-1 Nef protein has been reported to cause endothelial dysfunction via activation of the NF-*k*B pathway within endothelial cells, however, the mechanisms involved in this process are not clearly defined (Wang et al., 2014). In our study, we found the HIV-1 Nef amino acid residues 16V, H40 and 50T at the N-terminal domain, and 169N and 188Q at the C-terminal domain, to be linked with increased levels of ICAM-1, while the Nef residues 202F, 182K and 205D were linked with higher levels of VCAM-1, MCP-1 and TNF-α, respectively. All these variants were located at the N- and C-terminal domains of Nef. A study by Walker et al (2007) reported that mutations in HIV-1 *nef* were linked to a loss in MHC-1 downregulation and other functions involving cell signalling and these variants were present at the N- and C-terminal domains of the protein.

The N-terminal domain of HIV-1 Nef contains sequence regions involved in MHC-1 and CD4 downregulation, while the C-terminal domain contains sequence regions involved in the internalization of the Nef protein (Mandic et al., 2001). *In vitro* studies conducted by Mandic et al. (2001) using simian immunodeficiency virus (SIV) showed the amino acid residues 39Y, 194L, 195M, 204D and 205D to be a prerequisite for the cellular uptake of Nef by Jurkat and 293-T cells. Changes to any of the aforementioned consensus amino acid residues resulted in a loss in infectivity. A substitution to the amino acid residue 205D has thus been linked to a decreased uptake of the Nef protein by cells (Mandic et al., 2001), and slow disease progression (Walker et al., 2007). In the current study, the mutation 205D→ N was found to be associated with decreased plasma levels of TNF-α, and based on the study by Mandic et al. (2001) this may be related to reduced uptake by host immune cells.

In our study, the highest number of Nef variants were associated with ICAM-1 serum levels. Nef is reported to play a direct role in the upregulation of ICAM-1 via the extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) signalling pathway (Fan et al., 2010). Phosphorylation of ERK1 and ERK2 is enhanced by Nef, which activates the ERK/MAPK pathway resulting in the expression of the adhesion molecule ICAM-1. This is demonstrated by *in vitro* studies conducted by Fan et al. (2010), in which the HIV-1 Nef-induced overexpression of ICAM-1 by human vascular endothelial cells was inhibited by the kinase inhibitor, PD98059, a chemical known to inhibit the ERK/MAPK pathway. Studies have also shown associations between Nef and CVD in HIV-infected subjects (Wang et al., 2014, Stolp et al., 2012, Almodovar et al., 2011). Thus, in a study conducted in Europe by Almodovar et al. (2011), HIV-1 Nef sequences from 11 HIV-infected patients with pulmonary hypertension were compared against HIV-1 Nef sequences obtained from 7 HIV-infected subjects without pulmonary hypertension and polymorphisms in HIV-1 Nef were identified to be associated with pulmonary hypertension (Almodovar et al., 2012). The Nef mutations identified in the HIV-infected pulmonary hypertensive patients, which include R19K, R21Q, H40Y, A53P, L58V, E63G, M79I, T80N, Y81F and P150A,R,S,K,Q, were not influenced by age, ART or duration of HIV infection (Almodovar et al., 2012). However, in a validation cohort from U.S.A consisting of 11 HIV-infected patients with pulmonary hypertension compared against 22 HIV-infected subjects without pulmonary hypertension, only R21Q, H40Y, A53P, L58V, E63G, Y81F and P150A,R,S,K,Q, mutations were validated (Almodovar et al., 2012). Given that H40 was associated with pulmonary hypertension and was also associated with ICAM-1 levels in the current study, this variant could be a key player in the aetiology of CVD in HIV-infected subjects. The current study also showed that subjects carrying viruses with higher numbers of the 5 ICAM-

1-associated residues had higher serum levels of ICAM-1. This suggests an additive effect of the variants on ICAM-1 levels.

Inflammatory cytokines, such as IL-6, IL-8 and TNF-α, are known to activate the NF-*k*b pathway (Liao, 2013). Activation of the NF-*k*b pathway results in endothelial activation in which ICAM-1, VCAM-1 and E-selectin expression is upregulated, subsequently leading to endothelial dysfunction (Liao, 2013). In an *in vitro* study conducted by Mahlknecht et al. (2008), sequence variants in exon 1 and exon 2 of the *tat* gene were shown to activate the NF-*k*b pathway. Thus, lysine amino acid residues at positions 88, 89, and 90 and glutamic acid residues at positions 92, 94 and 96 of *tat* exon 2 were associated with NF-*k*b activation (Mahlknecht et al., 2008). Deletion of *tat* exon 2 resulted in a five-fold decrease in NF-*k*b activation by Tat. However, in our clinical isolates we found two amino acid residues, the consensus 58A and variant 12K in *tat* exon 1, to be associated with ICAM-1 and VCAM-1, respectively, although these associations just missed statistical significance. We observed no association of variants in exon 2 with any serum biomarker levels. The lack of agreement between our study and that of Mahlknecht et al. (2008) may be related to the fact that the latter study was investigating NF-*k*b expression in T cells, while we were assessing serum levels of relevant biomarkers released from the endothelium. Furthermore, the mechanism by which Tat may increase endothelial release of ICAM-1 and VCAM-1 is not fully understood and may not involve NF-*k*b activation.

A limitation to this study is the low number of HIV-1 Nef and Tat amino acid sequences analysed. In this study population, 26 % had viral load levels below the threshold for successful sequencing (Dudley et al., 2012). Several other factors such as poor sample quality, degraded viral RNA and insertions/deletions and sequence variants within the HIV genome targeted by the primers could possibly account for the low

sequencing success rate. A similar observation was reported by Rousseau et al (2006) in which near full-length genomes of HIV-1 subtype C was successfully sequenced from 67 % of samples. Nonetheless, in this study, sequencing of HIV-1 *nef* and *tat* from each patient was done in duplicates and phylogenetic analysis performed to compare sequences as a measure of quality control to eliminate possible contamination.

A sample size calculation was not performed because *nef* and *tat* are very polymorphic genes and we had no prior information on which of the *nef* polymorphisms could be associated with the serum biomarker levels. Another limitation is the discrepancy between the sequenced and unsequenced population. In comparison to the unsequenced population, the sequenced population had higher levels of viral load and VCAM-1, and lower levels of triglycerides, total cholesterol, LDL-C, CD4 cell count and IL-8. Thus, the results obtained from this study may not be a true representative of all HIV-infected subjects. This therefore warrants the study to be expanded with a larger cohort having a wider range of viral loads.

In conclusion, amino acid residues in HIV-1 Nef, and to a much lesser extent, HIV-1 Tat, were associated with endothelial dysfunction. This therefore provides evidence of HIV Nef and Tat as possible causative factors in the development of CVD in the HIV-infected population. This hypothesis was assessed in the following study by measuring the prevalence of Nef variants in subjects with low or high levels of carotid intima-media thickness (CIMT), which is a measure of sub-clinical atherosclerosis.

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Chapter 4: Association of *nef* Variants with Carotid Intima-Media Thickness (CIMT) in HIV-infected Subjects

4.1 Introduction

A number of studies has reported that measuring the thickness of the intima and the media, i.e the two inner layers of the carotid artery, is an appropriate non-invasive strategy for the assessment of cardiovascular risk (Owolabi et al., 2015, Den Ruijter et al., 2012, Zielinski et al., 2007).

Individuals infected with HIV have a greater predisposition to cardiovascular diseases (CVDs) compared to uninfected individuals. A study conducted by Utama et al. (2019) showed a significantly strong inverse correlation to exist between CD4/CD8 ratio and CIMT. A systematic review and meta-analysis conducted to investigate the effect of HIV infection and antiretroviral therapy (ART) on CIMT showed that HIV itself influenced CIMT progression, and therefore, could be a potential risk factor for cardiovascular events (Msoka et al., 2019).

In the previous chapter, HIV-1 *nef* and *tat* gene sequences obtained from a total of 35 HIV-infected individuals showed six *nef* polymorphisms to be significantly associated with levels of ICAM-1 and one polymorphism to be associated with VCAM-1, whereas only two *tat* variants were associated with ICAM-1 and VCAM-1. In addition, ICAM-1 levels increased with an increase in the number of these HIV-1 *nef* polymorphisms. We therefore hypothesised that if *nef* polymorphisms are associated with endothelial dysfunction, they may also be associated with downstream events which would include atherosclerotic plaque formation. The aim of the current study was therefore to determine the association of *nef* polymorphisms with CIMT in a group of HIV-infected individuals.

4.2 Materials and Method

4.2.1 Ethics

Ethical clearance for patient recruitment and sample collection was granted to Dr Michelle Moorhouse et al. by the Medical Human Research Ethics Committee of the University of the Witwatersrand under the ethical clearance number M160130 (Appendix 4). Participants were recruited and blood samples collected by Dr Alinda G. Vos. Ms Genevieve Mezoh was granted ethical clearance by the same ethics committee under the clearance number M150979 (Appendix 2) to carry out further analyses on these blood samples.

4.2.2 Patient population

The study consisted of a population of Black South African adults between the ages of 29 to 56 years recruited from the Wits Reproductive Health and HIV Institute (WHRI) in Johannesburg. One hundred and forty (140) HIV-infected patients were selected from the Ndlovu study (Vos et al., 2017) based on their CIMT measurement.

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. Participants were tested for HIV at the time of recruitment using a rapid HIV test kit (ADVANCED QUALITY™ Rapid HIV Test [InTec Products, China]), and positive results confirmed with a second rapid HIV test (ABON™ HIV 1/2/O Tri-Line HIV

Rapid Test Device [ABON Biopharm Hangzhou, China]). Samples that tested positive for HIV were validated by ELISA.

The current cohort included 70 HIV-infected subjects with CIMT < 0.50 (lower CIMT group) and 70 HIV-infected subjects with CIMT > 0.58 (higher CIMT group) and matched for age. A sample size of 140 participants was considered sufficient because several HIV-1 *nef* mutations associated with plasma ICAM-1 levels were identified in the study described in the previous chapter using an N of 65 (Chapter 3 section 3.3). Participants recruited into the HIV-infected groups were excluded if the HIV-infected individuals had known cardiovascular or pulmonary disease. Plasma levels of ICAM-1 were measured in these subjects.

4.2.3 Data and sample collection

Data and samples were collected as described by Vos et al., (2017). In brief, data was collected at baseline at the WRHI by the clinical investigators using the RedCap data management system. Information obtained by direct questioning following a standard questionnaire included age, gender, ethnicity, smoking, alcohol and drug usage, personal and family history of pulmonary and cardiovascular diseases, mental health and physical activity. Anthropometric data including height, weight, hip and waist circumference and blood pressure were recorded following a physical examination. An automated sphygmomanometer was used on both arms to record blood pressure of which readings were repeated thrice after a 5 minute interval rest and an average taken. Hypertension was defined as a blood pressure of >130/85 mmHg (Alberti et al., 2009). Hip and waist circumference were measured using a measuring tape with the participants in a standing position wearing light clothing. Hip circumference was recorded as the distance between the widest section of the buttocks, while waist circumference was recorded as the

length running from midway between the hip bone and the lower rib cage. Measurements were taken thrice and the last one used for subsequent analysis.

Blood serum and plasma samples were collected for routine clinical laboratory tests and storage. Levels of HIV-1 RNA, CD4 count, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides and fasting glucose were measured in the routine laboratory following standard protocols as desccribed in Chapter 2 section 2.2.4. A blood sample was collected in an extra 4 mL EDTA tube, centrifuged and stored as 1 mL aliquots of plasma and peripheral blood mononuclear cells (PBMCs) at -80°C for HIV RNA and DNA analysis.

To assess subclinical atherosclerotic disease and central vascular function, carotid intima-media thickness (CIMT) measurements were taken by trainied nurses following a standard protocol. Ultrasound measurements of the CIMT were taken at the WRHI using the Siemens Acuson p500 ultrasound (Siemens Healthcare (Pty) Ltd, South Africa), and scans obtained in B-mode with a ≥ 7.0 MHz linear probe, as described by Vos et al. (2019). After 15 minutes of bed rest, the end-diastolic images of the near wall and far wall of the common carotid artery (CCA) were captured from the right common carotid segment at angles of 90°, 120° and 150°, and from the left common carotid segment at angles of 210°, 240° and 270°, using a Meijer Arc with a broadband linear array transducer of ≥7.0 MHz. For image analysis, the transducer was positioned in line with the CCA, 1 cm from the carotid bifurcation, and CIMT measured semi-automatically with the Artery Measurement System software (Chalmers University, Götenburg, Sweden).

4.2.4 Sequencing of HIV-1 *nef* from peripheral blood mononuclear cells

4.2.4.1 Isolation of genomic DNA and amplification of HIV-1 *nef* by nested polymerase chain reaction (PCR)

Genomic DNA from PBMCs was extracted using the ABIOpure^{TM} Total DNA Blood/Cell Extraction Kit (AllianceBio, MA, U.S.A) according to the manufacturer's instruction. HIV-1 *nef* primers were designed using the PrimerDesign-M tool of the Los Alamos HIV database (Yoon and Leitner, 2015, Brodin et al., 2013). The *nef* gene was amplified in a nested PCR using Platinum^{TM} *Taq* DNA polymerase high fidelity (Invitrogen, CA, U.S.A) using an outer primer pair (Nef_F1: 5'- CAGTAGCTGARGGRACAGATAGG and Nef_R1: 5'-CTTTATTGAGGCTTAAGCAGTGGGTTC) and an inner primer pair (Nef_F2: 5'-AAGAATAAGACARGGCTTYG and Nef_R2: 5'- CAGCTGCTTATATGCAGCATC) (see Figure 4.1).

Amplicons were electrophoresed on a 1% agarose gel to confirm successful amplification of the target 1316 bp fragment. Thereafter, PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, CA, U.S.A) according to the manufacturer's instruction, prior to performing HIV-1 *nef* sequencing reactions.

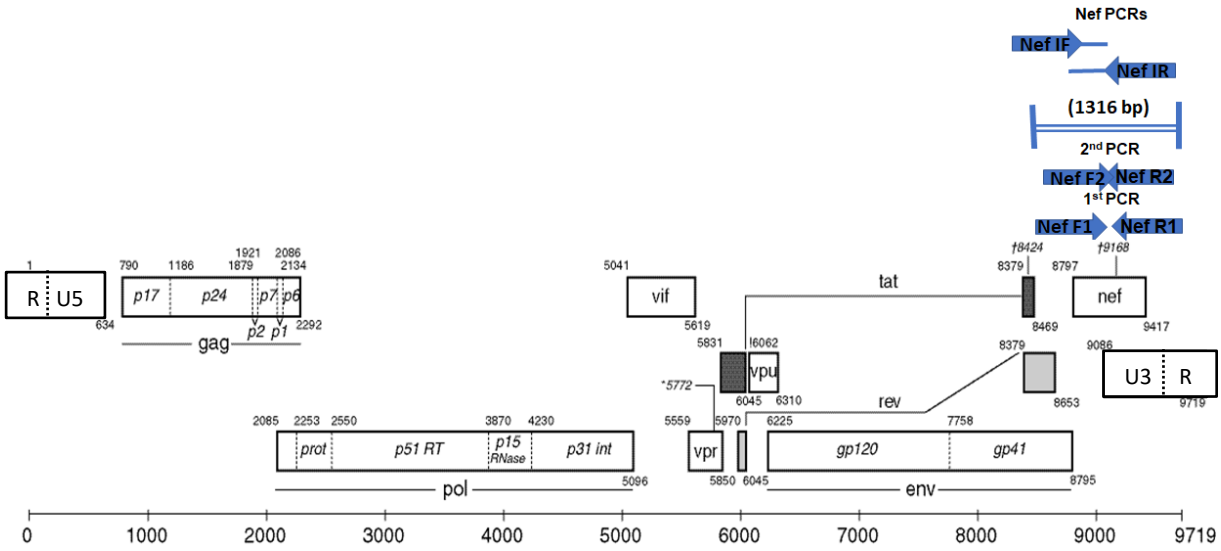


Figure 4.1: Schematic illustration of polymerase chain reactions (PCRs) carried out to obtain HIV-1 *nef* from clinical genomic DNA. HIV-1 gene map depicting the cDNA and HIV-1 genes obtained following reverse transcription of HIV-1 viral RNA. Outer primer pair used for the first round of PCR, NefF1 and NefR1, are located at position 8668 – 7422 and 10126 – 10152, respectively. The inner primer pair used in the second PCR, NefF2 and NefR2, are located at position 8741 – 8760 and 10037 – 10057, respectively. Primers used for sequencing HIV-1 *nef*, NefIF and NefIR, are located at position 8735 – 8756 and 9483 – 9502, respectively (adapted from Los Alamos National Laboratory, 2018).

4.2.4.2 Sequencing of HIV-1 *nef* genes

The *nef* encoding regions were sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, CA, U.S.A) according to the manufacturer's instruction. Sequencing reactions were performed using the Nef IF and Nef IR sequencing primers as detailed in Chapter 3, section 3.2.2.3

4.2.4.3 Bioinformatic analysis of sequencing data

Sequencing data was analysed and assembled using the CLC workbench (CLC Bio) to identify nucleotide sequence regions coding for HIV-1 *nef*, and edited with BioEdit (ver. 5.0.9), as detailed in Chapter 3, section 3.2.2.4. The HIV-1 *nef* gene sequences derived from PBMCs were aligned to the HIV-1 subtype C consensus sequence, retrieved from the Los Alamos sequence database (Los Alamos National Laboratory, 2018) and translated into Nef amino acid sequences using the Bio Edit translation tool. The HIV-1 Nef amino acid sequences for each individual were organised in increasing order of the associated CIMT and divided into two groups, lower (CIMT<0.50) and higher (CIMT>0.58) CIMT group. The correlation between Nef variants and CIMT was assessed by comparing the HIV-1 Nef multiple sequence alignments of the HIV-infected higher CIMT group against that of the lower CIMT group using the VESPA software (http://www.hiv.lanl.gov/content/sequence/VESPA/vespa.html). The MEME suite of VESPA calculates the frequency of each amino acid at each position in an alignment for the query and reference set, and selects the positions for which the most common character in the query set differs from that in the background set. The frequencies of characters at the distinguishing sites are also calculated (Korber and Myers, 1992).

4.2.5 Quantification of ICAM-1 by enzyme-linked immunosorbent assay (ELISA)

Plasma levels of ICAM-1 were measured by ELISA using the Human ICAM-1/CD54 allele-specific Quantikine ELISA kit (R&D Systems, Minneapolis, MS, USA) as described by the manufacturer. The assay is based on the quantitative sandwich enzyme immunoassay technique. Given the specificity of this technique, it can be used to accurately quantify ICAM-1 in clinical samples. Briefly, plasma samples were diluted 20-fold with the provided diluent, and 100 µL of each sample, standard and blank added in duplicates to the wells of a 96 well plate pre-coated with a monoclonal human ICAM-1 antibody. After

incubating and washing to remove unbound substances, a horseradish peroxidase-conjugated polyclonal human ICAM-1 antibody was added to the wells and incubated at room temperature. Thereafter, the plate was washed to remove any unbound antibody-enzyme reagent and the horseradish peroxidase substrate solution added, followed by another round of incubation, yielding a blue coloured complex. The colour development was directly proportional to the amount of ICAM-1 present in the test sample. The reaction was stopped using an acidic solution supplied with the kit, changing the colour of the reaction mixture from blue to yellow. Thereafter, samples were quantified by spectrophotometry using an ELISA plate reader as detailed in Chapter 2, section 2.2.5.2.

4.2.6 Statistical analysis

Statistical analysis was performed using the Statistica software v13.3 (Statsoft Inc., Tulsa, OK, U.S.A.) and GraphPad Prism 7 (GraphPad Software, Inc.). Continuous data that was normally distributed was compared across the two groups (HIV-infected lower CIMT and HIV-infected higher CIMT) using a Student's unpaired t test. For data that was not normally distributed, a non-parametric Mann-Whitney U test was used to compare the data between the two groups.

The frequency of the different Nef variants in subjects with higher compared to those with lower CIMT levels were calculated using the VESPA software, as described above and were tested for significant differences by using a contingency table and two-tailed Fisher's exact test. Each of these identified variants was then included in individual multivariable logistic regression models in which the dependent (output) variable was higher/lower CIMT to which possible confounding variables were then added. We limited the number of confounding variables due to the small sample size (N=60), which may lead to overfitting. The variables chosen were age, gender, BMI (as a lone indicator of anthropometry), LDL (as

a lone indicator of dyslipidaemia) and hypertension. Viral load and ICAM-1 were not included due to the high use of ART in the study population. Collinearity was assessed using the variance inflation factor (VIF) and any variables with VIF>5.00 were excluded from the regression model; none were found.

4.3 Results

4.3.1 Comparison of cardiometabolic variables between study groups

Out of the 140 selected HIV-infected participants, sufficient remnant plasma sample for use in this study was available for 123 subjects. Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) obtained from these samples and the amplicons obtained by nested PCR assessed by agarose gel electrophoresis (appendix 6). However, HIV-1 *nef* sequences were successfully amplified from genomic DNA extracted from the PBMCs of only 60 of these subjects. The mean age of this sub-group of 60 subjects was 39 years, of which 75% were on ART and had a mean CD4 T cell count of 526 cells/mm^3. Table 4.1 below shows the clinical and virologic characteristics of the sub-group compared to the group from which *nef* sequences were not obtained. The sub-group from which *nef* sequences were obtained was compared against the unsequenced group using either a Mann-Whitney U test with the non-transformed variables, or a Student's unpaired t test following normalisation of the variables. No significant differences were observed between the two groups with regards to any of the study variables.

Table 4.1: Characteristics and comparison of study participants from whom *nef* sequence was or was not successfully obtained

Variables	HIV-infected Unsequenced (n = 80)	HIV-infected Successfully sequenced (n = 60)
Demographic variables		
Age (years)	40.11 ± 5.35	39.26 ± 4.60
Gender, male n (%)	28 (35.00)	14 (23.33)
Anthropometric variables		
BMI (kg/m^2)	27.31 ± 6.65	27.43 ± 6.25
Waist circumference (cm)	92.53 ± 13.88	94.12 ± 12.63
Hip (cm)	106.60 ± 14.20	107.88 ± 12.44
Cardiovascular variables		
Diastolic BP (mmHg)	78.73 ± 12.03	79.99 ± 11.51
Systolic BP (mmHg)	123.33 ± 21.02	124.41 ± 18.70
CIMT (mm)	0.55 ± 0.08	0.57 ± 0.09
Endothelial marker		
ICAM-1 (ng/mL)	169.24 [123.82, 241.79]	220.20 [133.89, 276.18]
Biochemical variables		
Glucose (mmol/L)	4.66 ± 0.63	4.71 ± 0.89
Triglyceride (mmol/L)	1.09 [0.86, 1.71]	1.07 [0.78, 1.33]
Total cholesterol (mmol/L)	4.41 ± 0.98	4.60 ± 0.98
LDL-C (mmol/L)	2.66 ± 0.88	2.90 ± 0.88
HDL-C (mmol/L)	1.22 [1.00, 1.51]	1.36 [1.10, 1.54]
Immunological variables		
CD4+ cell count (cells/mm^3)	525.51 ± 257.49	525.90 ± 276.28
Viral load (copies/mL)	39.00 [1.00, 39.00]	35.00 [1.00, 1895]
ART (%)	60 (75.00)	44 (73.33)

Data given either as mean ± standard deviation or median [lower quartile, upper quartile]

The characteristics of the HIV-infected lower and higher CIMT groups from which HIV-1 *nef* sequences were obtained are shown in Table 4.2. On comparing across the two groups, no statistically significant differences were observed with regards to age, gender, anthropometry, blood pressure, plasma ICAM-1 levels, glucose, triglyceride, total cholesterol, LDL-C, HDL-C, CD4+ cell count and viral load. However, it was of note that the majority of cardiometabolic variables were higher in the higher compared to the lower CIMT group, with the diastolic blood pressure reaching statistical significance. Study participants of the HIV-infected higher CIMT group had significantly higher CIMT measurements compared to the HIV-infected lower CIMT group, as would be expected.

Table 4.2: Characteristics and comparison of HIV-infected participants across study groups

Variables	HIV-infected CIMT < 0.50 (n = 26)	HIV-infected CIMT > 0.58 (n = 34)
Demographic variables		
Age (years)	37.65 ± 4.44	39.32 ± 4.94
Gender, male n (%)	6 (23.00)	8 (23.53)
Anthropometric variables		
BMI (kg/m^2)	26.36 ± 6.67	30.27 ± 13.81
Waist circumference (cm)	90.84 ± 12.05	94.61 ± 15.51
Hip (cm)	103.39 ± 12.01	110.89 ± 13.76
Cardiovascular variables		
Diastolic BP (mmHg)	76.76 ± 8.18	82.36 ± 13.06[a]
Systolic BP (mmHg)	118.83 ± 13.68	128.52 ± 20.92
CIMT (mm)	0.47 ± 0.01	0.63 ± 0.06[aaa]
Endothelial marker		
ICAM-1 (ng/mL)	136.25 [41.14, 262.56]	89.96 [40.54, 238.45]
Biochemical variables		
Glucose (mmol/L)	4.69 ± 0.51	4.72 ± 1.10
Triglyceride (mmol/L)	0.98 [0.77, 1.24]	1.16 [0.80, 1.41]
Total cholesterol (mmol/L)	4.59 ± 0.95	4.61 ± 1.02
LDL-C (mmol/L)	2.94 ± 0.90	2.87 ± 0.88
HDL-C (mmol/L)	1.36 [1.24, 1.45]	1.32 [0.96, 1.67]
Immunological variables		
CD4+ cell count (cells/mm^3)	513.52 ± 256.41	536.44 ± 296.59
Viral load (copies/mL)	20.00 [1.00, 7177]	35.00 [1.00, 1895]
ART (%)	21 (80.78)	24 (70.59)

Data given either as mean ± standard deviation or median [lower quartile, upper quartile]; [a]p<0.0005, [aaa]p<0.0005 vs CIMT<0.50.

4.3.2 Sequence analysis of HIV-1 Nef in relation to CIMT and ICAM-1

Sequence analysis of the amino acid residues of HIV-1 Nef revealed associations of statistical significance between the frequency of sequence variants and CIMT (Table 4.3). Thus, results from the contingency table showed that for the H40Y, D177E and V180T variants, the consensus amino acids H40, D177 and V180 were found at a higher frequency in subjects with higher CIMT measurements. In addition, mutations at codons 39 and 143, from lysine to arginine (K39R) and phenylalanine to tyrosine (F143Y) respectively, were associated with higher CIMT. It should be noted that at each of these 5 sites multiple different amino acid residues were identified, as shown in Table 4.3, and in some cases no residue was present i.e. K39R, D177E and V180T. When naming these 5 polymorphic sites we chose to include the consensus amino acid and the most common of the variant amino acids in the name. When performing the statistical analyses we used a contingency table for each site that included all the amino acid residues and also a contingency table from which the rare residues were excluded. Exclusion of the rare residues led to stronger statistical associations, except for H40Y (see Table 4.3).

In addition, the combined effect of the 5 polymorphisms on CIMT was analysed by counting the number of Nef risk residues present in each individual producing a count that varied from 0 to 5 for each subject. These counts were split into 3 groups to improve the statistical power of the analyses. The 3 subject groups were those with 0 or 1 risk residue (n=12); those with 2 risk residues (n=20); those with 3-5 risk residues (n=28). The results are shown in Figure 4.2 and demonstrate that as the number of residues increase the prevalence of subjects with higher CIMT also rises in a significant stepwise manner.

Table 4.3: Association of HIV-1 Nef protein sequence variants with CIMT

Variant	Amino acid	Low CIMT (N)	High CIMT (N)	Lower CIMT Frequency (%)	Higher CIMT Frequency (%)	p value (p value if combine rare amino acids)*
K39R	K	22	20	84.62	58.82	0.083
	R	1	9	3.85	26.47	(0.049)
	E	0	1	0.00	2.94	
	F	1	0	3.85	0.00	
	None	2	4	7.69	11.76	
H40Y	H	13	27	50.00	79.41	0.007 (0.011)
	Y	12	4	46.15	11.77	
	R	0	3	0.00	8.82	
	N	1	0	3.85	0.00	
F143Y	F	23	21	88.46	61.76	0.063
	Y	3	12	11.54	35.29	(0.03)
	W	0	1	0.00	2.94	
D177E	D	5	16	19.23	47.06	0.071
	E	9	10	34.62	29.41	(0.036)
	H	6	7	23.08	20.59	
	A	1	0	3.85	0.00	
	None	5	1	19.23	2.94	
V180T	V	20	33	76.92	97.06	0.063
	T	2	0	7.69	0.00	(0.008)
	I	1	0	3.85	0.00	
	None	4	1	11.54	2.94	

*p-values from contingency tables with those in brackets produced by combining rare variants e.g. for K39R a 5x2 contingency table was used to take into account the 5 different residues (p-value without brackets) and a 3x2 table was used with residues E, F and none combined (p-value in brackets). For F143Y the single W residue was removed from the analysis. A rare residue was defined as one which occurred 5 times or less in both CIMT groups

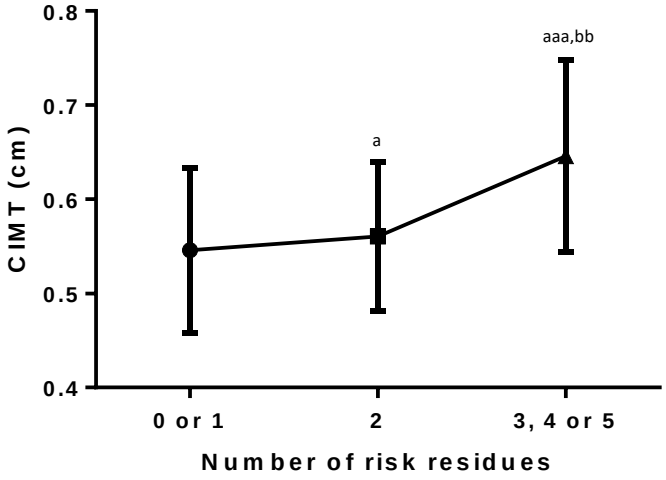


Figure 4.2: Prevalence of high CIMT across subject groups harbouring genome-integrated HIV DNA coding for varying numbers of Nef risk residues.

[a]p<0.05, [aaa]p<0.0005 vs 0 or 1 residues; [bb]p<0.005 vs 2 residues; for 0 or 1 residue n=12, for 2 residues n=20 and for 3, 4 or 5 residues n=28

The relationship of the 5 polymorphic sites with CIMT was analysed further using multivariable logistic regression models. This allows us to determine whether the significant associations observed in Table 4.3 are maintained after adjustment for possible confounding variables. The multivariable logistic regression models are shown in Table 4.4. Due to the low N of 60, adjustment was made for as small a number of confounding variables as possible. We therefore included age, gender, BMI, LDL-C and hypertension (see Statistics section of this chapter for a more detailed explanation of variable choices). The resulting models show that without adjustment, each of the 5 risk residues were associated with significantly greater odds for higher CIMT when compared to all the non-risk residues at the respective site. Furthermore, if we combined all the risk residues, as done for Figure 4.2, and compare subjects with >2 risk residues to those with <3 risk residues, a highly significant effect was observed. After adjustment for the possible

confounding variables, the odds for H40, Y143 and D177 were slightly attenuated with p-values decreasing to just below significance (see Table 4.4), while the odds for the combined residue score remained highly significant, and that for V180 strengthened slightly. The odds ratios for R39 increased dramatically, which may be a reflection of the small N and the small number of subjects (n=1) harbouring the R residue in the lower CIMT group.

Table 4.4: Logistic regression models analysing relationship of R39, H40Y, Y143, D177 and V180 residues with higher CIMT with and without adjustment for possible confounding variables

Dependent variable	Model number	Independent variable with OR (95% CIs); p-value (unadjusted)	Independent variable with OR (95% CIs); p-value (adjusted)[a]
Higher CIMT	**1**	R39 9.00 (1.01, 80.0); 0.044	R39 25.9 (2.05, 327.5); 0.009
	2	H40 3.86 (1.21, 12.3); 0.019	H40 3.26 (0.92, 11.5); 0.061
	3	Y143 4.18 (1.01, 17.4); 0.044	Y143 3.94 (0.89, 17.4); 0.064
	4	D177 3.73 (1.11, 12.5); 0.029	D177 3.26 (0.89, 11.9); 0.068
	5	V180 9.90 (1.06, 92.6); 0.040	V180 13.7 (1.13, 168.4); 0.035
	6	>2 residues 13.2 (3.51, 49.6); <0.0005	>2 residues 12.3 (2.80, 54.1); 0.0007

[a]Adjusted for age, gender, BMI, hypertension, LDL-C

Further analysis of the HIV-1 Nef sequences revealed the consensus amino acid residue, F143, to be significantly associated with higher levels of ICAM-1, while no significant association was observed between ICAM-1 levels and the Nef variants K39R, H40Y, D177E or V180T (Table 4.5).

Table 4.5: Association of Nef protein sequence variants with serum ICAM-1 levels

Variant		ICAM-1 levels (ng/mL)	P-value
K39R	**K**	114.3 [40.7, 241.2]	0.272
	R	232.5 [34.4, 309.1]	
	E, F & none	51.1 [18.8, 124.2]	
H40Y	**H**	127.7 [40.7, 252.7]	0.870
	Y	105.4 [39.0, 231.0]	
	R & N	93.9 [38.1, 218.7]	
F143Y	**F**	149.5 [42.0, 269.1]	0.042
	Y	44.1 [36.1, 138.0]	
D177E	**D**	73.8 [40.1, 205,6]	0.227
	E	83.6 [37.3, 220.8]	
	H	145.8 [40.7, 241.2]	
	A & none	244.8 [91.8, 291.5]	
V180T	**V**	122.8 [38.3, 256.6]	0.734
	T, I & none	165.3 [96.5, 226.6]	

Data given as median [lower quartile, upper quartile]

4.4 Discussion

The current study confirms the highly polymorphic nature of the *nef* gene and the resulting Nef peptide. Five polymorphic sites in Nef were found to be associated with CIMT and a score that combined these 5 variants demonstrated that the odds of higher CIMT increased in a step-like manner with an increasing

number of CIMT-associated (risk) amino acid residues. One of these polymorphic sites also associated with higher ICAM-1 levels.

No significant differences were observed for any of the study variables between participants from which HIV-1 *nef* sequences were obtained compared to those that could not be sequenced. This therefore implies the samples used for this sub-group were not substantially different from those of the other study participants and the results obtained from these samples may be applicable to the total cohort.

The study showed no statistically significant differences between participants with lower CIMT when compared to those with higher CIMT with regards to any of the study variables except diastolic blood pressure. Thus, individuals in the higher CIMT group did not exhibit higher cardiometabolic disease (CMD) risk compared to the lower CIMT group. The lack of association between CIMT and CMD risk factors in the sub-group could probably be due to the small n number used for comparative analysis, and it is noteworthy that the majority of CMD risk factors were higher in the higher CIMT group, but none of these differences reached statistical significance. Additionally, the higher CIMT group had a mean value of only 0.63 ± 0.06, of which measurements between 0.6 - 0.7 mm are considered normal (Jacoby et al., 2004). CIMT is known to be predictive of carotid artery disease at values > 1.0 mm (Madhuri et al., 2010).

Sequence analysis of HIV-1 Nef revealed five polymorphisms to be significantly associated with risk for higher CIMT; K39R (p=0.037), H40Y (p=0.004), D177E (p=0.031), F143Y (p=0.041) and V180T (p=0.036), and one polymorphism with ICAM-1; F143Y (p=0.042). The H40Y polymorphism associated with ICAM-1 levels in our previous study, reported in Chapter 3, was also associated with CIMT in the current study. These findings suggest that this *nef* gene variant may play an important role in the

development of atherosclerotic plaques in subjects with an HIV infection. However, in the present study, H40Y did not associate with ICAM-1 serum levels. This data therefore shows that the association of H40Y with CIMT may not be mediated by ICAM-1. However, the presence of a significant association of H40Y with ICAM-1 levels in the previous but not the current study may be due to the use of ART by the majority of subjects in the latter study, as ART is known to reduce ICAM-1 levels (Arildsen et al., 2013, van Vonderen et al., 2009), as also shown in Chapter 2. The association of F143Y with ICAM-1 levels must be interpreted cautiously given the high use of ART in this patient cohort and the fact that in the previous study F143Y was not associated with ICAM-1 levels.

The combined effect of the 5 polymorphic Nef sites on CIMT were analysed by counting the number of risk amino acid residues in each individual and then comparing the prevalence of higher CIMT across 3 groups of subjects: those with <2 risk residues, those with 2 risk residues and those with > 2 risk residues. This showed that the prevalence of higher CIMT increased across the groups. This finding was confirmed in a multiple logistic regression model adjusted for possible confounding variables. This finding suggests an independent, additive effect of these risk residues on CIMT. When the 5 individual polymorphic sites were analysed for their effects on CIMT in a multiple logistic regression model, adjustment for confounding variables did modify the odds ratios for higher CIMT. However, attenuation occurred for only 3 of the residues and this effect was small suggesting the association of these residues with CIMT is not substantially due to confounding from other variables.

A limitation of this study is that HIV-1 *nef* was sequenced from the host's genomic DNA and not circulating viral RNA. Administration of ART suppresses viral replication which leads to a concomitant decrease in circulating HIV, as observed in our study population in which 82 % of the HIV-infected

individuals had viral load levels of < 1000 cells/mm^3. Therefore, given the low number of virions in the circulation viral RNA could not be obtained and therefore HIV-1 *nef* was sequenced from PBMCs. During HIV replication, the viral DNA integrates within the host's genome (Diallo et al., 2012, Peterlin and Trono, 2003, Kirchhoff et al., 1999). The HIV sequence information must therefore be obtained by sequencing the host's genomic DNA. However, the sites of HIV integration are not known and the HIV DNA may not be representative of an intact provirus which can lead to problems with obtaining viral PCR products, mainly as a result of mismatches between the primers and target viral sequence (Diallo et al., 2012, Kirchhoff et al., 1999). Thus, we were only able to obtain 60 viral sequences from 123 samples. A further limitation of this study was the small sample size, which was compounded by the problem of obtaining viral sequences. Despite this we were able to identify HIV *nef* variants associated with CIMT, but it is possible that due to lack of power we may have missed other CIMT-associated viral polymorphisms. Further studies with a larger sample size must therefore be conducted to confirm the current results and to screen for other possible *nef* variants that associate with CIMT. An additional limitation of this study is that it is cross sectional and therefore we can only identify associations between variables and not ascribe causation. The use of samples obtained from subjects taking ART may also be another limitation of the study. Moreover, subjects used different ART regimens (both first- and second-line therapy). However, data from a number of investigations has clearly shown that the risk of CVD is heightened in subjects taking anti-HIV medication (Durand et al., 2011, Lang et al., 2010, Obel et al., 2007) and therefore it is important to perform studies specifically in such subjects. Furthermore, the molecular aetiology of endothelial dysfunction in indigenous African populations receiving ART is currently under-researched.

In conclusion, these results demonstrate that variations in the Nef amino acid sequence are associated with CIMT. The mechanisms involved in these associations are not known and will require further clinical and *in vitro* functional studies.

This study further suggests that Nef may be associated with endothelial dysfunction and downstream events such as plaque formation. However, to further characterise the possible role of Nef in HIV-associated cardiovascular pathology it would be necessary to quantify serum levels of the viral protein. The next chapter will discuss attempts to develop a sensitive and specific assay for the Nef peptide in human serum samples.

4.5 References

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Chapter 5: Development of a Targeted Multiple Reaction Monitoring (MRM) Method for the Quantification of HIV-1 Nef

5.1 Introduction

The prevalence of cardiovascular diseases (CVD) is reported to be higher in the HIV-infected population than the uninfected population (Durand et al., 2011, Chow et al., 2012, Freiberg et al., 2013). This may be due to the negative effect of viral proteins, such as Nef, on endothelial function (Wang et al., 2015). I*n vitro* studies showed that HIV viral proteins can affect endothelial function (Wang et al., 2015, Jiang et al., 2010, Dhawan et al., 1997), while *in vivo* studies have shown that HIV-infected subjects display some level of endothelial dysfunction (Matzen et al., 2004). However, no clinical studies have been conducted thus far to correlate endothelial function in HIV-infected subjects with serum levels of viral proteins. The measurement of Nef in biological fluids is hampered by the lack of availability of validated HIV-1 Nef antigen assays.

Quantification of proteins by ELISA is a traditional method routinely used in laboratories for clinical diagnostics. Enzyme-linked immunosorbent assays have been in existence for decades, and are based on antibody-antigen binding (Weiland, 1978). In-house HIV-1 Nef (Fujii et al., 1996) and Tat (Poggi et al., 2004) antigen assays have been developed by research groups for the quantification of HIV-1 viral proteins from human sera. Commercial HIV-1 Nef and Tat ELISA kits are readily available (Raymond et al., 2011, Santosuosso et al., 2009), however, these kits have not been validated for the measurement of HIV-1 Nef and Tat in human serum

as the manufacturers' indicate the kits are not for use for human samples (Ferdin et al., 2018). Moreover, sequence diversity within the *nef* (Bredell et al., 2007) and *tat* (Li et al., 2012) genes, among HIV-infected individuals, could lead to inaccurate quantification of the viral proteins by ELISA, given that this technique is based on specific antigen-antibody binding.

Mass spectrometric techniques, such as liquid chromatography mass spectrometry (LCMS), provide a possible alternative to immunoassays for measuring serum levels of HIV proteins. It is considered to be the gold standard method for validating ELISA assays (Harun et al., 2009, Gandhi et al., 2018). A LCMS instrument is a mass spectrometer (MS) coupled to an instrument that enables the separation of analytes by liquid chromatography (LC). On injecting a sample into the LCMS instrument, molecules first become separated based on their ability to be retained by the LC column, following which the molecules go into the mass spectrometer where they get bombarded with electrons in an ionisation chamber, resulting in the fragmentation of some of the molecules, as well as acquisition of a charge. Each resultant molecule, referred to as the parent or precursor ions, will therefore have a specific mass-to-charge ratio (m/z). The parent ions are separated in a second chamber, in which an electric or magnetic field is applied, to enable deflection of ions of the same m/z. At this stage, the parent ions are picked up by a detector and results displayed as a spectrum, indicative of the relative abundance of the charged molecules as a function of their m/z. In tandem mass spectrometry, the parent ions can be further fragmented into daughter or product ions. The target analyte can thus be identified based on the mass-to-charge ratio of either the parent ion (MS1), or both the parent and daughter ions (MS2). Several transitions, defined by the MS1 and resulting MS2 scans, can thus be monitored in parallel, referred to as multiple reaction monitoring (MRM). Measurement of analytes by LCMS is therefore based on both the physical and ionization property of the target analyte in the sample.

Sample preparation for the LCMS depends on the type of analyte being analysed and the matrix in which it resides. In the case of proteins in human biological fluids such as blood, they are generally precipitated out using organic solvents such as chloroform/methanol or cold acetone with or without trichloroacetic acid (Rogers and Bomgarden, 2016). Incubating the blood sample with an organic solvent compartmentalises the sample yielding a white pellet of endogenous proteins which can easily be recovered by centrifugation. This process aids to eliminate other interfering analytes such as lipids, and can also help eliminate high abundant proteins for in-depth analysis of low-abundant proteins (Wang et al., 2013). Thereafter, they are enzymatically digested into shorter fragments (peptides) to facilitate the ionisation process (Rogers and Bomgarden, 2016). Prior to digestion, the protein sample is denatured using chaotropic reagents, such as urea and thiourea, to solubilise the protein (Wong et al., 2016), reduced with dithiothreitol to break the disulphide bonds (Alliegro, 2000), and then alkylated with iodoacetamide to prevent random formation of disulphide bridges (Boja and Fales, 2001). The digestion step often entails introducing salts into the sample, such as ammonium bicarbonate, to increase enzyme efficiency and improve peptide detection by the LCMS (Honarvar and Venter, 2017). Salts are large particles that can crystalize and potentially damage the LCMS in the long run, thus, samples are often desalted using a reverse phase column, such as a C18 stationary phase, prior to instrument loading (Naldrett et al., 2005).

Quantification of proteins in biological fluids (such as serum, plasma, cerebrospinal fluid, saliva and urine) by mass spectrometry also has its limitations. These include the amount of time required for sample processing (Wiśniewski et al., 2009), and the large dynamic range of proteins (Anderson, 2005). Often, target proteins of clinical significance are in low-abundance, thereby rendering their detection by MS instruments more complex (Hanash et al., 2008).

Figure 5.1 illustrates the different steps involved in a typical proteomics workflow, however, there are variations to this workflow in clinical proteomics that involves the inclusion of enrichment steps. Several strategies have been developed to facilitate the detection of low abundance proteins, such as depletion of high abundant proteins by immunoaffinity chromatography (Bellei et al., 2011), fractionation of proteins or peptides by ion exchange chromatography (Kulak et al., 2014), and concentrating target peptides by antibody-based binding (Anderson et al., 2004). Although ELISAs require minimal sample processing compared to proteomics, thereby rendering it a more attractive method of choice, a lot of progress has been made to simplify serum proteomics. More recently, a simple and integrated spintip-based technology (SISPROT) has been developed that makes use of both strong cation exchange (SCX) and strong anion exchange (SAX) beads, and C18 plugs inserted into 200 ul pipette tips, which can be used for the rapid and cost effective processing of biological fluids (Lin et al., 2017, Chen et al., 2016). In this technology, the steps involving protein pre-concentration, reduction, alkylation, digestion, desalting, and fractionation have been amalgamated into a single unit. This could potentially be applicable in the clinical setting for processing several biological samples with minimal sample loss within a shorter time frame with more reproducibility and higher throughput (Lin et al., 2017).

An exploratory study was carried out by Nemeth *et al*. in which Nef peptides were identified and quantified by MS from *in vitro* and *in vivo* experiments (Nemeth et al., 2017). However, to date, there is no published MS method for the quantification of HIV viral proteins from clinical samples. In this study, we therefore sought to set up a LCMS method by which HIV-1 Nef could be measured from human serum samples.

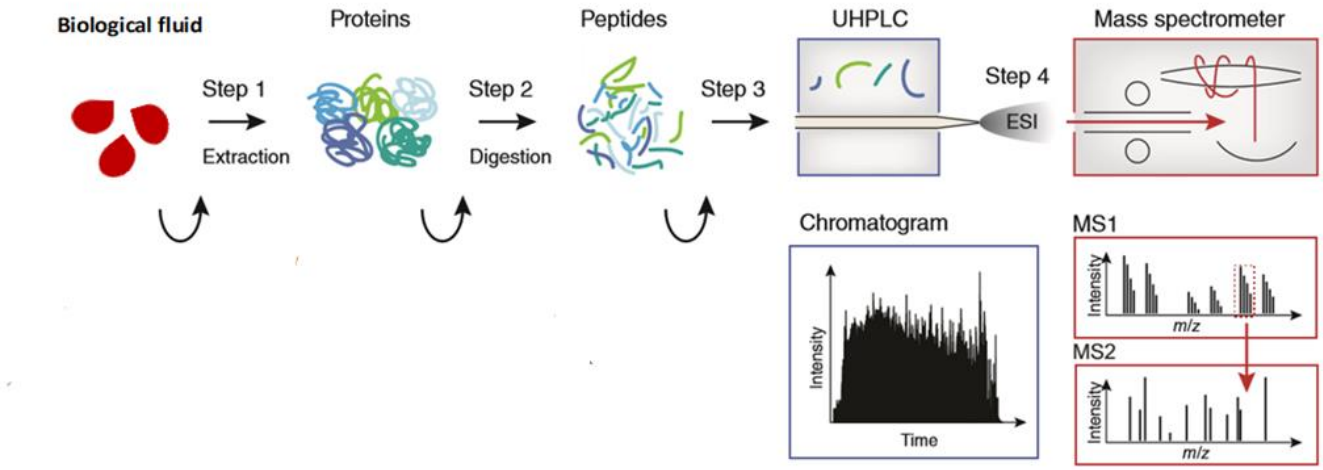


Figure 5.1: General workflow for proteomics by mass spectrometry. A typical serum proteomics workflow consists of four main steps. In step 1, proteins are extracted from the biological fluid. In step 2, the extracted proteins are enzymatically digested into peptides. In step 3, the peptides are separated by ultra-high-performance liquid chromatography (UHPLC) following which they are ionized by electrospray ionization (ESI) and picked up by a detector in the mass spectrometer according to their resulting mass to charge ratios (*m/z*). The operator can monitor reactions from both the UHPLC and mass spectrometer by viewing the chromatograms generated. Results are given as spectra of which the intensity of the ion correlates to the relative abundance of the charged ion as a function of its m/z. The precursor ions are identified in the first scan (MS1), while the product ions are identified in the second scan (MS2). Adapted with permission from Springer Nature: Nature America Inc., Nature Immunology (Meissner and Mann, 2014), Copyright 2014.

5.2 Materials and Method

5.2.1 Ethics

Ethical clearance for the study was obtained as detailed in Chapter 2, section 2.2.1.

5.2.2 Study population

The study population consisted of the subgroup of 30 HIV-infected ART-naïve subjects from which HIV-1 *nef* sequences were successfully obtained, as detailed in Chapter 3, section 3.3.1. However, from this study group, serum samples were available for only 26 of these subjects.

5.2.3 Quantification of HIV-1 Nef by enzyme-linked immunosorbent assay

The concentration of HIV-1 Nef in serum samples was measured using a commercially available HIV-1 Nef ELISA kit from ImmunoDX (Woburn, Massachusetts, U.S), according to the manufacturer's instruction, with slight modifications as described by Raymond et al. (2011). Briefly, immune complexes were dissociated by incubating plasma samples with HCl and neutralized by adding NaOH. Triton was subsequently added to the acid-treated serum samples to lyse the microvesicles and virions, prior to performing the ELISA. Samples were diluted 1,000-fold with the provided diluent, and 100 uL of each sample, standard and blank added in duplicates to a 96 well plate pre-coated with the HIV-1 Nef capture antibody. After incubating and washing to remove unbound substances, the anti-Nef-HRP-labelled antibody solution was added to bind the captured viral HIV Nef, followed by another round of incubation and washing procedures to remove unbound antibody-HRP. Incubation with the HRP substrate yielded a blue coloured complex. The reaction was stopped using an acidic solution supplied with the kit, changing the colour of the reaction mixture from blue to yellow, and absorbance read at 450nm and 540nm, and wavelength reading corrected by subtracting readings at 540 nm from the readings at 450 nm.

5.2.4 Selection of target Nef peptides

The HIV-1 *nef* gene was sequenced from the HIV-infected cohort as described in Chapter 3 section 3.3.2. An *in silico* tryptic digest of the consensus C Nef protein was conducted using the Mass-Digest tool of the ProteinProspector software v 5.24.0 (University of California, San Francisco, U.S.A) to generate potential peptides with their m/z that would be obtained following digestion with trypsin and MS analysis. To identify the most suitable target peptide for MS quantification, the peptides generated by the software were compared against the HIV-1 Nef amino acid sequences obtained from the clinical isolates. When the peptides generated by *in silico* trypsin digestion were aligned with the Nef sequences from clinical isolates, a target peptide that was 100% conserved across all the clinical isolates could not be identified. The most conserved peptide sequence identified was at position 134 – 144, which harboured three polymorphic sites, 10 amino acids in length. Analysis with a basic local alignment search tool (BLAST) revealed the peptide to be unique to HIV. Looking at this target tryptic Nef peptide, sequence analysis of the HIV-1 Nef sequences in our study population showed seven different peptide sequences to be present at position 134 – 144, and these are shown in Table 5.1.

Table 5.1: Endogenous Nef target peptides identified for the quantification and validation of HIV-1 Nef from human serum

Peptide	Sequence	Native Molecular weight (m)	Parent ion (m/z)
NEF 1	YPLTFGWCFK	1319 (+1)	659.82 (+2)
NEF 2	YPLTFGWCYK	1335 (+1)	667.82 (+2)
NEF 3	LPLTFGWCYK	1285 (+1)	642.83 (+2)
NEF 4	YPLTFGWLFK	1272 (+1)	636.35 (+2)
NEF 5	YPLTFGWPFK	1256 (+1)	628.33 (+2)
NEF 6	FPLTFGWCYK	1319 (+1)	659.82 (+2)
NEF 7	WPLTFGWCYK	1358 (+1)	679.32 (+2)

5.2.5 Sample preparation for liquid chromatography tandem mass spectrometry (LC-MS/MS)

Two methods were used to prepare the serum samples for MS proteomic analysis, a traditional method and a newly developed immuno-MRM method. HIV-1 Nef is present in serum samples in low abundance, thus lowering its degree of detectability by MS given the large dynamic range of proteins. Therefore, target capturing of HIV-1 Nef from serum samples using a HIV-1 Nef antibody prior to sample processing could address this problem.

5.2.5.1 Traditional method

Proteins (100 ug) were precipitated from serum samples using a standard acetone protein precipitation method, and re-solubilised in a denaturation buffer containing urea / thiourea. Thereafter, the proteins were reduced with 1 mM dithiothreitol (DTT) to break disulphide bridges between the cysteine amino acids, and alkylated with 5.5 mM iodoacetamide to block the SH group and prevent reformation of disulphide bridges. The pH of the sample was then brought up to at least pH 8 by diluting the sample 4-fold with 50 mM ammonium bicarbonate, prior to adding sequencing grade modified trypsin (Promega, WI, U.S.A). Samples were incubated with trypsin overnight to cleave the proteins at the C-terminal to arginine (R) and lysine (K). Subsequently, samples were desalted using the C-18 stop-and-go extraction (STAGE) tip protocol as described by Rappsilber et al. (2003), dried down with a speed vacuum and reconstituted in 10 % acetonitrile.

5.2.5.2 Immuno-MRM method

A customised immuno-MRM method was set up as illustrated in Figure 5.2 below. Briefly, activated epoxide beads (ResynBiosciences, R.S.A) were washed with water and incubated overnight with HIV-1 Nef antibody [3D12] (AB 42355, Abcam, England). Beads were then

blocked by incubating with 200mM ethanolamine for 4 hrs and washed with 1M NaCl. A 1M DTT solution made up in 50 mM ammonium bicarbonate was freshly prepared and added to 100 µL of serum sample to a final concentration of 10 mM and incubated for 1 hour at room temperature. Thereafter, 0.55 M iodoacetamide dissolved in 50 mM ammonium bicarbonate was prepared prior to use and added to the sample to a final concentration of 550 mM. Samples were incubated in the dark at room temperature for 1 hour. The Nef antibody-bead complex was washed with water and the reduced and alkylated protein samples (100 µL) added and incubated for 1 hour at room temperature with shaking. Beads were washed with 200 µL of digest buffer (50 mM ammonium bicarbonate). Prior to adding trypsin (Promega, WI, U.S.A) to the beads, pH was adjusted by adding 100 µL of 50 mM ammonium bicarbonate. Samples were incubated overnight at 37°C with shaking. Subsequently, samples were frozen at –80°C, dried down using a speed vacuum and reconstituted in 50 µL of 30 % acetonitrile.

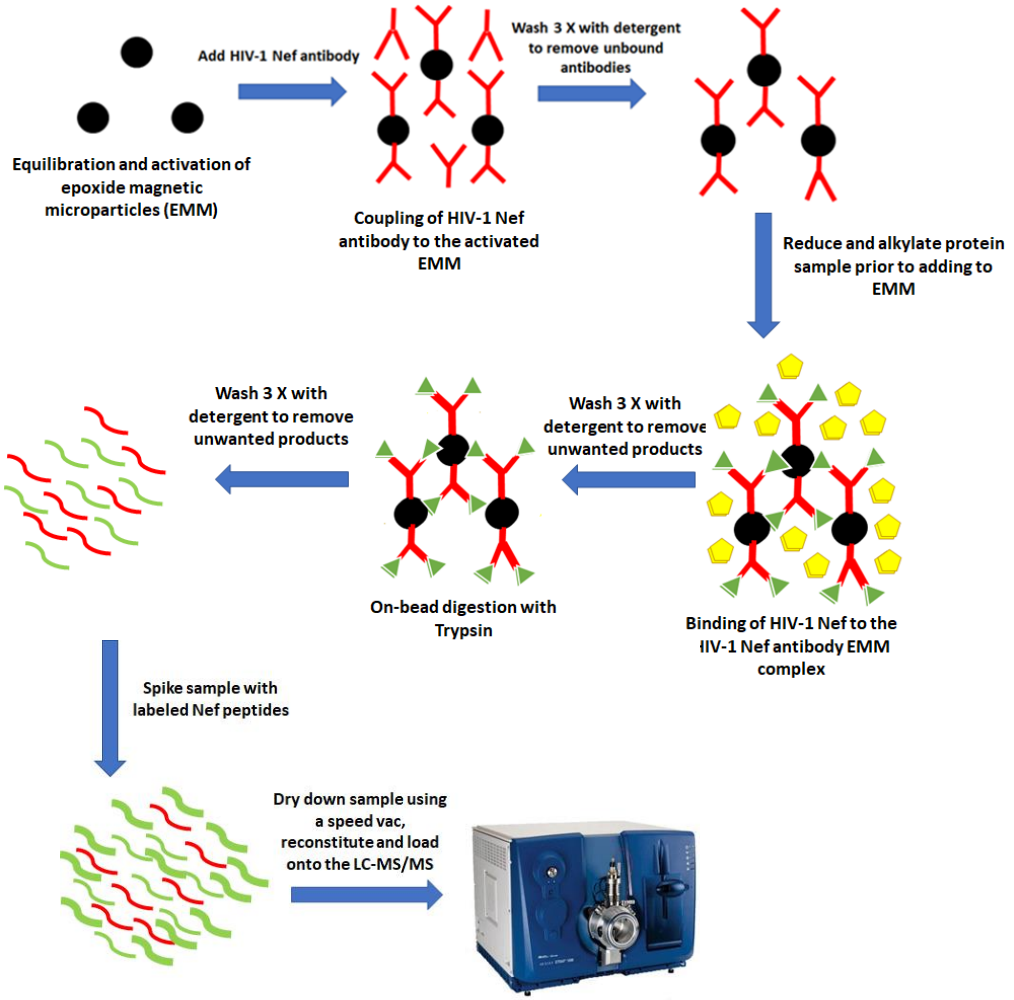


Figure 5.2: Schematic illustration of the immuno-MRM method established for the detection of HIV-1 Nef in serum samples. The HIV-1 Nef antibody is immobilised onto magnetic microparticles and added to a reduced and alkylated protein sample containing the target HIV-1 Nef protein. Beads are then digested with trypsin and the peptides generated loaded onto an LC-MS/MS instrument. Target Nef peptides are detected following an established MRM method developed on the instrument.

5.2.6 LC-MS/MS set up parameters

The QTrap 5500 mass spectrometer with Micro LC (Sciex, Framingham, MA, U.S.A) was used for data acquisition. For absolute quantification, a sample can be spiked with known amounts of a labelled version of the target peptide, and the intensity of the labelled peptide compared to the non-labelled (light) endogenous peptide (Bantscheff et al., 2007). Labelling the synthetic version of a target peptide enables it to be differentiated from the endogenous peptide on the MS. In our study population, out of the seven target endogenous Nef peptides (Nef 1 – 7), four Nef peptides (Nef A - D) were selected for labelling based on the prevalence of each of the polymorphisms in the tryptic fragment within the study population. The labelled Nef peptides were synthesised with the terminal lysine residue labelled with ^{14}C (New England Peptides, MA, U.S.A), resulting in a mass shift of 8 Daltons, thereby distinguishing the endogenous Nef peptide from the labelled Nef peptide through the electromagnetic field.

Instrument conditions were optimised by manually tuning the collision energy, ion source, declustering, entrance and collision cell exit potential with each of the Nef labelled peptides. The collision energy fragments the peptide at the peptide bond between two amino acid residues, while the declustering potential prevents the parent ions from clustering together. The entrance and collision cell exit potentials assists movement of the parent ions from the first chamber into the second chamber where they get further fragmented into daughter ions. Retention of the charge on either the N or C terminus of the fragmented peptide yields b and y ions, respectively. Prospective parent and daughter ions of the endogenous (light) and labelled (heavy) Nef peptides were generated using the Skyline software v 19.1 (MacCoss lab, University of Washington, WA, U.S.A) based on the parent and product y ions, and the information used to build the MRM method on the QTRAP for detection of the endogenous and labelled Nef peptides (Table 5.2 and 5.3). The quantifier ion was thus the target ion

characteristic of the target Nef peptide to be used for quantitation, which is distinguishable from other ions with a similar retention time, while the qualifier ion selected was a secondary ion relative to the target ion that gives the best signal to validate the identity of the target peptide.

Table 5.2: Labelled Nef peptides used for the HIV-1 Nef targeted-MRM assay and selected transitions for quantification and validation

Labelled Peptide	**Sequence**	**Molecular weight (m)**	**Precursor ion (m/z)**	**Quantifier** (parent > daughter)	**Qualifier** (parent > daughter)
Nef A	FPLTFGWCFK	1311 (+1)	655.8 (+2)	655.8 > 582.2	655.8 > 116.3
Nef B	YPLTFGWCFK	1327 (+1)	664.6 (+2)	664.6 > 582.2	664.6 > 183.2
Nef C	YPLTFGWCYK	1343 (+1)	671.7 (+2)	671.7 > 590	672 > 136
Nef D	YPLTFGWPFK	1263 (+1)	632.5 (+2)	632.5 > 550.8	632.5 > 550.8

Table 5.3: MRM assay and selected transitions for the quantification and validation of HIV-1 Nef from human serum

Peptide	Sequence	Native Molecular weight (m)	Parent ion (m/z)	Ions	MRM transitions (parent > daughter)
NEF 1	YPLTFGWCFK	1319 (+1)	659.82 (+2)	-	-
	PLTFGWCFK			y9	659.82 > 578.29
	LTFGWCFK			y8	659.82 > 529.76
	TFGWCFK			y7	659.82 > 473.22
NEF 2	YPLTFGWCYK	1335 (+1)	667.82 (+2)	-	-
	PLTFGWCYK			y9	667.82 > 586.28
	LTFGWCYK			y8	667.82 > 537.76
	TFGWCYK			y7	667.82 > 481.22
NEF 3	LPLTFGWCYK	1285 (+1)	642.83 (+2)	-	-
	PLTFGWCYK			y9	642.83 > 586.28
	LTFGWCYK			y8	642.83 > 537.76
	TFGWCYK			y7	642.83 > 481.22
NEF 4	YPLTFGWLFK	1272 (+1)	636.35 (+2)	-	-
	PLTFGWLFK			y9	636.35 > 554.81
	LTFGWLFK			y8	636.35 > 506.29
	TFGWLFK			y7	636.35 > 449.75
NEF 5	YPLTFGWPFK	1256 (+1)	628.33 (+2)	-	-
	PLTFGWPFK			y9	628.33 > 546.80
	LTFGWPFK			y8	628.33 > 498.27
	TFGWPFK			y7	628.33 > 441.73
NEF 6	FPLTFGWCYK	1319 (+1)	659.82 (+2)	-	-
	PLTFGWCYK			y9	659.82 > 586.28
	LTFGWCYK			y8	659.82 > 537.76
	TFGWCYK			y7	659.82 > 481.22
NEF 7	WPLTFGWCYK	1358 (+1)	679.32 (+2)	-	-
	PLTFGWCYK			y9	679.32 > 586.28
	LTFGWCYK			y8	679.32 > 537.76
	TFGWCYK			y7	679.32 > 481.22

Micro LC conditions were optimised on a 2.7 μm, HALO Fused-Core C18, 90Å, 150 x 0.3 mm column (Separations, R.S.A) given that the flow rate of the LC system used is in the microlitre range and the column permits separation of analytes based on their hydrophobicity. A combination of two mobile phases were optimised for the LC runs; mobile phase A which consisted of 0.01 % formic acid and 0.5 mM ammonium formate in water, and mobile phase B which consisted of 0.05 % formic acid and 0.5mM ammonium formate in acetonitrile. Various percentages of the mobile phases and run conditions were tested and the chromatogram assessed for a good peak shape and intensity of the target Nef peptide.

5.2.7 Data analysis

Statistical analysis was performed using the Statistica software v13.3 (Statsoft Inc., Tulsa, OK, U.S.A.). The MS data was analysed using MS Analyst and the Skyline software (MacCoss Lab, University of Washington, WA, U.S.A). A non-parametric univariate correlation analysis (Spearman) was performed to investigate if serum levels of HIV-1 Nef correlated with serum biomarkers of endothelial dysfunction and inflammation, as well as age, gender, anthropometry, BMI, lipids, glucose, viral load and CD4 counts.

5.3 Results

5.3.1 Subject characteristics and correlations with serum Nef levels

The clinical characteristics of the study participants are shown in Table 5.3. The study group consisted of 26 HIV-infected ART-naïve individuals of which 27 % (7/26) were males, with a mean CD4 count of 223 cells/mm^3 and a mean viral load of 92888 copies/mL. HIV-1 Nef was measured using an ELISA and was detected in 58 % (15/26) of the study population. Nef concentrations of <1.3 ng/mL was regarded as undetectable.

Table 5.3: Clinical characteristics of study participants

Sample ID	Gender	Age (Years)	BMI (kg/m^2)	CD4 ($cells/mm^3$)	Viral Load (copies/mL)	Nef (ng/mL)
EFH0002	M	45	21.7	248	152000	28.8
EFH0006	M	37	20.1	146	299000	<1.3
EFH0010	M	44	21.0	201	3440	24.0
EFH0011	F	50	28.7	86	103000	11.5
EFH0013	F	34	22.4	59	81800	6.8
EFH0014	M	38	28.8	338	301000	2.6
EFH0021	F	37	32.3	203	151000	15.7
EFH0030	F	46	38.9	155	189000	13.1
EFH0032	M	34	22.7	288	89100	<1.3
EFH0037	F	42	23.5	282	1500	<1.3
EFH0038	F	30	18.5	130	292000	<1.3
EFH0040	F	37	27.7	24	102000	14.1
EFH0046	F	41	31.7	219	88100	3.1
EFH0050	M	39	18.4	138	35100	<1.3
EFH0051	F	50	30.2	295	76900	<1.3
EFH0058	F	30	30.8	320	208000	41.3
EFH0060	F	46	34.2	345	4250	<1.3
EFH0067	F	37	23.5	345	0	5.3
EFH0068	F	47	30.1	298	0	15.2
EFH0071	F	31	31.8	116	31400	37.4
EFH0076	F	33	20.7	118	10100	<1.3
EFH0081	F	47	22.2	337	86	<1.3
EFH0123	F	38	27.6	294	6010	26.6
EFH0125	F	37	30.5	205	0	<1.3
EFH0132	F	38	18.2	272	32300	<1.3
EFH0147	M	41	19.7	342	158000	16.2
Mean	-	40	26.0	223	92888	-

Correlation analysis with ICAM-1 and VCAM-1 showed no association between the markers of endothelial function and HIV-1 Nef ($p>0.05$). Moreover, in a correlation analysis conducted with all variables, no associations were seen between plasma Nef with any of the demographic, anthropometric, cardiometabolic or immunologic variables (Table 5.4). BMI correlated positively with plasma Nef, though not strongly ($p=0.046$).

Table 5.4: Association between serum levels of HIV-1 Nef with demographic, anthropometric, cardiometabolic and immunological variables

Variables	Spearman correlation coefficient	p
Demographic variables		
Age (years)	-0.3158	0.163
Gender, male n (%)	-0.2895	0.203
Anthropometric variables		
BMI (kg/m^2)	**0.4391**	**0.046**
Waist circumference (cm)	0.3012	0.185
Hip (cm)	0.2445	0.286
Cardiovascular variables		
Diastolic BP (mmHg)	-0.0844	0.716
Systolic BP (mmHg)	0.0141	0.952
Endothelial and inflammatory markers		
TNF-α (pg/mL)	-0.0214	0.927
IL-6 (pg/mL)	-0.0459	0.843
IL-8 (pg/mL)	0.0210	0.928
ICAM-1 (ng/mL)	-0.1517	0.511
VCAM-1 (ng/mL)	0.1335	0.564
E-selectin (ng/mL)	0.1050	0.651
vWF (µg/mL)	0.0172	0.941
MCP-1 (pg/mL)	-0.1297	0.575
Biochemical variables		
Glucose (mmol/L)	0.2420	0.291
Triglyceride (mmol/L)	0.1338	0.563
Total cholesterol (mmol/L)	0.0680	0.770
LDL-C (mmol/L)	0.0021	0.993
HDL-C (mmol/L)	0.0996	0.667
Immunological variables		
CD4+ cell count (cells/mm^3)	-0.0113	0.961
Viral load (copies/mL)	0.0948	0.683

5.3.2 Identification of a Nef peptide for targeted proteomics

In Figure 5.3, the HIV-1 Nef amino acid sequences successfully obtained from 30 participants are represented in the logogram. Potential Nef peptides obtained from performing an *in silico* tryptic digest were compared against the 30 aligned sequences. A target region, RYPLTFGWCFK, was identified which was relatively conserved within the HIV-infected cohort.

On comparing the HIV-1 Nef sequences of subjects with detectable levels of Nef against those from whom Nef could not be detected, a mutation at codon 35 from Q to R was seen in participants with undetectable levels of Nef. The 35 Q→R mutation was observed in 33 % of the HIV-infected subjects with undetectable plasma Nef levels ($p=0.0005$).

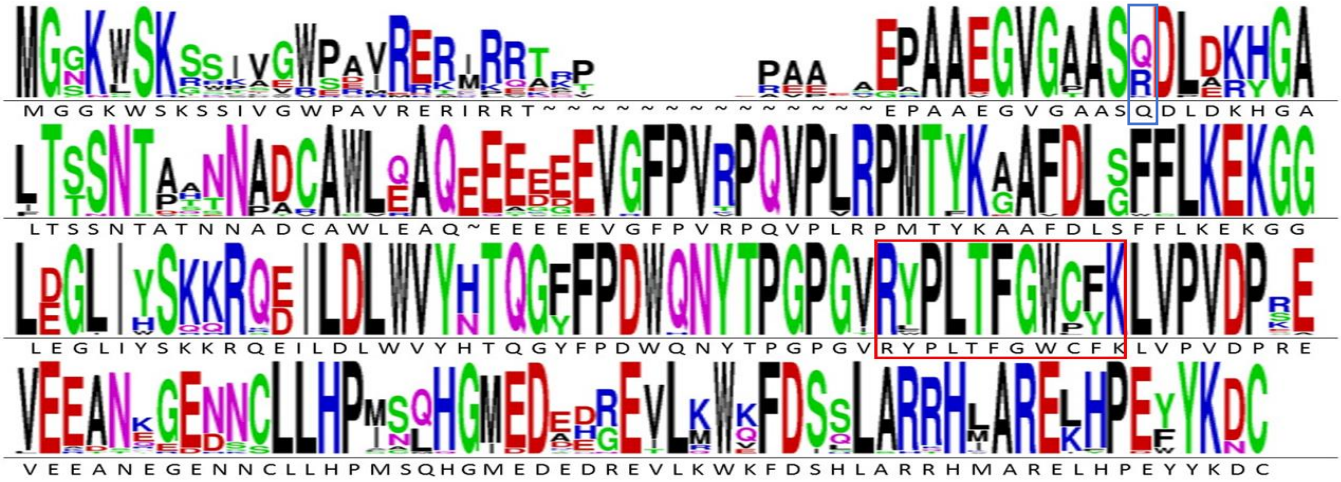


Figure 5.3: Logogram of the multiple sequence alignments of HIV-1 Nef amino acid sequences from clinical isolates. The amino acid residues are colour coded with the acidic residues in red, basic residues in blue, nonpolar and hydrophobic residues in green, polar residues in pink and other nonpolar amino acids in black. The sequence region in the red box represents the relatively conserved target tryptic region for the quantification of HIV-1 Nef by MRM. Digestion with trypsin cleaves exclusively C-terminal to R and K residues resulting in several variable peptides. The amino acid residues in the blue box represent the Q35R mutation that was associated with the non-detection of plasma Nef.

5.3.3 LCMS optimisation for the detection of HIV-1 Nef

The labelled Nef peptides were directly injected into the MS, and detection of each of the labelled Nef peptides on the QTRAP was optimized by manually tuning the ion source, collision energy (CE), entrance potential (EP), declustering potential (DP) and collision cell exit potential (CXP). Optimised parameters for the selected MRM transition of each Nef peptide are shown in Table 5.5.

Table 5.5: LC-MS/MS optimized parameters for the labelled Nef peptides A-D

Labelled Peptide	EP	DP	CE	CXP
Nef A	10	109	31	16
Nef B	10	140	34	12
Nef C	4	102	32	12
Nef D	11	100	27	17

After tuning the MS instrument, the micro LC was optimised for the separation and identification of the labelled Nef peptides (Table 5.6). Optimal conditions identified was a sample injection volume of 2 µL, with an isocratic flow of 10 % B (0.05 % formic acid and 0.5 mM ammonium formate in acentonitrile) and 90% A (0.01 % formic acid and 0.5 mM ammonium formate in water) at a rate of 10 µL/min.

Table 5.6: MicroLC optimized parameters for the labelled Nef peptides A-D

MicroLC optimized conditions	
Flow Rate	10 µL/min
Injection Volume	2 µL
Mobile phase A	0.01% FA + 0.5mM AF in H_20
Mobile phase B	0.05% FA + 0.5mM AF in ACN
LC method	Isocratic 10 % B
Run time	5 mins

5.3.4 Detection of HIV-1 Nef by targeted proteomics

The extracted ion chromatograms of the MRM of a 500 fmol/µL mixture of the four labelled Nef peptides are shown in Figure 5.4. The retention time of the peptides Nef A-D, was 1.06, 1.05, 1.05 and 1.04 min, respectively.

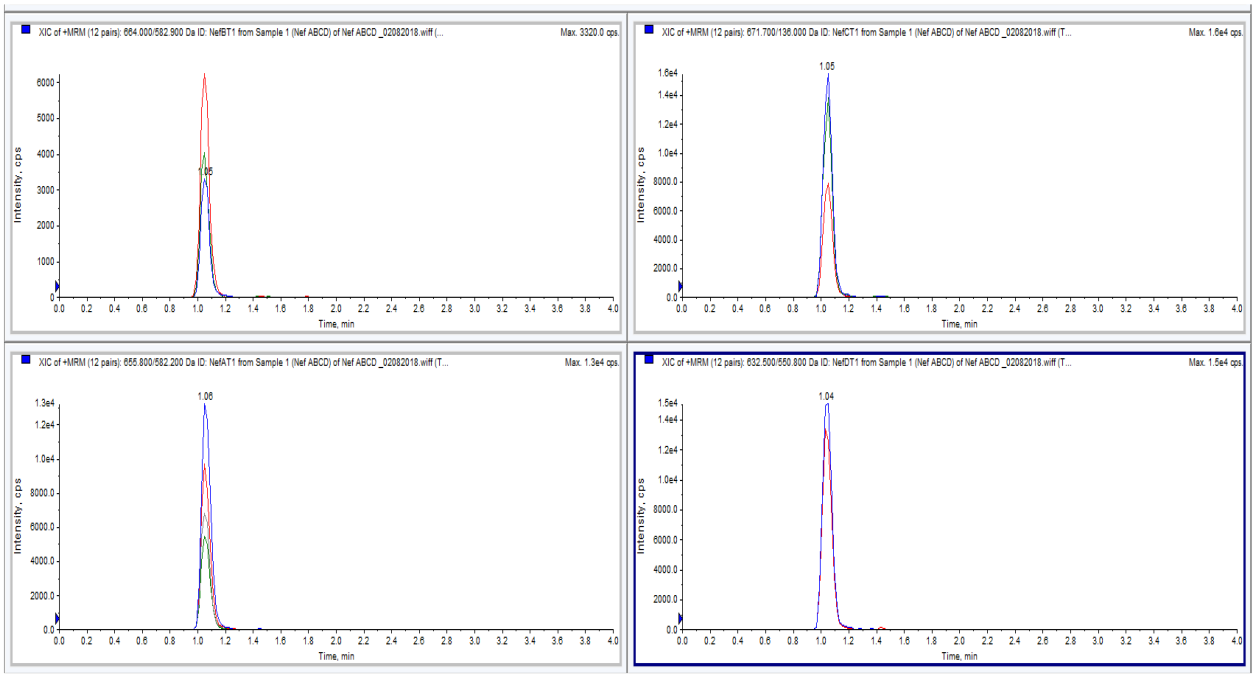


Figure 5.4: MRM chromatogram of labelled target Nef peptides. The four panels show the extracted ion chromatograms of the MRM of each individual Nef peptide. Each colour represents a single reaction for a selected y ion.

As a trial run, peptides were extracted from an HIV-infected serum sample (subject EFH0058) with high viral load (208,000 copies/mL) using the traditional method, and the sample processed on the LCMS using the MRM method developed in this study. Figure 5.5 shows the extracted ion chromatogram of the MRM of the Nef peptide identified in an HIV-infected serum sample, which corresponds to the peptide sequence obtained from the patient sample. Given the low signal obtained on the MS, a new method (Immuno-MRM) for sample preparation was developed to enrich for the Nef protein so as to obtain a higher signal. However, the method requires optimization and is yet to be compared to the traditional sample preparation method.

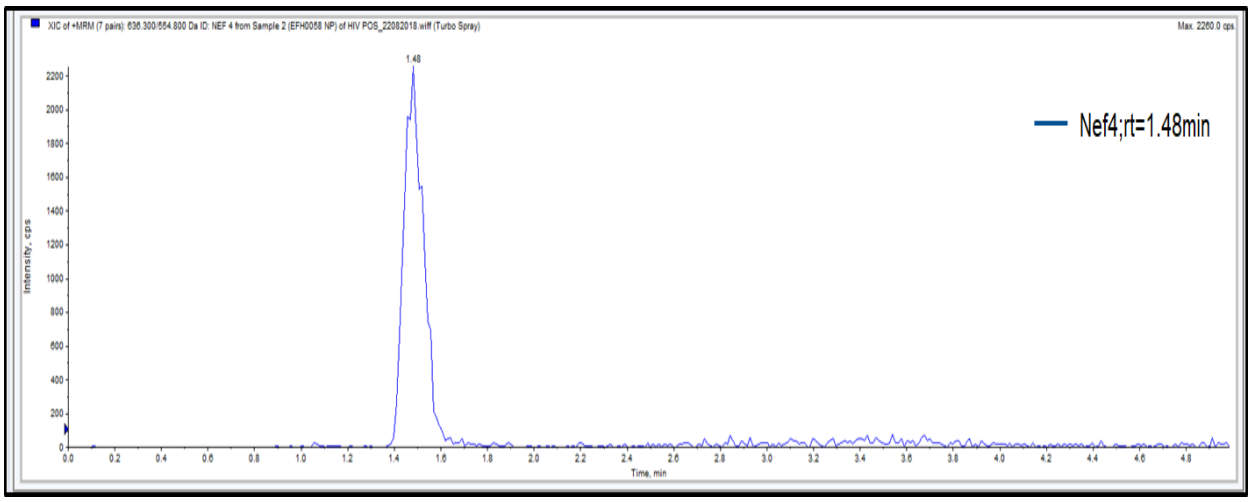


Figure 5.5: The extracted ion chromatogram of the MRM of the Nef peptide identified in an HIV-infected serum sample. The blue line represents the total ion count (XIC) of Nef 4. This corresponds to the peptide sequence obtained from the patient sample.

5.4 Discussion

In this study, HIV-1 Nef was detected in the serum samples of HIV-infected ART-naïve individuals using an ELISA method. Statistical analysis of serum levels of Nef did not show any correlation with CD4 count or viral load, however, this could possibly be due to the small sample size used in the study. Nonetheless, this observation is supported by reports from a study conducted by Wang et al. (2015) in which no associations were found between plasma levels of Nef and clinical characteristics, such as CD4 and viral load in an HIV-infected ART-naïve cohort of 17 subjects. An HIV-infected non-controller with very high viral load (85,800 copies/mL) had very low levels of plasma Nef (Wang et al., 2015). In addition, similar results were obtained by Ferdin et al. (2018) in an HIV-infected ART-naïve cohort of 28 subjects. However, contrary to our findings, Ferdin et al. (2018) observed a positive correlation between plasma levels of Nef and viral load.

Within our study cohort, Nef was detected in two of the HIV-infected ART-naïve participants (EFH0067 and EFH0068) that had undetectable viral load. This observation was also reported by Wang et al. (2015) in which an HIV-infected ART-naïve subject had undetectable viral load (<50 copies/mL) and CD4 cell count of 840 cell/µL but had detectable levels of Nef. Viral load in non-controllers is anticipated to be above the limit of detection (>50 copies/mL), however, lack of detection could possibly be due to haemolysis during storage or poor quality of the sample (Wan Azman et al., 2019). In this study, no associations were also found between plasma levels of Nef with markers of endothelial dysfunction such as ICAM-1, VCAM-1 and CIMT, however, levels of Nef correlated positively with BMI. To the best of our knowledge, this is the first study to look at the association of plasma levels of Nef with markers of endothelial dysfunction, inflammation and CVD risk factors.

Of note, mutations in a protein sequence can potentially hinder binding to its target antibody. As an example, the K29M mutation in the human ICAM-1 sequence can affect the performance of the Human ICAM-1/CD54 Allele-specific Quantikine ELISA kit (R&D Systems, Minneapolis, MS, USA) as the detection antibody is allele-specific (Fernandez-Reyes et al., 1997). Given that the HIV-1 Nef sequence contains a number of mutations, it is possible to have false negative readings with the HIV-1 Nef ELISA kit, as the HIV-1 Nef antibody used was monoclonal. In this study, all the HIV-infected subjects with undetectable levels of HIV-1 Nef possessed arginine at codon 35. Of those with measurable Nef, only 33% possessed Arginine (R). It is possible that the Q35R mutation in the HIV-1 Nef sequence possibly impaired detection of plasma Nef in HIV-infected subjects. Information on the epitope to which the HIV-1 Nef antibody binds was not made available by the manufacturer of the HIV-1 Nef ELISA kit. Moreover, epitopes to which the HIV-1 Nef antibodies bind are reported to be located at the N and C-terminal domains of the Nef protein, which have polymorphic sites (Lülf

et al., 2014, Siakkou et al., 1993). As an example, the HIV-1 Nef antibody [3D12] (AB 42355, Abcam, England) supplied by Abcam is mapped to the amino acids at position 35-50 of the HIV-1 Nef protein, of which codons 35, 38, 39, 40, 43, 45, 49 and 50 are polymorphic sites.

Mass spectrometry offers an alternative approach to ELISAs for the identification and quantification of polymorphic proteins as it is based on the mass-to-charge ratio (m/z) of a target analyte (Peterman et al., 2014). Moreover, liquid chromatography tandem mass spectrometry (LC-MS/MS) methods have a higher limit of detection and quantification, sensitivity, accuracy and precision, compared to immunoassays (Harun et al., 2009). The MRM method developed in this study was able to detect HIV-1 Nef in a clinical sample, however, there are ongoing investigations to validate this method. It is important to note the effect of variation in *nef* on the reliability of the MRM assay. The HIV population present within an infected individual consists of a mixture of heterogeneous strains (Meyerhans et al., 1989). The selected target tryptic Nef peptides used in the development of the MRM assay may not be present in all HIV-infected subjects. Moreover, a mutation to the arginine and lysine residue at positions 134 and 144, respectively, would greatly affect the MRM assay as the resulting peptide obtained upon digestion with trypsin would not fall within the target Nef region. Therefore, due to intra-patient and inter-patient variation in HIV-1 Nef amino acid sequences, serum HIV-1 Nef levels measured by the MRM assay may not be a true reflection of the total amount of circulating Nef present in the blood.

Our previous studies have shown that Nef variants are related to serum levels of biomarkers of endothelial dysfunction and also with CIMT. Thus, it is possible that the Nef sequence is more important than the Nef serum concentration in modulating endothelial function. However, no studies of sufficient size have been undertaken to test this hypothesis, and once the Nef assay

is fully operational, such analyses will be conducted. It is also possible that Nef peptides that do not contain these endothelial dysfunction-related variants may still be able to affect the endothelium, but at a lower level. Thus, measuring serum levels of Nef would still be important in subjects infected with such a virus.

A limitation to this study is the low number of samples used (26). In addition, HIV-uninfected samples were not subjected to the HIV-1 Nef ELISA kit. Thus, we have no data on false positive results. Nonetheless, similar results in relation to the lack of correlation between plasma levels of HIV-1 Nef and viral load was obtained in an independent study as described above (Wang et al., 2015), in which five HIV-uninfected samples were included in the study for comparison.

In conclusion, HIV-1 Nef is present in serum of HIV-infected individuals. HIV-1 Nef can be measured by ELISA, however, an MS method is required to validate the HIV Nef ELISA kit for clinical research use. The MS method developed in this study shows potential but is yet to be validated, and further analysis is required with HIV-infected serum samples.

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Chapter 6 Discussion and Conclusions

In chapter 2, 80 HIV-infected ART-naïve patients, including 38 subjects with CD4 count ≤ 350 cells/mm^3 (low CD4 group) and 42 subjects with CD4 count > 350 cells/mm^3 (high CD4 group) were compared with 60 HIV-uninfected healthy individuals. Collectively, the HIV-infected cohort had higher levels of ICAM-1, VCAM-1 and vWF, and lower levels of HDL-C, compared to the HIV-uninfected cohort (all $p<0.05$). Administration of HIV treatment decreased plasma ICAM-1 and VCAM-1 levels well below pre-treatment levels and below the levels observed in the HIV-uninfected cohort. This, therefore, confirms that endothelial dysfunction is present in the HIV-infected population in South Africa and that HIV treatment appears to attenuate the dysfunction. Nonetheless, inflammation continued to increase despite ART administration, represented by the continuous increase in serum levels of TNF-α, IL-6 and IL-8 post-treatment. Cytokines such as TNF-α, IL-6 and IL-8 activate endothelial cells and their upregulation results in endothelial dysfunction (Liao, 2013). Although HIV is seen to upregulate ICAM-1 expression, inhibition of signalling pathways, such as the ERK/MAPK and Rac1 pathways, can inhibit ICAM-1 expression in the presence of HIV-1 Nef (Chelvanambi et al., 2019, Fan et al., 2010), suggesting that this may be one of the pathways through which the viral peptide may modulate endothelial function. However, this needs to be confirmed in future studies. A direct relationship was found to exist between markers of endothelial dysfunction and HIV infection. Thus, serum levels of VCAM-1 and vWF correlated positively with low CD4 counts and high viral load, respectively. This suggests that HIV infection may affect the endothelium by direct effects of the virus and indirectly by its effects on the immune system.

Data from Chapter 2 therefore suggests there is a relationship between Nef and the endothelium. We therefore sought to explore this potential relationship by examining the

association of variations in the viral *nef* and *tat* genes with serum levels of biomarkers of endothelial dysfunction. Chapter 3 describes results from sequencing HIV-1 *nef* and *tat*. Analysis of the HIV-1 *nef* and *tat* gene sequences showed a stronger link to exist between HIV-1 Nef and endothelial dysfunction compared to HIV-1 Tat. In this study five Nef polymorphisms (V16I, H40Y, T50H,A, S169N, H188Q,S) were identified to be significantly associated with levels of ICAM-1 and one polymorphism each was associated with VCAM-1 (Y202F), MCP-1 (K182M) and TNF-α (D205N) levels while no association of statistical significance was found between HIV-1 Tat amino acid residues and biomarkers of endothelial dysfunction and inflammation. Moreover, ICAM-1 levels rose with an increase in the number of HIV-1 Nef ICAM-1-associated amino acid residues. These data further confirm the possibility of a role for Nef in the aetiology of HIV-associated vascular disease.

In Chapter 4 we therefore decided to determine whether *nef* polymorphisms were associated with a clinical measure of atherosclerotic plaque formation i.e, CIMT. The study cohort consisted of 140 HIV-infected patients on ART. Using CIMT as a marker of sub-clinical atherosclerosis, 5 HIV-1 *nef* gene polymorphisms were found to be significantly associated with high CIMT (K39R, H40Y, D177E, F143Y and V180T), and one polymorphism with ICAM-1 (F143Y). The latter association, however, must be treated with caution as the HIV-infected participants in this study had been on ART for more than 2 years. However, of note, the *nef* H40Y variant identified in Chapter 3 as being associated with higher ICAM-1 levels was also found to be associated with a higher risk of increased CIMT. The H40Y polymorphism has also been linked to pulmonary hypertension (Almodovar et al., 2012). This *nef* variant therefore warrants further investigation.

In order to investigate the association of Nef with endothelial dysfunction it is necessary to develop an accurate and sensitive assay for serum Nef levels. Immunoassays do exist but because of the polymorphic nature of the *nef* gene these assays may not be accurate. Furthermore, these assays have not been validated for use with human serum or plasma. Therefore, in Chapter 5 we sought to develop a specific and sensitive assay for serum Nef using LCMS technology and to use this method to validate a commercial Nef ELISA. Unfortunately, we were not able to develop this assay to a stage where it could be used for accurately measuring Nef in human serum. However, this methodology was able to detect pure Nef standard and to detect a Nef signal in human serum. Further refinements in methodology are required to improve the assay sensitivity. The Nef protein was measured by ELISA in 26 of the HIV-infected, ART-naïve subjects from Chapter 3 and from whom the *nef* gene had been sequenced. Interestingly, in all subjects in which Nef serum levels were below the detection limit of the assay a Q to R variant at codon 35 was identified, which was present in only 33% of subjects in whom Nef could be measured. This suggests that Nef sequence variations may affect the ability of immunoassays to accurately quantify Nef in human serum, but this finding must be confirmed in a larger group of subjects.

Several weaknesses were identified in these studies. These include the small sample size used to identify potential HIV-1 Nef amino acid residues associated with endothelial dysfunction and sub-clinical atherosclerosis, which were largely due to the low success rate for *nef* sequencing. Also, there was a large loss to follow-up of the longitudinal study conducted in Chapter 2. The small sample size also introduces a higher chance of bias, as shown by the differences observed between the *nef*-sequenced and the non-sequenced subjects in Chapter 3. In addition, a positive control group, such as a cohort of HIV-infected patients that have experienced a cardiovascular event, was not included in the studies conducted.

Despite the weaknesses listed above, the strength of this study is that different strategies were employed to investigate the effect of HIV on endothelial function in HIV-infected subjects. Moreover, results obtained in this study were supported by findings from other independent studies. Although the sample sizes were small significant associations were observed and these results can be used to direct our future studies. Such studies will include an *in vitro* functional study to determine the effect of the *nef* variants observed in the current study on cultured endothelial cell function, including ICAM-1 secretion. In addition, HIV-infected subjects with confirmed coronary artery disease (CAD) will be recruited from the Cardiology Department at Charlotte Maxeke Johannesburg Academic Hospital and the *nef* gene will be sequenced and compared to that isolated from subjects without CAD. Lastly, the Nef LCMS assay will be further refined and developed to a stage where it can be used for measuring serum levels of Nef. This will then allow us to determine if serum Nef correlates with markers of endothelial dysfunction and with clinical atherosclerotic endpoints such as CAD, peripheral arterial disease or stroke.

Our study showed evidence of endothelial dysfunction in the Black South African ARV-naïve HIV-infected population and further suggests that the virus could lead to endothelial dysfunction, which may in turn increase CVD risk in this population. However, a longitudinal study with a much larger cohort is required to support this finding. Commencement of HIV treatment is thus recommended as soon as an individual is tested HIV-infected, regardless of CD4 cell count and viral load, as endothelial function is seen to improve with ART administration (Francisci et al., 2009, Arildsen et al., 2013). Nonetheless, persistent immune activation which continues in patients taking ART, especially in those that start treatment late (Ghislain et al., 2015, De Pablo-Bernal et al., 2014), is a cause for concern and requires

thorough investigation to understand the cause of this phenomenon. Such immune activation may lead to endothelial dysfunction and may partially explain the higher prevalence of CVD observed in subjects receiving ART (Freiberg et al., 2013, Chow et al., 2012).

The increasing trend of non-communicable diseases, such as CVD, in the HIV-infected population warrants further investigation to devise strategies that could attenuate this high CVD risk. To the best of our knowledge, this is the first study to look at HIV-1 Nef and Tat amino acid sequences from clinical isolates in the context of CVD. We found amino acid residues in HIV-1 Nef that were associated with endothelial dysfunction. Therefore, it is possible that blocking Nef activity, specifically in subjects carrying the Nef variants identified in this study, could be a potential therapeutic strategy to alleviate endothelial dysfunction and associated vascular disease. To achieve the latter, potential receptors to which HIV-1 Nef bind that mediate endothelial function need to be identified, and antagonists developed to hinder binding of Nef to these receptors. Ligand-receptor pairs could be identified using display technologies such as phage display, cell display, ribosomal display, mRNA display and covalent DNA display. This generally involves generating combinatorial peptide libraries and screening to map cell surface receptor proteomes (Wu et al., 2016). Thus far, HIV-1 Nef is reported to bind to the inositol triphosphate (IP3) receptors (Manninen and Saksela, 2002). The IP3 receptors mediate the release of Ca^{2+} from intracellular stores and are classified into three types; IP3 receptor type 1, 2 and 3. The type 1 receptors are predominantly expressed in the brain, while the type 2 receptors are predominantly expressed in the liver, pancreas, lung, testis and spleen, and the type 3 receptors are mainly expressed in the pancreas, intestine, lung, kidney and brain (Yoshida and Imai, 1997). Future work in this field would also include the development of Nef inhibitors, possibly anti-Nef antibodies, and using such agents in *in vitro* and *in vivo* HIV-1 Nef inhibitory studies, to assess if inhibiting this viral protein results in

decreased endothelial cell activation. This would equally provide more insight into the mechanisms surrounding HIV-1 Nef associated endothelial dysfunction.

The LC-MS/MS method that was developed to measure Nef was able to identify this protein using both labelled peptides and a patient serum sample. Thus, the use of mass spectrometry could potentially allow the accurate identification and quantification of the polymorphic Nef protein in human serum. However, further studies need to be conducted to validate the HIV-1 Nef MRM assay for limit of detection, limit of quantification, precision and accuracy. It has been shown that Nef can be detected in serum from subjects receiving ART (Ferdin et al., 2018, Raymond et al., 2011) and this 'leakage' of Nef from HIV reservoirs may partially explain higher levels of CVD observed in subjects receiving ART. Thus, the measurement of HIV Nef could potentially be used as a marker of translationally active HIV reservoirs in aviraemic individuals and such subjects could be targeted for anti-Nef therapy in the absence of mechanisms for destroying these reservoirs.

Overall, this study demonstrates that the aetiology of CVD in black African subjects with HIV infection may involve specific variants of the viral peptide Nef which induce endothelial dysfunction and sub-clinical atherosclerosis. The mechanisms involved in this process may be due directly to Nef or its modulation of the immune system of the host. In addition, HIV infection is associated with inflammation that is not attenuated by anti-viral therapy, and this may partially explain the high prevalence of CVD in subjects receiving ART. Continual production of viral peptides from HIV reservoirs may also be involved. Anti-CVD strategies should be focused on subjects carrying virus with specific Nef sequence variants. Future studies are required to confirm these findings and to develop strategies that reduce the effects of viral peptides in subjects receiving ART.

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Appendices

Appendix 1: Ethical clearance granted to Dr Nereshni Lutchman to recruit HIV-infected and HIV-uninfected subjects to investigate endothelial dysfunction in the HIV-infected Black South African population

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Nereshni Lutchman

CLEARANCE CERTIFICATE	**M10408**
PROJECT	An Investigation of Enothelial Function and Cardiovascular Risk in the Human Immunodeficiency Virus (HIV-Infected South Africans Black Population
INVESTIGATORS	Dr Nereshni Lutchman.
DEPARTMENT	departmentof Chemical Pathology
DATE CONSIDERED	30/04/2010
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 16/07/2010 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof N Crowther

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

Appendix 2: Ethical clearance granted to Ms Genevieve Mezoh to take over Dr Nereshni Lutchman's study and investigate the effect of HIV viral proteins on endothelial function in the HIV-infected population

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

Appendix 3: Univariate regression models for markers of endothelial dysfunction in HIV-infected subjects

Independent variables		Dependent variables				
		E-selectin	ICAM-1[a]	VCAM-1[a]	MCP-1[a]	vWF [b]
Male vs female	β-value	,3716	,0077	,1677	,1724	,0179
	p-value	p=,003	p=,952	p=,189	p=,177	p=,889
Age	β-value	,1985	,2044	,1245	,3014	,2083
	p-value	p=,119	p=,108	p=,331	p=,016	p=,101
Waist	β-value	-,0131	-,0205	-,1125	-,0291	,0886
	p-value	p=,919	p=,873	p=,380	p=,821	p=,490
Hip	β-value	-,1802	-,0328	-,1993	-,0899	,0152
	p-value	p=,158	p=,799	p=,117	p=,484	p=,906
BMI	β-value	-,1425	-,0108	-,1333	-,1151	,0580
	p-value	p=,265	p=,933	p=,298	p=,369	p=,652
Systolic BP	β-value	,1336	,0980	,2255	,1330	-,1386
	p-value	p=,297	p=,445	p=,076	p=,299	p=,279
Diastolic BP	β-value	,2532	,1038	,1677	,2639	,0617
	p-value	p=,045	p=,418	p=,189	p=,037	p=,631
Glucose	β-value	,0615	-,1113	,0020	,0437	,0354
	p-value	p=,647	p=,406	p=,988	p=,745	p=,792
Triglyceride	β-value	,1269	-,1336	-,3029	,0620	-,1524
	p-value	p=,322	p=,296	p=,016	p=,629	p=,233
LDL-C	β-value	-,1991	-,0254	-,3256	,0755	-,0605
	p-value	p=,118	p=,843	p=,009	p=,556	p=,638
HDL-C	β-value	-,0793	,2342	-,1238	-,0523	-,1613
	p-value	p=,536	p=,065	p=,334	p=,684	p=,207
Total cholesterol	β-value	-,1590	,0214	-,3648	,0564	-,1468
	p-value	p=,213	p=,868	p=,003	p=,660	p=,251
TNF	β-value	,0628	,1110	,1858	,2571	,1753
	p-value	p=,625	p=,386	p=,145	p=,042	p=,169
IL6	β-value	-,1218	,0198	,3230	,2294	,2284
	p-value	p=,342	p=,877	p=,010	p=,071	p=,072
IL8	β-value	,0488	,0146	-,1859	,1557	,0524
	p-value	p=,709	p=,911	p=,151	p=,231	p=,688
CD4	β-value	-,1454	-,1959	-,4832	-,1826	,0339
	p-value	p=,256	p=,124	p=,000	p=,152	p=,792
Viral load	β-value	,2565	-,1624	,1191	,0904	,3526
	p-value	p=,064	p=,245	p=,396	p=,520	p=,010

[a]Square root and [b]logged values used; p-values marked in red are those at p<0.20 and these signify the independent variables that were included in the multivariable model for the appropriate dependent variable.

Appendix 4: Ethical clearance granted to Dr Michelle Moorhouse to recruit HIV-infected subjects to investigate the effect of HIV infection and antiretroviral treatment on the cardiovascular profile of HIV-infected individuals, and ethical clearance granted to Ms Genenvieve Mezoh to use samples collected in the study

R14/49 Dr Michelle Moorhouse et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M160130

NAME: (Principal Investigator)	Dr Michelle Moorhouse et al
DEPARTMENT:	Clinical Medicine Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	The Influence of Human Immunodeficiency Virus Infection and Antiretroviral Treatment on Cardiovascular Profile and Pulmonary Condition in HIV-Infected Individuals in an Urban Setting in Sub-Saharan Africa. CaPuT Prevalence - and Cappuchino Study
DATE CONSIDERED:	29/01/2016
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	01/06/2016

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 in Room 10004,10th floor, Senate House/2nd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the the conditions under which I am/we are authorised 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 to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. in this case, the study was initially review in January and will therefore be due in the month of January each year.

Principal Investigator Signature

Date 20 JUNE 2016

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

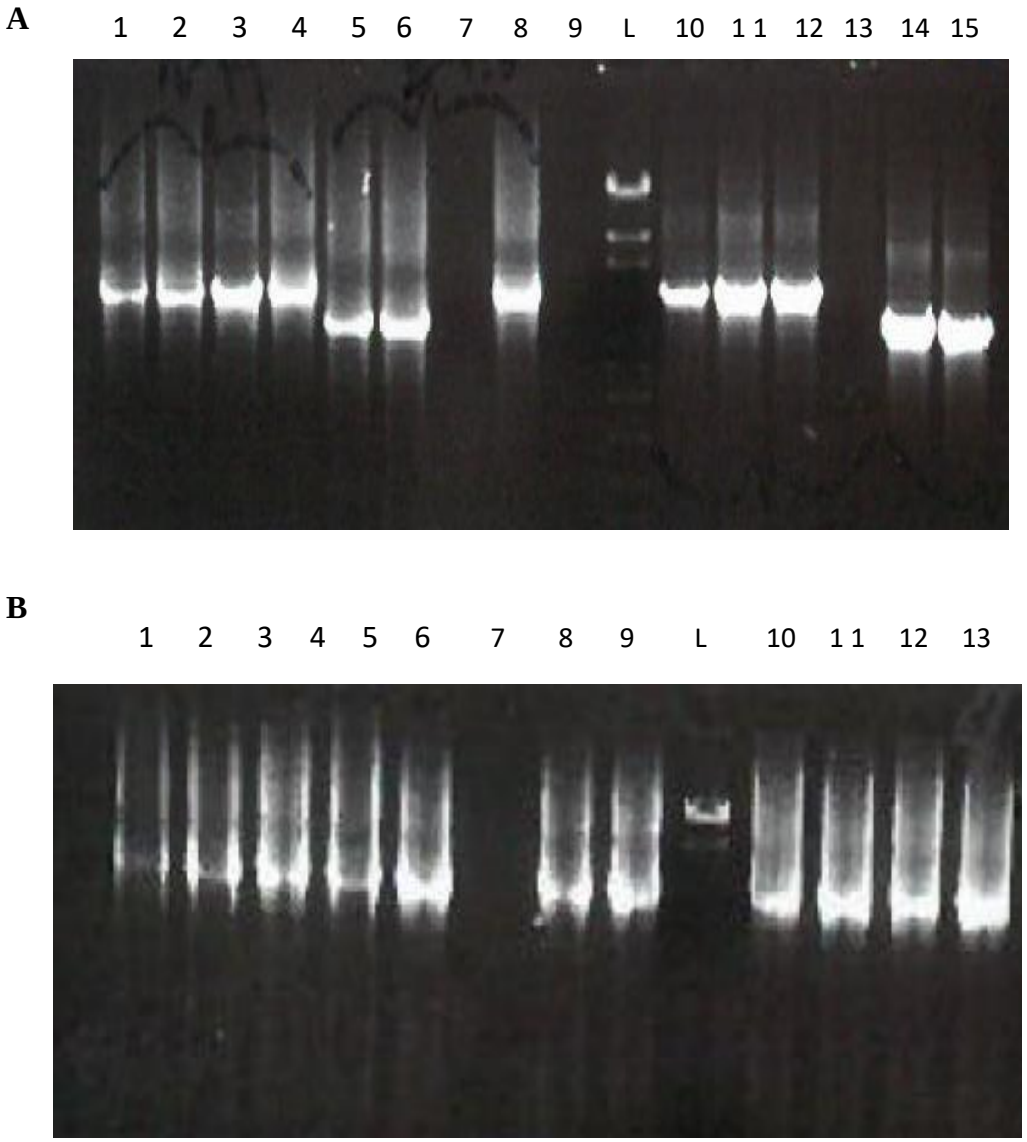
Thank you for keeping us informed and updated.

Yours Sincerely,

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

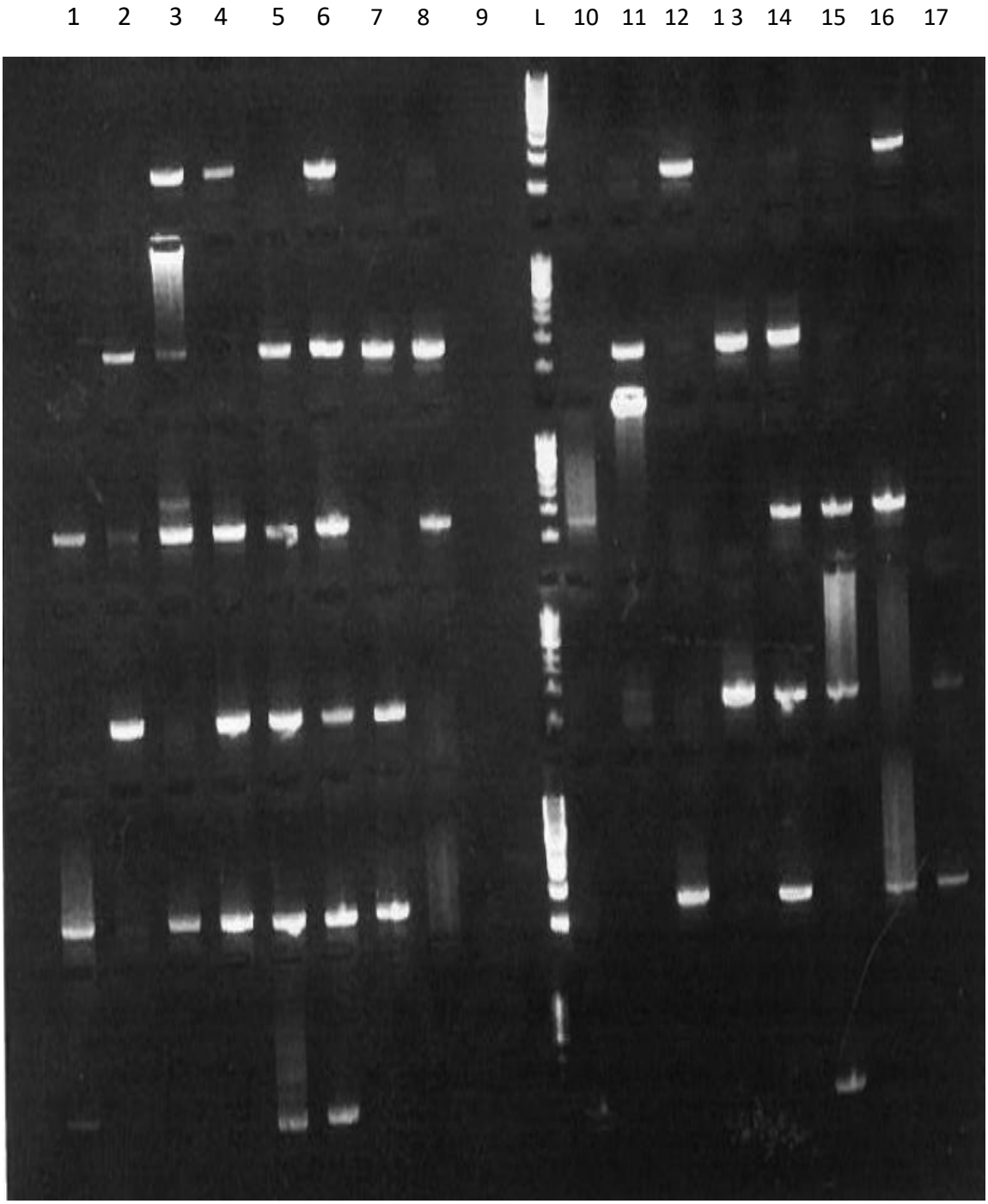
Research Office Secretariat: Faculty of Health Sciences, Phillip Tobias Building, 3rd Floor, Office 304, Corner York Road and 29 Princess of Wales Terrace, Parktown, 2193 Private Bag 3, Wits 2050 I **T**+27 (0)11-717-1234/2656/2700/1252 **E**: Rhulani.Mkansi@wits.ac.za I **Office E** HREC-Medical.ResearchOffice@wits.ac.za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

Appendix 5: Assessment of HIV-1 Nef and Tat DNA, obtained from viral RNA, by agarose gel electrophoresis



(A) DNA agarose gel showing the 4054bp HIV-1 subtype C amplicon obtained from nested PCR with the outer primer pair Vif1: 5'-GGGTTTATTACAGGGACAGCAGAG-3' and OFM19: 5'-GGTAGGATCTCTACAATACTTGGCACTG-3' and inner primer pair, Tat1F2: 5'-GGTAGGATCTCTACAATACTTGGCACTG-3' and NefIR: 5'-CTTATATGCAGCATCTGAGG-3', are in lanes 1-4 and 10-13, while that obtained with the outer primer pair Vif1: 5'-GGGTTTATTACAGGGACAGCAGAG-3' and OFM19: 5'-GGTAGGATCTCTACAATACTTGGCACTG-3' and inner primer pair, ENV A: 5'–GGCTTAGGCATCTCCTATGGCAGGAAGAA and ENV N: 5'–CTGCCAATCAGGGAAGTAGCCTTGTGT, are in lanes 5-8 and 13-14. The negative control and DNA ladder are in lanes 9 and L, respectively. (B) The 4054bp amplicon obtained from nested PCR with the primer pair Nef and TatIF2 are in lanes 1-13, while the DNA ladder is in lane L.

Appendix 6: Assessment of HIV-1 Nef DNA, obtained from genomic DNA, by agarose gel electrophoresis



DNA agarose gel showing the 1,316 bp HIV-1 Nef amplicon obtained from individual patient samples using an outer primer pair (Nef_F1: 5'- CAGTAGCTGARGGRACAGATAGG and Nef_R1: 5'-CTTTATTGAGGCTTAAGCAGTGGGTTC) and an inner primer pair (Nef_F2: 5'- AAGAATAAGACARGGCTTYG and Nef_R2: 5'- CAGCTGCTTATATGCAGCATC) are in lanes 1-8 and 10-17, while the negative control and DNA ladder are in lanes 9 and L, respectively.

Appendix 7: Deciphering Endothelial Dysfunction in the HIV-Infected Population

Chapter 11
Deciphering Endothelial Dysfunction in the HIV-Infected Population



Genevieve Mezoh and Nigel J. Crowther

Abstract Cardiovascular disease (CVD), as a possible consequence of endothelial dysfunction, is prevalent among HIV-infected patients despite successful administration of antiretroviral drugs. This warrants the routine clinical assessment of endothelial function in HIV-positive patients to circumvent potential CVD events. Several different non-invasive strategies have been employed to assess endothelial function in clinical research studies yielding inconsistencies among these reports. This review summarises the different techniques used for assessing endothelial function, with a focus on proposed blood-based biomarkers, such as endothelial leukocyte adhesion molecule-1 (E-selectin), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), von Willebrand factor (vWF), TNF-α, interleukin 6 (IL6) and soluble thrombomodulin (sTM). The identification of suitable blood-based biomarkers, especially those that can be measured using a point-of-care device, would be more applicable in under-resourced countries where the prevalence of HIV is high.

Keywords Endothelial dysfunction · HIV · Biomarkers · Inflammation · Cardiovascular disease

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11.1 Introduction

There are currently more than 36 million people in the world living with HIV, with sub-Saharan Africa accounting for up to 53% of this population [1].The introduction of highly active antiretroviral therapy (HAART) has increased the life expectancy of HIV-positive patients, and presently, cardiovascular disease (CVD) has emerged as an important cause of mortality among infected individuals. Despite effective antiretroviral therapy, HIV-positive patients have been observed to have a higher risk of myocardial infarction and cardiovascular death, than age-matched uninfected controls [2, 3]. An increased prevalence of traditional risk factors such as dyslipidaemia and smoking, among individuals infected with HIV, is thought to contribute to this increased cardiovascular morbidity. However, growing evidence suggests that HIV infection and treatment may lead to endothelial dysfunction, likely through inflammation and immune dysregulation [4, 5].

The endothelium is constantly in contact with foreign particles that circulate within the bloodstream [6], and is one of the first points of contact between tissues and circulating blood-bourne pathogens. Therefore, the endothelium is subjected to immune system defence strategies that can lead to endothelial damage [7]. This in turn can initiate repair mechanisms that lead to a cascade of events that may culminate in further vascular damage and plaque formation [8, 9]. This review seeks to explore the association between HIV infection and endothelial dysfunction, with an emphasis on the serum biomarkers that have been used to assess the level of endothelial dysfunction in such disease states.

11.2 Endothelial Dysfunction

The endothelium constitutes a single layer of cells that runs throughout the body internally lining the walls (intima) of the blood vessels. It is therefore the principal barrier separating blood from tissues. The endothelial cells secrete a number of substances that confer upon the endothelium its ability to regulate numerous blood vessel functions including vascular tone, cell adhesiveness, and coagulation [10]. An imbalance in the levels of these mediators can result in endothelial dysfunction [11]. This systemic pathological state of the endothelium is the earliest clinically detectable stage of cardiovascular disease [7].

Cytokines are soluble proteins secreted by a variety of cells, mainly leukocytes, as well as endothelial, stromal and mast cells, in response to foreign entities [12]. They therefore play a vital role in the regulation of innate and adaptive immunity, hematopoiesis and lymphocyte development [13]. Endothelial cells express transmembrane adhesion proteins such as endothelial leukocyte adhesion molecule-1 (E-selectin), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). The expression of these adhesion molecules by the endothelium is increased in response to proinflammatory cytokines such as tumour

necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [14] and in response to turbulent blood flow. These factors increase the expression of endothelial adhesion molecules by activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [15]. This phenomenon, referred to as endothelial cell activation, enables the transmigration of monocytes across the endothelium, which is a primary factor in the initiation of atherosclerotic plaque formation [14, 16].

Endothelial dysfunction is defined as the decreased bioavailability of nitric oxide (NO) derived from the endothelium, and is caused by a number of factors including hypercholesterolaemia, smoking and oxidative stress [16]. The synthesis of NO in endothelial cells is catalysed by the enzyme endothelial nitric oxide synthetase (eNOS), which is activated by shear stress brought about by the continuous pulsatile flow of blood [17]. Endogenous NO has several functions which include inhibition of white cell and platelet activation, inhibition of vascular smooth muscle cell proliferation, smooth muscle relaxation and vasodilation [18]. Levels of NO are reduced by its interaction with superoxide, which is produced from oxygen by the action of NADPH oxidase [19]. Failure of the endothelium to respond to shear stress would therefore lead to a decrease in the endothelium-derived NO with a concomitant build-up of superoxide which further decreases levels of NO. Given the vital role NO plays in smooth muscle relaxation, a prerequisite for vasodilation, a decrease in NO therefore leads to vasoconstriction [19, 20]. A decrease in the bioavailability of NO also leads to greater endothelial activation which results in increased platelet aggregation, vascular smooth muscle cell proliferation, greater monocyte adhesion and transmigration and oxidative stress, which promote atherosclerosis [8].

Post-mortem studies show that the atherosclerotic process starts in individuals as early as childhood, and by the early third decade of life, 20% will have advanced lesions present [21, 22]. Core factors known to influence endothelial function include age [20], gender [23], family history of diabetes and CVD [24], physical activity [25], insulin resistance [26], hyperglycaemia [27], active and passive smoking [28], visceral fat [29], obesity [26], hypertension [30] and dyslipidaemia [31]. Infection with HIV has been proposed as a further risk factor for endothelial dysfunction [32, 33] (Fig. 11.1).

The endothelium is exposed to shear stress induced by turbulent blood flow, which is common in areas where the vasculature branches, and are sites at which atherosclerotic plaques are often formed [34]. Endothelial damage at such sites initiates a repair process, which involves the localization of circulating cells originating from the bone-marrow. These cells include circulating angiogenic cells (CACs) and endothelial progenitor cells (EPCs). Endothelial damage equally results in the release of circulating endothelial cells (CECs) and endothelial micro-particles (EMPs) into the blood circulation [35]. As a consequence, CACs are recruited to the site of endothelial damage for vascular repair [36]. Angiogenic cytokines are produced from the site of endothelial damage, and induce movement of EPCs from the bone marrow, to the damaged endothelium, where they are integrated into the sites of active angiogenesis [15, 37]. The release of EPCs from the bone marrow is modulated by the activation of matrix metalloproteinase (MMP)-9, in a mechanism

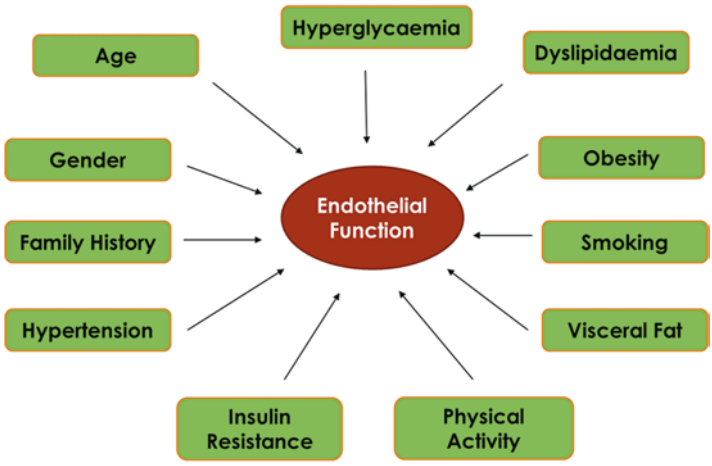


Fig. 11.1 Main factors that affect endothelial function. Several factors are known to have an impact on the endothelial cell state. This includes hyperglycaemia, dyslipidaemia, obesity, smoking, visceral fat, physical activity, insulin resistance, hypertension, family history of endothelial dysfunction, gender and age

coordinated by the bioavailability of NO [38, 39]. Therefore, reduction in the levels of NO, as observed during endothelial dysfunction, hinders recruitment of EPCs to the site of endothelial damage [38]. A recent study has further shown that in an animal model of atherosclerosis, treatment with EPCs reduces the level of atherosclerosis [40].

The formation of an atherosclerotic plaque can therefore be seen as a complex process, involving both endothelial activation and dysfunction. Reduced production and/or activity of endothelial-derived NO appear to be central to this process, but the molecular pathways involved are only partially understood. In addition, recruitment of EPCs to the site of endothelial damage seems to be essential to the attenuation of plaque formation. Many factors can initiate endothelial dysfunction and activation, and HIV infection may be one such agent.

11.3 HIV Infection and Endothelial Dysfunction

Infection with HIV has been associated with the development of CVD [2, 3], which may occur via the induction of endothelial dysfunction brought on either by the initiation of an inflammatory response or by the direct action of the HIV proteins Nef [42] and gp120 [42]. In vitro and in vivo studies conducted using human microvascular endothelial cells and C57BL/6 rats respectively, showed that adhesion of leukocytes to the endothelium was selectively activated by the synergistic action of HIV-1 Tat and TNF-α. In a study conducted by Dhawan and colleagues [44] using human umbilical vein endothelial cells, HIV-1 Tat was found to induce expression of E-selectin, ICAM-1 and VCAM-1. Research conducted by Wang et al. [41] using

human coronary arterial endothelial cells (HCAECs), and Indian rhesus macaque heart tissue, showed that HIV-1 Nef contributes to endothelial dysfunction via two distinct pathways; an NADPH oxidase-dependent mechanism resulting in the apoptosis of endothelial cells, and by the expression of monocyte chemoattractant protein-1 (MCP-1) through the NF-κB signalling pathway. In addition, gp120 with TNF-α pre-treatment was found to cause endothelial dysfunction by downregulating the expression of endothelial nitric oxide synthase and upregulating ICAM-1 expression in HCAECs [43]. Hence, research conducted thus far shows that the circulating viral proteins act individually via different mechanisms to induce endothelial dysfunction, with HIV Tat and gp120 acting in synergy with TNF-α, and HIV Nef being secreted in vesicles and entering the endothelial cells by cell-to-cell transfer [41] (Fig. 11.2).

Elevated plasma levels of endothelial cell derived markers such as endothelial leukocyte adhesion molecule-1 (E-selectin), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), von Willebrand factor (vWF), and the inflammatory cytokines TNF-α, interleukin 6 (IL6) and interleukin 8 (IL8), have all been observed in HIV-infected individuals with elevated CVD risk factors (Table 11.1). In addition, an association between

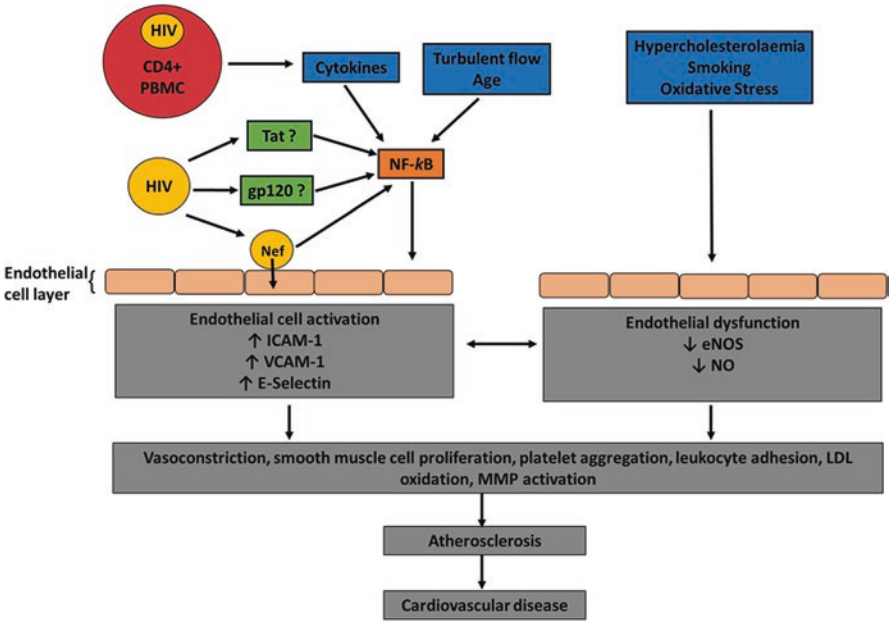


Fig. 11.2 Schematic illustration of the potential mechanisms behind CVD in the HIV-infected population. HIV-infected individuals are more predisposed to endothelial dysfunction. This occurs not only as a result of inducers such as hypocholesterolaemia, smoking, oxidative stress, turbulent blood flow and age, but also as a result of immune activation upon HIV infection and direct interaction between HIV viral proteins such as Nef, Tat and gp120 with endothelial cells. (Adapted from Liao [16])

Table 11.1 Studies comparing markers of endothelial dysfunction in HIV-infected and non-infected subjects

Authors	Study populations	Effectors and markers of endothelial function analysed	Results of comparison of cases versus controls	Associations
[72]	106 HIV-infected children, of which 86% were on HAART, against 55 normal control children aged 12–15 years	CRP IL6 MCP-1 sICAM-1 sVCAM-1 E-Selectin	Increase Increase Increase Increase Increase Increase	Correlation between all endothelial markers with higher waist:hip ratio, low CD4 counts and high viral load.
[148]	83 HIV-infected children on HAART against 83 uninfected healthy children aged 7–13 years	CIMT CRP	Increase Increase	Positive association between HIV infection with CIMT and elevated CVD risk factors. Negative association between CIMT with total cholesterol and HDL-C
[146]	A cross-sectional study of 39 HIV-infected ARV-naïve patients aged 29–45 years compared with three different groups; (i) 26 age-matched (27–37 years) and (ii) 26 elderly (64–81 years) uninfected healthy controls; and (iii) 26 non-survivor elderly subjects (74–87 years).	CRP TNF-α INF-γ IL-1β IL-6 IL-8 IL-17	Increase Increase Increase Below detection Increase Increase Below detection	No association between any markers of inflammation and HAART.
	A longitudinal study of 39 HIV-infected ARV-naïve patients at baseline, and after 48 and 96 weeks of ARV administration.	CRP TNF-α INF-γ IL-1β IL-6 IL-8 IL-17	No change Decrease Decrease Below detection Decrease Decrease Below detection	
[115]	494 HIV-infected individuals, of which 82.4% were on HAART, compared with 5386 healthy controls aged 45–76 years; and 287 HIV-infected individuals, of which 72.5% were on HAART, compared with 3231 participants aged 33–44 years.	CRP IL-6	Increase Increase	Positive association between endothelial markers and HIV infection. No association between levels of endothelial markers and HAART.

(continued)

Table 11.1 (continued)

Authors	Study populations	Effectors and markers of endothelial function analysed	Results of comparison of cases versus controls	Associations
[46]	300 ARV-naïve HIV-infected participants compared with 300 uninfected controls aged 36–52 years.	IL-6 CRP sICAM-1 sVCAM-1 Carotid radialis pulse wave velocity (crPWV)	Increase Increase Increase Increase No difference	Positive association between crPWV and age in the HIV-infected group. Positive association of HIV-infection with levels of ICAM-1 and VCAM-1.
[145]	66 HIV-positive subjects on HAART compared with 165 HIV-negative controls.	IL-6 CRP sICAM-1 sVCAM-1 crPWV	No difference No difference Increase Increase No difference	Positive association between HIV-infection with ICAM-1 and VCAM-1. Inverse association between VCAM-1 with CD4 count.
[99]	90 HIV-positive subjects on HAART versus 90 uninfected controls	vWF sTM E-selectin TNF-α IFN-α	Increase Increase Increase Increase Increase	Negative correlation between sTM and CD4+ cell count. Positive correlation of vWF with TNF-α and IFN-α Higher level of markers of endothelial dysfunction in HIV-positive subjects.
[149]	112 HIV-infected adults on long-term HAART, 35–45 years of age, compared with 84 HIV-uninfected controls aged 33–43 years.	IL-6 sICAM-1 sVCAM-1 E–selectin CIMT	No difference No difference Increase No difference No difference	Positive correlation between HIV infection with increased levels of VCAM–1 but not ICAM–1 or E–selectin. No association of IL-6 with markers of endothelial dysfunction.

markers of inflammation such as C-reactive protein (CRP) and TNF-α with CVD in the HIV-positive population has also been reported [45, 46].

Although traditional risk factors such as hypercholesterolemia, smoking, oxidative stress and age cause endothelial dysfunction, HIV-infected individuals have an additional increased risk of endothelial dysfunction due to the action of HIV (Fig. 11.2). Invasion of cells of the immune system by HIV initiates immune responses that involve an upregulation of cytokine production [47]. In addition, studies have shown immune reconstitution inflammatory syndrome (IRIS) to occur upon ARV administration, characterised by excessive proinflammatory cytokines

[48]. Cytokines, as well as age and turbulent blood flow are factors known to activate the NF-κB pathway resulting in endothelial cell activation, which subsequently leads to endothelial dysfunction [16]. The HIV viral protein, Nef, is suggested to cause endothelial dysfunction via activation of the NF-κB pathway within endothelial cells [41]. Endothelial activation and dysfunction put into motion a series of events which include vasoconstriction, smooth muscle cell proliferation, platelet aggregation, leukocyte adhesion, LDL oxidation and matrix metalloproteinase (MMP) activation, which initiates atherogenesis, and as consequence, the development of CVD.

11.4 Assessment of Endothelial Function

There are several methods by which endothelial function can be assessed, which can be either invasive or non-invasive (Table 11.2) [49]. Non-invasive strategies are more applicable in terms of cost, patient compliance and are easier to conduct compared to invasive strategies [49, 50], and therefore, only these methods will be described.

The quantification of cells involved in the process of endothelial repair, such as the endothelial micro-particles (EMPs) and endothelial progenitor cells (EPCs), by flow cytometry, has been used as a strategy to investigate endothelial dysfunction [51]. However, this technique is limited by the fact that the circulating EPCs are present in low numbers, and there is no specific marker currently available that can be used for the identification of EPCs [52]. To address this problem, a combination of markers has been utilised to measure EPC numbers [51, 53]. Researchers have also proposed the use of CECs and EMPs as prognostic markers in evaluating atherosclerotic vascular disease [54]. However, there are several drawbacks to the use of these flow cytometric methods for the assessment of endothelial dysfunction such as the requirement of highly specialised equipment, trained personnel, and low turnaround times.

Another non-invasive strategy is the measurement of flow-mediated dilation (FMD). This involves the ultrasound measurement of the diameter of the brachial arteries following a brief period of occlusion and reflow in which reactive hyperaemia is induced. Reduction of endothelial function as demonstrated by a decrease in flow-mediated vasodilation of the brachial artery has been observed in HIV-infected subjects [32, 55]. However, this procedure is not subject to automation, is time consuming and requires the availability of specialised equipment and skilled personnel. In addition, significant variation has been observed in day-to-day measurements, most likely due to changes in the biological circadian rhythms [50].

A more recent non-invasive approach is the plethysmographic recordings obtained from the fingertip using the EndoPAT (endothelial peripheral arterial tonometry) device [56, 57]. The finger arterial pulse wave amplitude is recorded at rest, following which the forearm is cuffed for 5 min to induce reactive hyperaemia, and plethysmographic readings taken in 30 s intervals for 4–5 mins following occlu-

Table 11.2 Methods used for the assessment of endothelial function

	Technique	Principle	Reference
Invasive strategies	Quantitative coronary angiography	Infusion of vasodilator (e.g. acetylcholine, bradykinin or serotonin) followed by measurement of changes in vascular diameter. Patients with good endothelial function show a vasodilatory response whereas either vasoconstriction or no change in vascular diameter is observed in those with endothelial dysfunction	[11, 139]
	Intracoronary doppler	Infusion of an endothelium-dependent vasodilator (such as acetylcholine) and direct dilator of vascular smooth muscle (such as papaverin) via a Doppler catheter followed by measurement of intracoronary blood flow velocity and cross-sectional arterial area	[140]
	Strain-gauge venous impedance plethysmography	Direct brachial artery administration of agonists of vasodilation in the forearm, followed by measurement of change in blood flow	[141, 142]
Non-invasive strategies	Brachial artery ultrasound (flow mediated vasodilation)	Brachial artery diameter measured using high-resolution ultrasound transducer before and after brief occlusion. Reflow after occlusion results in raised shear stress that causes endothelial dependent dilation in patients with healthy endothelial function. This function is impaired in patients with endothelial dysfunction	[23, 143]
	Peripheral arterial tonometry (EndoPAT)	Arterial blood volume measured in fingertip using plethysmograph before and after a brief period of occlusion. Reflow results in arterial volume changes which increases the measured signal	[56, 144]
	Immunoassays including enzyme-linked immunosorbent assays (ELISAs)	Formation of an antibody-antigen complex, with antigen concentration determined via the quantification of light emitted by a conjugated fluorescent protein (antibody orantigen)	[145, 146]
	Flow cytometry	Quantification of forward and scattered-light emitted by labelled cells	[51, 147]
	Immuno-magnetic separation	Antibody-antigen binding of CECs (similar to ELISA) coupled with fluorimetry for quantification of the selected autofluorescent CECs	[126, 132]

sion. Advantages of the EndoPAT over FMD include the fact that it is easier to perform, less variability in day-to-day readings and higher sensitivity [49]. However, this technique is more expensive compared to FMD as it requires the use of disposable finger probes. Therefore, as with FMD, this approach may not be easily adopted in resource-limited countries. Moreover, EndoPAT readings have been associated with several traditional and metabolic cardiovascular risk factors, thus, non-endothelial factors can influence the signal [58, 59].

Ultrasound measurement of the thickness of the two inner layers of the carotid artery (the intima and the media), referred to as carotid-intima media thickness (CIMT), has been proposed as a non-invasive strategy to assess endothelial dysfunction as it provides a quantitative measure of sub-clinical atherosclerosis. Several studies report a positive correlation to exist between CIMT and CVD risk [60, 61]. However, a lack of correlation between CIMT and markers of endothelial function has been observed [62, 63].

The use of circulating plasma markers for the assessment of endothelial function in HIV-infected patients would be a more practical and sustainable approach in developing countries. Given that this technique uses immunoassays to analyse the level of targeted analytes in blood samples, it can easily be applied to automated systems, thereby enabling the processing of hundreds of samples with a short turn-around time. However, this technique may lack specificity in that some of these markers are elevated under conditions not associated with endothelial dysfunction [64, 65], and do not necessarily originate from endothelial cells [66]. This problem may be partially overcome by measuring multiple blood markers. The following section of this review will discuss each of these blood markers of endothelial function in more detail.

11.5 Blood-Based Markers of Endothelial Function

11.5.1 Adhesion Molecules

The ICAM-1 and VCAM-1 molecules are trans-membrane proteins belonging to the immunoglobulin superfamily often found on the surface of leukocytes and endothelial cells [66, 67]. Though attached to the membrane, they also exist in a soluble form in biological fluids [66, 68]. These adhesion proteins are similar in structure and function. They play a major role in cell-cell adhesion by stabilizing cell-cell interactions [69], facilitate the migration of leukocytes across the endothelium, and are expressed upon cytokine stimulation [67]. Thus, cytokines such as TNF-α and IL-6 activate the signal transducers and activators of transcription (STATs) signalling pathway, and the STATs bind to NF-κB in the ICAM-1 promotor leading to upregulation of expression [70]. Reactive oxygen species (ROS) are required for the binding of STATs to NF-κB [71]. Therefore, increases in the levels of ROS as a result of endothelial dysfunction would result in further increased levels of ICAM-1. Increased expression of ICAM-1 on the endothelial cells results in the recruitment of inflammatory immune cells [72, 73].

In addition, studies show ICAM-1 to be the entry point of viruses including the human rhinoviruses [74, 75], and the malarial parasite Plasmodium falciparum [76, 77]. The ICAM-1 and VCAM-1 molecules are not simultaneously expressed in all diseases involving an inflammatory process [78]. Thus, a study conducted by Mason et al. showed the levels of sICAM-1 and sVCAM-1 to be significantly higher in

patients with rheumatoid arthritis compared to healthy controls, however, in patients with lupus erythematosus, only sVCAM-1 was elevated and not sICAM-1 [79]. Therefore, different inflammatory diseases may be associated with specific adhesion or inflammatory molecules.

Elevated levels of ICAM-1 and VCAM-1 in blood would imply an increase in their expression on the cell surface, and thus, an increase in the transmigration of leukocytes across the endothelial cells. This increase in movement across the endothelium results in the build-up of pressure at the surface of the endothelial cells [80]. Eventually, endothelial shedding will occur, resulting in the detachment of the endothelial trans-membrane proteins and their shedding into the blood circulation [15].

Blood flow through the blood vessels is generally laminar. However, obstruction to blood flow as a result of atherosclerotic plaques generates rhythmic patterns. In vitro studies show that laminar and oscillatory shear stress upregulates ICAM-1 expression [81, 82]. However, up-regulation of VCAM-1 is either to a much lesser extent compared to ICAM-1 or non-existent [80, 83], and prolonged exposure to shear stress appears to down-regulate both adhesion molecules [81, 84].

Endothelial leukocyte adhesion molecule-1, often referred to as E-selectin or ELAM-1, is similar to VCAM-1 and ICAM-1 in that it is also a trans-membrane adhesion molecule that facilitates cell-cell interaction, and the transmigration of leukocytes into tissues following cytokine activation. However, E-selectin differs from VCAM-1 and ICAM-1 in that it is solely expressed on endothelial cells. In addition, it promotes the adhesion of resting CD4+ memory cells to endothelial cells [81, 85]. This adhesion molecule could therefore be regarded as a plasma marker of choice for the assessment of endothelial function given that it is endothelium specific. However, continuous blood flow and influx of leukocytes to inflamed tissues results in shear stress, which has been shown to inhibit E-selectin expression in vitro [81, 86]. Levels of sICAM-1, sVCAM-1 and E-selectin have been found to be elevated in other conditions not associated to endothelial dysfunction such as multiple sclerosis [87] and cancer [88, 89]. Interestingly, sICAM-1 and sVCAM-1 levels have been shown to be influenced by other factors such as ethnicity [90] and high-density lipoprotein cholesterol (HDL-C) [91, 92]. The latter down regulates expression of the adhesion molecules and upregulates endothelial cell migration and proliferation [92].

11.5.2 von Willebrand Factor (vWF)

von Willebrand factor (vWF) is a glycoprotein present in blood plasma that plays a role in haemostasis [93]. It is synthesized by endothelial cells [94] as well as megakaryocytes [95]. It is thought to be an excellent blood-based marker for the assessment of endothelial function, with several studies demonstrating a strong inverse correlation between vWF and FMD [96, 97]. Thus, a study conducted by Felmeden et al. [96] in which 89 hypertensive subjects were compared against 43 healthy subjects, before and

after 6 months of cardiovascular risk management, revealed a significant ($r = -0.517$, $P < 0.001$) inverse correlation between vWF and FMD. Following 6 months of antihypertensive treatment and intensive cardiovascular risk factor management, vWF levels decreased with a simultaneous increase in FMD. Moreover, both vWF and FMD were found to correlate significantly with 10-year cardiovascular risk. In contrast, another study in which 32 obese children were compared against 20 healthy controls with regards to measurements of E-selectin, vWF, thrombomodulin, FMD and carotid intima-media thickness (CIMT), no association was found to exist between FMD and plasma endothelial markers [98]. Within the context of HIV infection, a study performed by Seigneur et al. involving 90 HIV-positive subjects versus controls revealed the HIV-positive cohort to have higher levels of vWF, sTM (soluble thrombomodulin), E-selectin, TNF-α and IFN-α compared to the HIV-negative cohort [99]. Levels of vWF correlated positively with TNF-α and IFN-α while sTM correlated negatively with CD4+ cell count.

11.5.3 Thrombomodulin

Similar to E-selectin, thrombomodulin (TM) is a trans-membrane protein specifically expressed on the surface of endothelial cells [100]. However, it differs in its function in that it acts as a protein C co-factor and possesses anti-coagulant activity [100]. Thrombomodulin has been found to exist in a soluble form within the blood circulation, thus, easily quantified by ELISA [101, 102]. Clinical studies revealed a positive association between levels of sTM and endothelial damage, thereby warranting its use as a plasma marker of endothelial damage [101, 103, 104]. However, a positive correlation between plasma levels of sTM with CECs, but not with E-selectin, was observed by Strijbos et al. [105]. On the other hand, a strong negative correlation has been reported to exist between levels of sTM with CD4+ cell count [99]. The authors suggested increased levels of sTM to be a true representative of endothelial dysfunction, as this marker was found to be elevated only in patients with more severe HIV infection, whereas vWF is elevated largely as a result of an inflammatory stimulus [99]. Moreover, results originating from the measurement of sTM to assess endothelial function could be more reliable, as plasma level of sTM is not generally influenced by age. However, women within the age range of menopause are observed to have higher levels of sTM compared to younger women [106].

11.5.4 Markers of Inflammation

C-reactive protein (CRP) is a pentameric protein found in plasma and largely synthesised by hepatocytes in response to acute inflammation [107, 108]. However, expression of CRP in other cells such as neurons, monocytes, lymphocytes and foam cells have been reported, though, most likely at insignificant plasma levels [109, 110]. The

C-reactive protein stimulates phagocytosis by activating the complement pathway, and binds to immunoglobulin receptors, thereby playing a role in innate immunity as a defence mechanism against infections [108]. Expression of CRP is principally induced by the inflammatory cytokine, interleukin-6 (IL-6), which can be enhanced by interleukin-1 (IL-1) [111]. C-reactive protein has gained acknowledgement as an independent marker of inflammation and can be used in the clinical evaluation of endothelial function and CVD risk [112, 113]. The C-reactive protein has been observed to be high in HIV-positive patients, despite long periods of HAART administration [114, 115]. C-reactive protein can decrease endothelial nitric oxide synthase mRNA [7], and upregulate the adhesion molecules, ICAM-1 and VCAM-1, and chemoattractant chemokines such as monocyte chemoattractant protein 1 (MCP-1) [50, 116]. In addition, in vivo and in vitro studies have shown that CRP upregulates angiotensin type 1 receptor in vascular smooth muscle cells, thereby increasing proliferation and migration of these cells, as well as increasing restenosis and the production of reactive oxygen species [117]. These mechanisms would ultimately lead to a decrease in NO levels, and as consequence, endothelial dysfunction.

Interleukin-6 is a soluble protein secreted by a variety of cells such as monocytes, macrophages, fibroblasts, vascular smooth muscle cells, stromal cells and endothelial cells [118, 119]. It acts not only as a pro-inflammatory cytokine, but also has anti-inflammatory effects, mediated by its ability to inhibit TNF-α and IL-1, and activate the IL-1 receptor antagonist (IL-1Ra) and IL-10 [118]. Interleukin-6 activates the NF-κB pathway, resulting in the expression of ICAM-1, VCAM-1 and E-selectin, and inhibits NO production by downregulating eNOS expression [120]. Interleukin-6 also plays a role in the development of atherosclerotic plaques. Thus, after migration of monocytes across the endothelium, these cells develop into foam cells as they absorb modified lipoproteins, and secrete IL-6 and TNF-α. Both of these cytokines further mobilize immune cells, leading to the progressive development of the atherosclerotic plaque [121].

Interleukin-8 (IL-8) is a cytokine produced not only by macrophages, but also by other types of cells, such as epithelial, smooth muscle and endothelial cells [119, 122]. Interleukin-8 also plays a role in atherogenesis, however, to a much lesser extent when compared to IL-6 [119].

Tumour necrosis factor alpha (TNF-α), is a trans-membrane cell signalling protein primarily expressed by activated macrophages in response to acute systemic inflammation [123]. During inflammation, TNF-α and IL-1 are released by the macrophages in the inflamed tissue, which in turn induces overexpression of the adhesion molecules, VCAM-1, ICAM-1 and E-selectin [124, 125]. In a study performed in HIV-infected subjects, TNF-α was found to correlate strongly with levels of vWF [99].

11.5.5 Circulating Cells

Studies conducted by da Silva et al. [51] demonstrated FMD and the systemic levels of EPCs to be lower, and EMPs to be higher, in HIV-infected ARV-naïve individuals when compared to healthy controls. A report from López et al. revealed the numbers

of EPCs to be significantly lower, and CACs to be significantly higher, in HIV-positive ARV-naïve subjects versus controls [126]. The authors hypothesized that direct infection of EPCs by HIV could account for the reduced levels of EPCs in HIV-positive patients, as these cells may possess the chemokine receptors (CCR5 and CXCR4) that are used by HIV to infiltrate host cells [127]. This is evident by the observation that administration of HAART to HIV-positive patients fully restores the level of EPCs [128]. This observation was confirmed by Costiniuk et al. [129], who observed reduced levels of EPCs in HIV-infected versus HIV-negative controls, in a study conducted with 30 ARV-naïve HIV-infected men against 36 HIV-uninfected men. However, no correlation was found to exist between EPC levels and CD4+ cell count or viral load [129]. In contrast, a cross-sectional and a longitudinal study conducted with 50 and 66 chronic HIV-infected subjects respectively, in which both cohorts were receiving ARVs, showed EPC levels in HIV-infected subjects to be significantly higher compared to negative controls [128]. Moreover, a correlation was found to exist between EPC numbers and CD4+ cell count, but no association could be seen between EPC numbers or change in EPC numbers over time with CIMT measurements.

Circulating angiogenic cell (CAC) levels and their migration to the site of endothelial damage have been shown to correlate with endothelial function [130, 131]. However, levels of CACs can be influenced by factors such as age [130] and dyslipidemia [132]. To date, no study has been conducted to assess levels of CACs in HIV-infected patients under conditions of continuous viral replication or suppressed viremia.

Studies have revealed a significant positive correlation between levels of CECs with vWF levels [132, 133], and a significant negative correlation ($r = -0.423$, $P = 0.002$) between levels of CECs and FMD measurements. Also, HIV-infected ARV-naïve patients are reported to possess higher EMP levels [51] and CEC levels [126] compared to healthy individuals. These data therefore suggest that HIV infection results in significant endothelial injury. However, to date, there are no studies to show if controlled viremia on administration of HAART reduces levels of EMPs and CECs.

11.5.6 Metabolites of Endothelial Nitric Oxide Synthetase

As previously discussed, the enzyme, eNOS, plays a vital role in maintaining the body's vasculature in a healthy state. Endothelial dysfunction is characterised by a decrease in eNOS with the concomitant decrease in NO and increase in ROS. An increase in ROS results in the increase oxidation of NO to NO_2^-, which is further oxidised to NO_3^-. This increase in NO_3^- and NO_2^-, upon oxidation of NO, can be measured in human biological fluids [134]. Given that NO has a short half-life in blood of about 0.1 s [135], the measurement of the NO metabolites, NO_3^- and NO_2^-, as biomarkers for the assessment of endothelial dysfunction, has been proposed by Lomelí and colleagues [134, 136]. A study conducted on 32 subjects with Marfan

syndrome, a connective tissue disorder, with 35 healthy controls, revealed an inverse correlation to exist between NO_3^- / NO_2^- and NO_2^- with FMD [136]. However, although a significant difference was observed between the two groups with regards to NO_3^- / NO_2^- ($p = 0.002$), NO_2^- ($p = 0.03$) and VCAM-1 ($p = 0.03$), no significant difference was observed between the groups with regards to markers of inflammation IL-6, IL-8 and TNF-α, and a marker of endothelial function, ICAM-1 [136]. In addition, no studies on these molecules have been performed in HIV-positive subjects.

11.6 Conclusions

Although a standard method for the assessment of endothelial function in HIV-positive or HIV-negative subjects is yet to be established, the use of plasma biomarkers, including vWF, VCAM-1 and ICAM-1, remains a promising option. Furthermore, these protein-based biomarkers can easily be adopted for use in point-of-care devices [137, 138]. One such example is the development of an integrated fluorescence correlation spectroscopy point-of-care device by Olson and colleagues [137] that can be used for the measurement of vWF. The advantages of using point-of-care devices include the fact that they are user-friendly, allow for the simultaneous measurement of multiple analytes, they give fast and precise results, are easily transported, do not require specialised equipment and have a low cost of production [138]. Therefore, these methodologies could be more easily sustained in developing countries. However, more research is warranted in this field to reach a consensus on the best biomarker, or a panel of biomarkers, with accurate diagnostic and predictive values, for the clinical assesment of endothelial dysfunction, to monitor the development of CVD in individuals infected with HIV.

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