The role of the HIV-1 Tat protein in acute stroke: more than just a transactivator of transcription?

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Declaration

I, *Kate Elisabeth McMullen*, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Ethics approval

The original study on HIV infection and young stroke was approved in 2010. (HREC REF: 178/2010). The permission to do this research as a sub-study has been approved by the Human Research Ethics Committee of the Faculty of Health Sciences at the University of Cape Town (HREC REF: 086/2017) and by Groote Schuur Hospital.

Please see Appendix A.

Objectivity

Every effort has been made to ensure the highest level of objectivity in the discussion and primary data analyses of this research project.

“We live in a completely interdependent world, which simply means we cannot escape each other. How we respond to AIDS depends, in part, on whether we understand this interdependence. It is not someone else's problem. This is everybody's problem.”

Former U.S President, Bill Clinton

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Abstract

Background: Individuals infected with the human immunodeficiency virus (HIV) are at increased risk of developing ischaemic stroke. The reasons for this are multifactorial, but HIV-associated vasculopathy is a potentially important cause. HIV-induced chronic inflammation may initiate endothelial dysfunction or accelerate vascular injury from other disease processes. Viral proteins such as the transactivator of transcription (Tat) are emerging role-players in HIV disease pathogenesis and have a putative role in HIV-associated endothelial dysfunction. Tat has paracrine pro-inflammatory effects, but its role in HIV-related stroke has not yet been investigated.

Aims: The primary aim of this study was to determine whether specific Tat amino acid variants are associated with ischaemic stroke and biomarkers of inflammation and endothelial dysfunction in a group of HIV-1 subtype-C-infected individuals. In order to do so, I first determined the aetiology of stroke in these participants using clinical, biochemical and neuro-imaging data. A secondary aim of the study was to identify any HIV-related and/or other traditional stroke-related risk factors that might independently or cumulatively increase stroke risk. For comparison, these putative risk factors were also determined in a group of age-matched HIV-infected non-stroke controls. Finally, I aimed to identify any HIV-related factors and/or Tat amino acid variants that might distinguish strokes due to HIV-associated vasculopathy from other mechanisms of stroke.

Methods: A case-control study was performed on 58 Subtype-C HIV-infected individuals with acute ischaemic stroke and 71 HIV-1 Subtype-C-infected non-stroke controls. Clinical, demographic, laboratory and imaging data were used to determined baseline differences between groups and to distinguish different stroke aetiologies. Exon 1 of the HIV-1 Tat protein was sequenced from peripheral blood samples of stroke participants and controls and amino acid variants were identified using viral epidemiology signature pattern analysis. Regression

analyses were used to examine the correlation between residues at signature positions with biomarkers of inflammation and endothelial activation.

Results: Stroke and control groups were mostly young (mean age 33 years) females (62.1% & 71.8%), and of Black African ancestry. The strokes showed a higher prevalence of some traditional cardiovascular risk factors. Individuals with strokes had a higher prevalence of antiretroviral treatment interruption (25.9% vs 0.0%, p= 0.003), lower CD4 nadir (112 *vs* 177.5 cells/μl, p=0.008) and CD4 count (208.5 *vs* 322.5 cells/μl, p=0.012) than controls. Median viral loads were elevated in both strokes and controls (4.58 & 4.13 $\log_{10}$ copies/ml, p=0.28). The most common causes of stroke were HIV-associated vasculopathy (43.1%) and opportunistic infections (22.4%). Two amino acid variants (proline at position 21 and histidine at position 29) were associated with acute ischaemic stroke. These positions were also associated with modulation of plasma interleukin 6 and monocyte chemoattractant protein 1 levels. Threonine at position 58 distinguished strokes due to alternative mechanisms from strokes due to HIV-associated vasculopathy.

Conclusions: Two Tat protein amino acid variants are associated with stroke in HIV. The precise mechanisms by which these associations occur are not known. However, they are likely to be part of a multiple-hit phenomenon in HIV stroke pathogenesis. Tat-mediated inflammation with endothelial dysfunction, HIV disease severity, treatment interruption and conventional cardiovascular risk factors probably all contribute to stroke aetiology. Thus, a multi-modal approach is needed to reduce ischaemic stroke risk in HIV infection.

Acknowledgements

There are a great many people without whom this project would not have been possible.

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To Dr Alan Stanley and Dr Sameera Allie, who recruited and enrolled the participants who formed a part of the original study. This sub-study would not have been possible without the enormous amount of work that they put into the overall project looking into HIV infection and stroke. Specific thanks must go to Dr Alan Stanley, who gave me permission to explore the Tat protein in the context of the original study, and who also performed all of the cytokine assays for the endothelial biomarkers which were used in part of my analysis.

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Lastly, and most importantly, the greatest of thanks must go to the individuals who participated in this study. It was my intention to honour their selfless contributions that kept me motivated throughout this project.

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List of abbreviations used in the text

Abbreviation	Explanation
AA/aa	Amino acids
ADMA	Asymmetric dimethylarginine
AIDS	Acquired immunodeficiency syndrome
AP-1	Activator protein-1
ART	Antiretroviral therapy
BBB	Blood brain barrier
CA	California
CD	Cluster differentiation
CLAT	Crytpococcal latex antigen test
CMV	Cytomegalovirus
CNS	Central nervous system
CREB	cyclic adenosine monophosphate response element-binding protein
DALYs	Disability adjusted life years
DNA	Deoxyribonucleic acid
dNTP's	Deoxynucleotide triphosphates
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetraacetic acid
ET-1	Endothelin-1
HAND	HIV-associated neurocognitive disorders
HIV	Human immunodeficiency virus
HIV-1	HIV type 1
HLA	Human leukocyte antigen
hsCRP	Highly sensitive C-Reactive protein
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
ICAM-1	Intercellular adhesion molecule 1
IgG	Immunoglobulin G
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-1β	Interleukin 1 beta
IRIS	Immune reconstitution inflammatory syndrome
LTR	Long terminal repeat
MAFFT	Multiple alignment using Fast Fourier Transform
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
NADPH	Nicotinamide adenine dinucleotide phosphate
Nef	Negative factor
NF-κB	Nuclear factor-kappa B
NRTIs	Nucleoside reverse transcriptase inhibitors
PCR	Polymerase chain reaction
PKR	Protein kinase RNA-activated

PMNL	Polymorphonuclear leukocyte
Rev	Regulator of expression of virion proteins
RGD	Tripeptide Arg-Gly-Asp
RNA	Ribonucleic acid
SA	South Africa
SIV	Simian immunodeficiency virus
Tat	Transactivator of transcription
TAE	Tris acetate EDTA
TAR	Transactivator response element
Tm	Primer melting temperature
TNF-α	Tumour necrosis factor alpha
TOAST	Trial of Org 10172 in Acute Stroke Treatment
UK	United Kingdom
USA	United States of America
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
Vpr	Viral protein R
VZV	Varicella Zoster virus
WI	Wisconsin

CHAPTER ONE: INTRODUCTION

There are currently 36.7 million people living with the human immunodeficiency virus (HIV) worldwide. In 2016, one million individuals succumbed to Acquired Immunodeficiency Syndrome (AIDS)-related illnesses. As a result of the increasing effectiveness and availability of antiretroviral therapy, AIDS-related deaths have fallen by 48% since their zenith in 2005 (UNAIDS, 2017). However, the advent of antiretroviral therapy (ART) has changed the natural progression of the disease. As the HIV-infected population ages, and the incidence of AIDS-defining conditions declines, non-communicable diseases, such as HIV-associated cardiovascular disease, have emerged as new challenges.

HIV-infected individuals are at increased risk of cardiovascular disease and ischaemic stroke in comparison to the HIV-uninfected population. The pathogenesis of stroke in HIV is multi-factorial, and stroke aetiology has been associated with traditional cardiovascular risk factors, opportunistic infections, cardio-thromboembolism and coagulopathies. In recent years, HIV has been observed to be an independent risk factor for stroke. The chronic persistent inflammatory state seen in most HIV-infected individuals may play an important role in endothelial dysfunction and the development of ischaemic stroke.

HIV-associated vasculopathy is a term used to describe endothelial damage and dysfunction caused directly or indirectly by HIV (Benjamin et al., 2012). HIV-induced inflammation disrupts the physiology of normal endothelium, increasing the risk of vessel thrombosis and occlusion. HIV-associated vasculopathy is not yet well-understood, but HIV type 1 (HIV-1) proteins are postulated to contribute to the chronic persistent inflammation that results in endothelial dysfunction (Kline & Sutliff, 2008; Benjamin et al., 2012; Pillay, Ramdial & Naidoo, 2015).

Research into various HIV-1 proteins has revealed that each may have unique and synergistic mechanisms of promoting disease pathogenesis (Kline & Sutliff, 2008). Furthermore, the secretion of some HIV-1 proteins is less affected by antiretroviral therapy (Annunziata, 2003; Mediouni et al., 2012), which may account for the persistently elevated risk of cardiovascular disease in effectively treated individuals. Work on the Viral protein R (Vpr) and Transactivator of Transcription (Tat) proteins in HIV-associated neurocognitive disorders (HAND) has highlighted that viral proteins may have a prominent role in HIV-associated disease pathogenesis (Tilghman et al., 2014; Dampier et al., 2017).

Viral protein toxicity is a putative mechanism for the direct action of HIV on vascular endothelium. The Tat protein has multiple extra-cellular effects which could contribute to HIV-associated vasculopathy. Induction of pro-inflammatory molecules, upregulation of arterial wall remodelling, and promotion of oxidative stress are all mechanisms which may disturb the physiology of normal endothelium and alter stroke risk. Amino acid mutations in this protein could enhance these functions and alter the susceptibility of certain individuals to cardiovascular disease and stroke. There is a need to explore the possible contribution of the Tat protein to the inflammation and endothelial dysfunction seen with HIV infection.

South Africa (SA) is an ideal context in which to investigate the stroke risk posed by the virus itself. The largest burden of HIV is borne by individuals aged 15-49 (UNAIDS, 2016), most of whom have a relative paucity of conventional cardiovascular risk factors. Just over 50% of HIV-infected individuals in SA are on antiretroviral treatment (UNAIDS, 2016), which allows for the study of individuals who are either treatment-naïve, recently initiated, or well-established on treatment. The most prevalent HIV-1 subtype in the population is Subtype-C, which also accounts for 52% of HIV infections globally (Ariën, Vanham & Arts, 2007), making this research potentially relevant to many HIV-infected individuals worldwide.

Following recent work demonstrating that certain amino acid variations in Vpr and Tat distinguish cognitively-impaired and normal participants in HAND (Tilghman et al., 2014; Dampier et al., 2017), I decided to determine the amino acid profile of the Tat protein in young HIV-infected individuals with acute ischaemic stroke and to compare it with the Tat protein sequences found in HIV-infected non-stroke controls. At the time of writing, I believe that this is the first study examining the Tat protein in the context of acute ischaemic stroke.

I undertook a case-control study of young HIV-infected individuals with acute ischaemic stroke, comparing them with HIV-infected non-stroke controls. My intention was to analyse both the clinical and virological characteristics of this cohort, in order to further the understanding of endothelial dysfunction in HIV, and the multiple factors that may determine the progression of endothelial dysfunction to the critical threshold of thrombosis and occlusion. The primary focus of this study was to examine the genetic attributes of the Tat protein in this cohort, to see if there were amino acid differences that distinguished the stroke group from the controls, as well as to determine the effect of any distinguishing amino acids on pro-inflammatory biomarkers measured in these individuals.

CHAPTER TWO: BACKGROUND AND RATIONALE FOR STUDY

2.1 Introduction to the rationale for the study

This chapter provides an overview of the relationship between ischaemic stroke and HIV infection. It explains the current understanding of the pathogenesis and role of HIV-related endothelial dysfunction and HIV-associated vasculopathy in HIV-associated young stroke. The importance of biomarkers of inflammation and endothelial activation as a reflection of endothelial dysfunction is introduced, as well as the profile of these biomarkers in HIV infection. The specific focus of this chapter is the potential role of the Transactivator of Transcription protein in promotion of endothelial dysfunction, and I cover the mechanisms by which the Tat protein could contribute to elevation of specific biomarkers, endothelial injury, and stroke. Furthermore, I look at genetic variation in HIV, specifically the pathogenic effects of amino acid variations in HIV-1 proteins. I note also that this research took place in the South African context, which is the epicentre of the HIV epidemic, and has a high prevalence of HIV-1 Subtype C.

2.2 Ischaemic Stroke

2.2.1 Epidemiology of stroke worldwide and in South Africa

Stroke is a global health problem. It is the second leading cause of death and the third leading cause of Disability-Adjusted Life Years (DALYs) worldwide (Murray et al., 2012). It is also a growing problem in South Africa. Stroke was the second leading cause of natural death in SA in 2014, and in 2008, contributed 564 000 DALYs to the country's burden of disease (Bertram et al., 2013; Statistics South Africa, 2015). Ischaemic stroke accounts for the majority of the stroke burden. In recent years, ischaemic strokes comprised 54%-85%

and 73%-90% of all recorded strokes in developing and high-income countries respectively (Feigin et al., 2009).

2.2.2 Pathogenesis of ischaemic stroke

Ischaemic stroke results from thrombo-embolic occlusion of extra- and intra-cranial arteries supplying the brain. Embolic occlusion includes that from a cardiac source, or artery-to-artery embolism from a diseased or dissected artery. Risk factors for ischaemic stroke initiate a process that leads to injury and dysfunction of vascular wall endothelium. This culminates in thrombosis and occlusion of vessels. Intact and healthy endothelium maintains vascular integrity and homeostasis, exerting major protective effects by regulating vasoconstriction, fibrinolysis, vasodilation, smooth muscle proliferation, and thrombogenesis (Bonetti, 2003).

2.2.3 Endothelial dysfunction in stroke

The impairment of endothelial function often results from physical shear stress, damage by reactive oxygen species and exposure to pro-inflammatory cytokines (Poggesi et al., 2016). Consequently, the endothelium can no longer balance “endothelium-derived relaxing factors”, which promote vasodilation and prevent thrombus formation (Furchgott & Zawadzki, 1980; Flammer & Lüscher, 2010), and “endothelium-derived contracting factors” (Luscher et al., 1992), which have the opposite effect. Endothelial dysfunction results in ischaemia and stroke when the imbalance reaches a threshold that produces pathological vasoconstriction, thrombosis and occlusion.

2.2.4 Traditional cardiovascular risk factors in stroke pathogenesis

In HIV-uninfected individuals, traditional cardiovascular risk factors, such as hypertension, diabetes, dyslipidaemia, smoking and obesity are pro-atherogenic, and the principal contributors to ischaemic stroke (Sacco et al., 2006; O'Donnell et al., 2016). The primary mechanisms for stroke in the presence of these risk factors are inflammation and oxidative stress. Endothelial dysfunction results from the initiation and maintenance of chronic inflammatory processes and production of reactive oxygen species (Libby, 2002; Widlansky et al., 2003). Traditional cardiovascular risk factors promote high levels of free radicals and other oxidising molecules. This causes structural damage to cellular components, alters secondary messenger systems and activates various transcription factors. Oxidative stress also depletes nitric oxide, which is crucial for vasodilation, downregulates adhesion molecule expression, and inhibits platelet aggregation and leukocyte adhesion to the vascular wall (Cai & Harrison, 2000; Li, Horke & Förstermann, 2014). The most common stroke subtypes in individuals with these risk factors are large artery atherosclerosis and small vessel occlusion (Chung et al., 2014).

2.3 HIV infection and stroke

2.3.1 Epidemiology of HIV infection in South Africa

South Africa is the epicentre of the global HIV epidemic. The number of infected individuals in the country constitutes 19% of the total number of people with HIV worldwide. 15% of new infections and 11% of all AIDS-related deaths occur in SA (UNAIDS, 2016).

HIV-related mortality and morbidity are a significant burden to the health care system in South Africa. In 2014, HIV infection was the fifth leading cause of natural death in SA (Statistics South Africa, 2015).

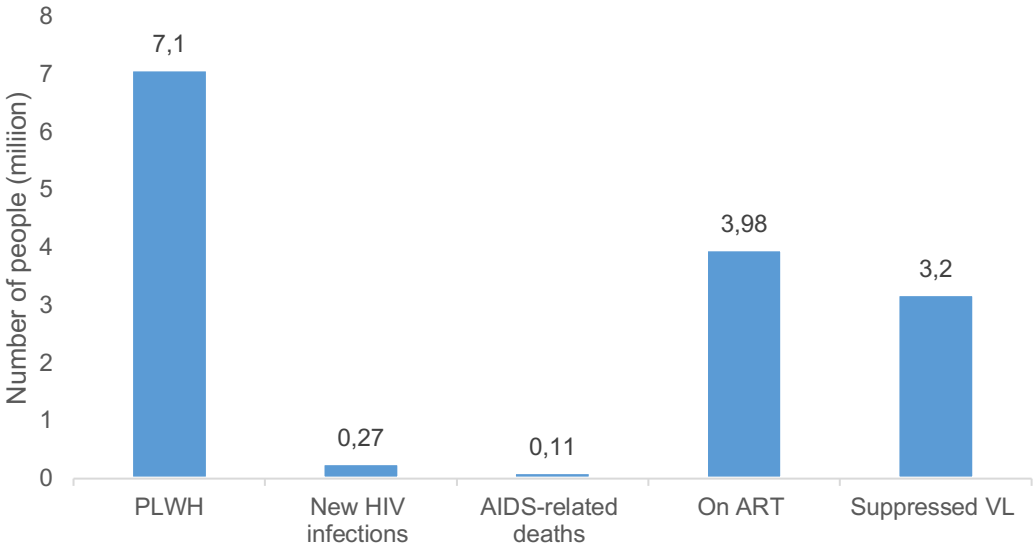


Figure 2.1. Epidemiology of HIV infection in South Africa. Statistics adapted from *(UNAIDS, 2016)*. Abbreviations: PLWH, people living with HIV; ART, antiretroviral therapy; VL, viral load

2.3.2 Indicators of HIV disease severity

Measures of the severity of HIV disease include the peripheral blood cluster of differentiation 4 (CD4) T-lymphocyte count and the HIV-1 ribonucleic acid (RNA) viral load. The CD4 count is a measure of the stage and progression of disease. The viral load quantifies the concentration of peripheral blood HIV-1 RNA, in copies/millilitre (ml). Viral load provides an estimation of the degree of systemic viral replication in addition to demonstrating the effectiveness of antiretroviral therapy.

2.3.3 The link between HIV and stroke

SA is in the midst of a transition between health problems common to developing countries and those of the developed world. Two components of the quadruple disease burden unique to SA are infectious disease, including HIV, and non-communicable disease, which includes stroke (Mayosi et al., 2012; South African Department of Health, 2014). Research has demonstrated a connection between HIV and an increased risk of cardiovascular disease

(CVD) (Islam et al., in press; Tripathi et al., 2014). Furthermore, there is a growing body of evidence showing an association between HIV and stroke.

Stroke in HIV-infected individuals was initially reported in the 1980s (Anders et al., 1985). Studies from the both the pre- and post-antiretroviral era demonstrate that HIV-infected individuals have an increased risk of stroke, especially ischaemic stroke, in comparison with HIV-uninfected controls (Engstrom, Lowenstein & Bredesen, 1989; Qureshi et al., 1997; Cole, 2003; Ovbiagele & Nath, 2011; Rasmussen et al., 2011; Chow et al., 2012; Mateen et al., 2013; Sico et al., 2015). Incidence-rate estimates (1.25 vs 0.74; 2.79 vs 2.24 and 5.27 vs 3.75 per 1000 person years), are consistently higher in HIV-infected persons compared to HIV-uninfected individuals (Chow et al., 2012; Marcus et al., 2014; Sico et al., 2015), with the same studies reporting hazard ratios for stroke of 1.17 and 1.40.

With improved availability of ART, the prevalence of cardiovascular conditions associated with advanced HIV disease, such as dilated cardiomyopathy, has declined. However, the relative prevalence of stroke has increased (Subsai et al., 2006; Ovbiagele & Nath, 2011; Feinstein et al., 2016). Some authors estimate that the rate of stroke in people well-established on ART may be similar to the pre-ART era (Arentzen et al., 2015).

Stroke and central nervous system (CNS) complications are a significant and complex problem in people living with HIV (Power et al., 2012; McArthur & Smith, 2013; Bhatia & Chow, 2016). Cardiovascular events, including cerebrovascular disease, pose the greatest risk of mortality to people on ART (Reisler et al., 2003). Consequently, much research in recent years has focused on the risk factors and aetiology of stroke in HIV. Development of effective adjunctive treatment which complements antiretroviral therapy may prove essential to combat the cardiovascular and neurological disease burden seen in HIV-infected individuals.

2.3.4 Aetiology and pathogenesis of stroke in HIV infection

The pathogenesis of ischaemic stroke in HIV-infected people, in the absence of easily identifiable causes such as meningitis and cardiac disease, is complex and multifactorial. Ischaemic stroke in HIV is likely due to the synergistic effect of multiple factors. Research into HIV and stroke, including prior research on individuals from this cohort, suggests that continual or cumulative exposure to a variety of risk factors may cause progression of endothelial dysfunction to a threshold at which thrombosis and occlusion occurs (Maggi, Ingrassia & D'Annunzio, 2008; Allie, 2013). The overview of the predominant risk factors presented below highlights a common underlying mechanism: inflammation.

2.3.4.1 Contribution of HIV to the metabolic syndrome

Untreated HIV infection is associated with adverse metabolic profiles such as dyslipidaemia and impaired glucose metabolism. In treated individuals, the metabolic effects of HIV, in combination with traditional risk factors and certain antiretroviral agents, may promote metabolic syndrome, which in turn increases the risk of stroke (El-Sadr et al., 2005; Alberti et al., 2009; Krishnan et al., 2012; Willig & Overton, 2016; Non, Escota & Powderly, 2017).

2.3.4.2 Antiretroviral therapy and stroke risk

In the past, antiretroviral therapy was associated with metabolic side effects and increased risk of cardiovascular disease (The Data Collection on Adverse Events of Anti-HIV Drugs (DAD) Study Group, 2003; Venter & Sanne, 2003). However, newer evidence suggests that this risk may be confined to older protease inhibitors, and is not increased in those on newer nucleoside reverse transcriptase inhibitors (NRTIs) such as Tenofovir and Lamivudine (Worm et al., 2010; Eholié et al., 2015). Furthermore, a specific association between modern ART and stroke is less well-proven than the association of ART with cardiovascular disease as a whole (Chow et al., 2012; Vinikoor et al., 2013).

2.3.4.3 Traditional cardiovascular risk factors and the concept of accelerated atherosclerosis in HIV-associated stroke

In the developed world, the increased incidence of HIV-associated stroke may partly be explained by an ageing population of HIV-infected individuals, in whom there is a high prevalence of cardiovascular risk factors (Escárcega et al., 2014). In higher income countries, hypertension, dyslipidaemia, smoking, diabetes and substance use are common in HIV-infected populations (Triant et al., 2007; Corral et al., 2009). Researchers in developed countries find that the aetiology of HIV-associated stroke more frequently mirrors that of ischaemic stroke in the general population: an adverse conventional cardiovascular risk profile resulting in atherosclerotic vasculopathy. In an American cohort of 2515 HIV-infected individuals, two-thirds of those with stroke were over the age of 40. Atherosclerosis and small vessel disease accounted for 77% of ischaemic strokes (Vinikoor et al., 2013).

However, whilst traditional cardiovascular risk factors do indeed play a role, their contribution may be less prominent than that of HIV itself. The Framingham Risk Score for Stroke (Framingham Heart Study, 2017), which uses classical cardiovascular risk factors to predict the 10-year risk of stroke in asymptomatic individuals, has been shown to underestimate the stroke risk in HIV-infected people (Mateen et al., 2013). Even after adjustment for traditional risk factors, HIV-infected individuals have an increased rate of CVD compared to those without HIV infection (Triant et al., 2007; Chow et al., 2012). Furthermore, many young people with HIV develop stroke in the absence of hypertension, hyperlipidaemia, smoking, diabetes and concomitant CVD (Connor, 2007; Ortiz et al., 2007). The Data-collection on Adverse Effects of Anti-HIV Drugs (D:A:D) study has more recently developed a model which includes CD4 count and ART exposure, thus improving model prediction compared to more conventional risk scores (Friis-Møller et al., 2016). This suggests that additional mechanisms other than traditional cardiovascular risk factors play a more prominent role in the pathogenesis of HIV-related stroke.

Increasing recognition of the role of the virus is evident in recent reviews of HIV-associated cardiovascular disease. There is an acknowledgement that whilst traditional risk factors are important, HIV itself contributes significantly to stroke and other cardiovascular diseases (Escárcega et al., 2014; Feinstein et al., 2016). A “two-step hypothesis” has been proposed for cardiovascular disease in HIV. The first step involves various degrees of inflammation initiated by HIV itself, which then progresses to a second step of accelerated atherosclerosis, from the synergistic effect of more classical and well-recognised risk factors, such as hypertension, smoking and diabetes (Maggi, Ingrassia & D’Annunzio, 2008).

2.3.4.4 HIV as an independent risk factor for stroke

HIV is now recognised as an independent risk factor for stroke. This association may be even stronger for HIV-infected individuals under the age of 45 years, in whom conventional cardiovascular risk factors are less prominent (Engstrom, Lowenstein & Bredesen, 1989; Chow et al., 2012; Benjamin, Corbett, et al., 2016). More advanced HIV disease, with low CD4 counts and high viral loads, is positively correlated with risk of incident CVD and stroke (Siedner, in press; The Data Collection on Adverse Events of Anti-HIV Drugs (DAD) Study Group, 2003; Lichtenstein et al., 2010; Chow et al., 2012; Sico et al., 2015). In developing countries, well-recognised causes of HIV-associated stroke in young people are coagulopathy, opportunistic infections and cardio-embolism, the mechanisms for which are fairly well understood. However, HIV-associated vasculopathy is emerging as an important stroke phenotype in HIV, and there is still much to be explored with regards to its aetiology and pathogenesis (Benjamin et al., 2012).

2.3.5 HIV-associated vasculopathy

In HIV-infected individuals with stroke, HIV-associated vasculopathy is a term used to define “any abnormality” of cerebral vasculature that can be attributed “directly or indirectly to HIV” (Connor, 2007; Benjamin et al., 2012). It has recently been defined to encompass a range of phenotypes, including accelerated atherosclerotic vasculopathy, HIV-associated vasculitis, small vessel disease and non-atherosclerotic vasculopathy. Notably, HIV-associated vasculopathy occurs in the absence of vasculitis due to opportunistic infections. The various phenotypes can be diagnosed with a combination of imaging, serology, cerebrospinal fluid (CSF) analysis and histopathological examination of brain tissue, once other important known causes of stroke have been excluded (Benjamin et al., 2012, 2017; Benjamin, Bryer, et al., 2016).

2.3.5.1 Histopathology of HIV-associated vasculopathy

HIV-associated vasculopathy affects extra- and intra-cranial vessels of all sizes, producing a range of pathological changes. One of the best-characterised is aneurysmal enlargement and occlusion of large- and medium-sized vessels, which may be accompanied by cerebral infarction (Chetty, Batitang & Nair, 2000; Chetty, 2001).

Other documented pathological changes include leucocytoclastic vasculitis of the vasa vasorum and peri-adventitial vessels, stenosis and thrombotic occlusion, elastic lamina fragmentation, neovascularisation and accelerated atherosclerosis (Benjamin et al., 2012).

Small vessel changes, thought to be asymptomatic, have been characterized on autopsy of HIV-infected individuals. Histopathological features include cerebral micro-infarcts, hyaline wall thickening of small vessels, dilatation of perivascular spaces, pigment deposition with vessel wall mineralization and perivascular inflammatory cell infiltrates (Connor et al., 2000).

2.3.5.2 Proposed mechanisms for HIV-associated vasculopathy

The pathogenesis of HIV-associated vasculopathy is thought to be related to virus-induced endothelial damage and dysfunction, which contributes to the pathogenesis of thrombosis and occlusion. Figure 2.2 illustrates the proposed mechanisms for pathological changes seen in HIV-associated vasculopathy. These processes induce a pro-inflammatory state that disrupts the physiology of normal endothelium.

In HIV infection, the vascular endothelium is subjected to direct and indirect injury from the virus and its sequelae. Mechanisms of direct injury include exposure to virions and toxic viral proteins, pro-inflammatory cytokines, and infected HIV-tropic cells such as monocytes, macrophages, and CD4+ T-lymphocytes. This culminates in chronic persistent inflammation, similar to that propagated by conventional cardiovascular risk factors. Activation of endothelium increases permeability and permits monocyte invasion. There is "production of reactive oxygen species, expression of cell adhesion molecules (CAMs), and…release of chemoattractants" (Kline & Sutliff, 2008; Benjamin et al., 2012) The severity of HIV infection has been associated with a greater degree of endothelial dysfunction (Maggi, Ingrassia & D'Annunzio, 2008). Poorly controlled viral replication, signified by a high viral load or low CD4 count, would likely enhance the exposure of vascular endothelium to direct damage from viral particles.

Indirect mechanisms maintain this inflammatory state. Circulating infected leucocytes and monocytes continually release pro-inflammatory molecules. Trapped sub-endothelial monocytes release cytokines and chemokines. Another source of inflammation could be the immune reconstitution inflammatory syndrome (IRIS), after the introduction of antiretroviral therapy.

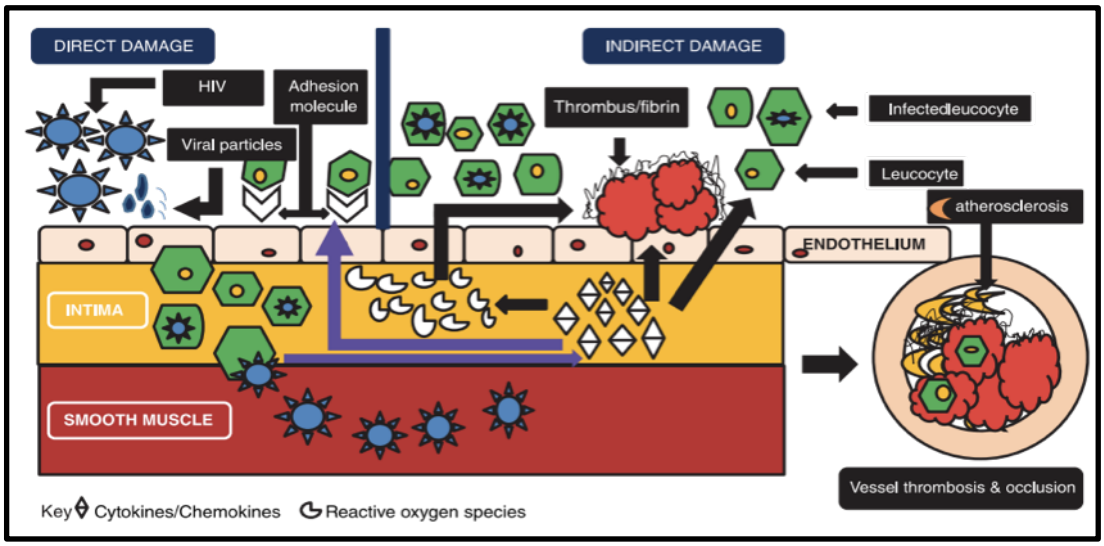


Figure 2.2. Direct and indirect damage from HIV, leading to endothelial dysfunction, thrombosis and occlusion.(Benjamin et al., 2012; Pillay, Ramdial & Naidoo, 2015)

The combination of direct and indirect mechanisms results in persistent inflammation. This produces a cycle of endothelial activation and remodelling, promoting thrombosis and occlusion (Monsuez et al., 2009; Benjamin et al., 2012; Paiardini & Müller-Trutwin, 2013; Pillay, Ramdial & Naidoo, 2015).

2.4 Biomarkers of inflammation and endothelial dysfunction

2.4.1 Biomarkers as a measure of inflammation and endothelial damage

Measurement of biomarkers in the peripheral blood of individuals provides an indication of the degree of inflammation, endothelial dysfunction and vessel injury. The severity of dysfunction is positively associated with the risk of a cardiovascular event (Widlansky et al., 2003). Levels of pro-inflammatory and endothelial activation biomarkers in people with HIV may therefore indicate the extent of endothelial dysfunction present at the time of sampling.

2.4.2 Biomarkers of inflammation and endothelial dysfunction in HIV

Biomarkers of endothelial activation, dysfunction, inflammation and haemostasis are persistently elevated in people with HIV. This would suggest that all HIV-infected individuals are subject to a spectrum of endothelial dysfunction, which could range from sub-clinical changes to overt vessel wall disease.

These biomarkers include highly sensitive C-reactive protein (hsCRP), D-dimers, interleukin-6 (IL-6), serum CD163, asymmetric dimethylarginine, monocyte chemoattractant protein 1 (MCP-1), adhesion molecules, and soluble CD14 (Coll et al., 2006; Melendez et al., 2008; Neuhaus et al., 2010; Burdo et al., 2011; Armah et al., 2012; Baker et al., 2012; Graham et al., 2013; Wada et al., 2015). These international studies showing persistent elevation of pro-inflammatory and endothelial activation biomarkers reflect the chronic pro-inflammatory milieu that is characteristic of HIV infection. The African and South-African context is no different. High levels of inflammatory biomarkers have been detected in both treated and treatment-naive African and South-African HIV-infected individuals (Fourie et al., 2011, 2015; Graham et al., 2013).

Chronic inflammation with persistent elevation of biomarkers likely contributes to HIV-associated vascular disease. Elevated IL-6, for example, is independently associated with an increased risk of cardiovascular disease and stroke in HIV-infected individuals (Duprez et al., 2012; Tenorio et al., 2014). IL-6 and tumour necrosis factor-alpha (TNF-α) enhances transcription and replication of HIV (Breen, 2002). This creates a positive-feedback mechanism that increases endothelial exposure to viral stimuli.

2.4.3 Inflammation and endothelial dysfunction in the antiretroviral era

HIV disease severity is positively correlated with biomarkers of endothelial dysfunction and inflammation (De Larrañaga et al., 2003). However, complete

normalisation of various biomarkers does not occur with initiation of antiretroviral therapy (Torriani et al., 2008; Arildsen et al., 2013; Paiardini & Müller-Trutwin, 2013; O'Halloran et al., 2015).

In fact, systemic inflammation, immune activation and vascular injury persist, albeit at lower levels, in virally-suppressed HIV-infected individuals (French et al., 2009; d'Ettorre et al., 2016; Longenecker, Sullivan & Baker, 2016). Whilst ART does reduce cardiovascular risk in treated individuals, compared with those who are treatment-naïve, an elevated CVD risk remains (The Strategies for Management of Antiretroviral Therapy (SMART) Study Group, 2006; Corral et al., 2009; Chow et al., 2012).

This suggests that endothelial damage and inflammation in HIV is not completely dependent on active viral replication. This is where the role of viral proteins may come to the fore (Kline & Sutliff, 2008; Pillay, Ramdial & Naidoo, 2015). Whilst ART suppresses active viral replication, it may not significantly reduce viral protein secretion. Thus, continual exposure of vascular endothelium to toxic viral proteins may be a plausible explanation for the increased cardiovascular risk seen in both treated and untreated individuals (Annunziata, 2003; Mediouni et al., 2012).

2.5 HIV-1 proteins and endothelial dysfunction

It is currently unclear whether endothelial cells can be directly or productively infected by HIV (Bissel & Wiley, 2004). However, secreted HIV-1 proteins interact with endothelium, promoting endothelial damage, dysfunction and remodelling (Kanmogne, Kennedy & Grammas, 2002; Mu et al., 2007; Kline & Sutliff, 2008). Several HIV-1 proteins have been implicated in endothelial injury. Of particular interest are the regulatory and accessory proteins, such as the Tat protein, Negative factor (Nef) and Viral protein R (Kline & Sutliff, 2008; Wang et al., 2015).

Although all three proteins have plausible mechanisms for promoting endothelial injury, the activation of inflammatory pathways in endothelial cells has been primarily attributed to the effects of the Tat protein (Mu et al., 2007).

2.5.1 Classification and function of HIV-1 proteins

The HIV-1 genome consists of nine genes, encoding fifteen viral proteins. Structural proteins that make up the virion core and envelope are encoded by gag. The pol proteins provide the enzymatic functions necessary for reverse transcription, integration and proteolytic processing. The remaining six proteins are classified as regulatory or accessory, depending on their individual functions (Frankel & Young, 1998).

Table 2.1. Functions of HIV-1 proteins (adapted from *(Wilson et al., 2013)*

	Gene	Protein	Description/Function
Structural	*gag*	MA	Matrix protein
		CA	Core capsid protein
		NC	Nucleocapsid protein
	env	SU (gp120)	Surface glycoprotein
		TM (gp 41)	Transmembrane protein
Viral enzymes	*pol*	Protease	Virus maturation
		Reverse Transcriptase	Reverse transcribes viral RNA
		Integrase	Integrates proviral DNA into host DNA
Regulatory & Accessory	*tat*	Tat	Transactivator of HIV gene expression
	rev	Rev	Expression of structural proteins
	vif	Vif	Virion maturation & infectivity
	vpr	Vpr	Inhibits cell division & promotes nuclear localization
	vpu	Vpu	Extracellular release of viral particles
	nef	Nef	Down-regulates CD4 & MHC Class I receptors, increases viral infectivity

2.6 The HIV-1 Transactivator of Transcription Protein

2.6.1 Introduction to the Transactivator of Transcription protein

I chose to focus on the Tat protein as a potentially important contributor to endothelial dysfunction in HIV. This is due to its critical role in the proliferative capacity of the virus, as well as its paracrine pro-inflammatory effects that have been demonstrated *in vitro*. The paracrine effects on vascular endothelium *in vivo* may work synergistically with various risk factors to produce clinical effects such as ischaemia.

2.6.2 Structure and primary function of the Tat protein

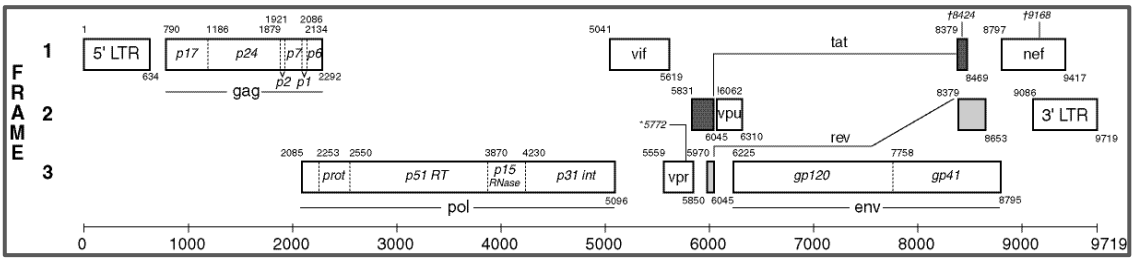


Figure 2.3. The HIV-1 Genome Map. The tat exon 1, position 5831-6045, is highlighted in charcoal grey, on the second reading frame. The tat exon 2 is also in charcoal grey, on the first reading frame (http://www.hiv.lanl.gov).

The Tat protein initiates and promotes full elongation of HIV-1 RNA transcripts. The protein is an 86 to 101 amino acid (aa) polypeptide encoded by two exons. The first exon is a 72-aa peptide that is necessary for transactivation (Caputo et al., 1995; Chang, Gallo & Ensoli, 1995). The second exon, containing a tripeptide Arginine-Glycine-Aspartic acid (RGD)-motif, confers structural stability and supports interaction with transcriptional factors. It may support improved binding of the Tat protein to endothelial cells and other cell types, and contribute to attenuation of the innate immune response to the virus (Brake, Debouck & Biesecker, 1990; Jeang, Xiao & Rich, 1999; Lopez-Huertas et al., 2010; Kukkonen et al., 2014).

In the absence of Tat, the 5' long terminal repeat (LTR) is a "defective promoter" (Karn, 2000). Premature termination of transcript elongation is common. Without Tat, many nascent RNA transcripts terminate within the first 500 nucleotides (Marciniak & Sharp, 1991). Tat is one of the first proteins to be expressed by infected cells, and binds to the transactivator response element (TAR) at the 5' end of new HIV-1 RNA transcripts (Rana & Jeang, 1999; Bagashev & Sawaya, 2013). Binding of Tat to TAR then recruits several transcription factors. Cooperation with these factors stimulates initiation and full elongation of transcripts by RNA polymerase II. During early transcription elongation, Tat abrogates promoter-proximal pausing of RNA-polymerase II and dramatically enhances synthesis of full-length RNA transcripts. This effectively upregulates the rate of HIV replication (Feng & Holland, 1988; Rana & Jeang, 1999; Zhou & Rana, 2002; Campbell & Loret, 2009; Reeder et al., 2015).

2.6.3 Functional domains of the HIV-1 Tat protein

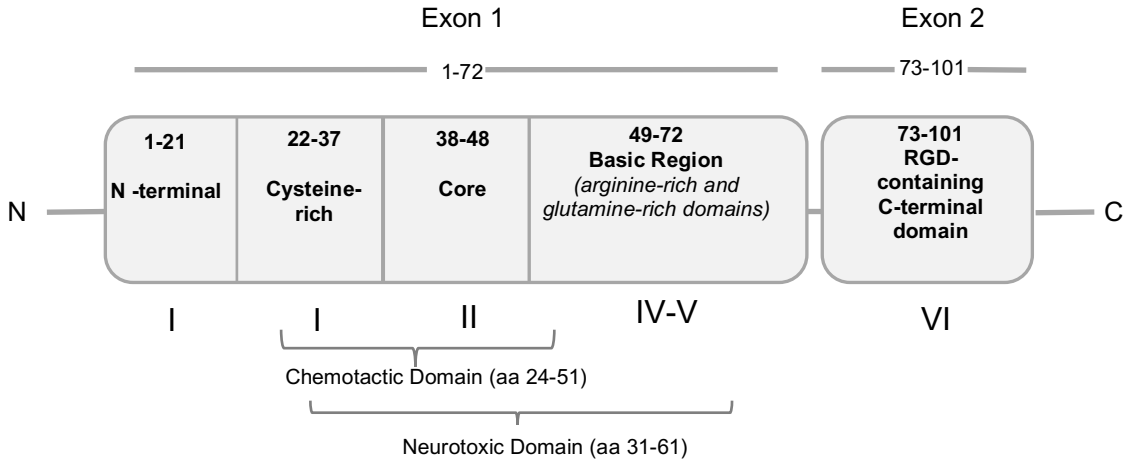


Figure 2.4. Functional domains of the Tat protein. Adapted from (Cowley et al., 2011; Roy et al., 2015; BioAfrica, 2017)

The amino acid sequence encoded by exon 1 has several functional domains, as depicted in Figure 2.4. Domain I (aa 1-21) is the N-terminal, which is a proline-rich, acidic region. Domains II (aa 22-37) and III (aa 38-48) are the cysteine-rich and core regions respectively. Domain IV (aa 49-57) and Domain V (aa 58-72), are the arginine- and glutamine-rich domains. Domains IV and

V (aa 49-72) are together considered to be the basic region (Jeang, Xiao & Rich, 1999; Li et al., 2012; Roy et al., 2015). Possibly due to nomenclature, the border between domains IV and V is unclear in the literature. Some papers cite Domain IV as residing between amino acid residues 49 and 57 (Demarchi, Gutierrez & Giacca, 1999; Mitola et al., 2000; Shojania et al., 2010; Roy et al., 2015), whilst others extend Domain IV to include residues 58 and 59 (Rana & Jeang, 1999; Grégoire et al., 2001; Strebel, 2003; Campbell et al., 2004; Campbell & Loret, 2009; Cowley et al., 2011; Musinova et al., 2016).

There are two additional regions thought to be primarily responsible for Tat's chemoattractant and neurotoxic properties, referred to as the chemotactic (aa 24-51) and neurotoxic (aa 31-61) domains respectively. In 1996, Nath *et al.* determined that a peptide fragment, containing aa 31-61 of Tat, was cytotoxic to human neurons, causing activation of excitatory amino acid receptors, intracellular influx of calcium (Ca^{2+}) and neuronal apoptosis (Nath et al., 1996). In 1998, Albini *et al.* identified that Tat residues 24-51 had potent chemotactic activity, proposing that this was the predominant region with which Tat mediated chemoattraction (Albini, Benelli, et al., 1998; Albini, Ferrini, et al., 1998).

Table 2.2. Functional domains of the Tat protein exon 1

	Residues	Description	Proposed Functions
Domain I	1-21	N-terminal Acidic, proline-rich Highly conserved sequence	• Contributes to initiation of transcription (Siderovski et al., 1992) • Mediates binding to cellular response element binding protein (CREB), CBP/p300 complex, which cooperates with Tat to activate transcription factors (Deng et al., 2000; Bagashev & Sawaya, 2013; Davey et al., 2014) • May suppress antigen-induced activation of T-lymphocytes (Mitola et al., 2000)
Domain II	22-37	Cysteine-rich	• Dimerization of Tat (Frankel et al. 1988) • Works synergistically with basic region to form stable homodimers mediating chemoattraction and migration of lymphocytes (Urbinati et al., 2009) • Supports transactivation of LTR (Jeang, Xiao & Rich, 1999)
Domain III	38-48	Core	• Mitogen-activated protein kinases (MAPK) & protein kinase R (PKR) activation in combination with basic region (Rusnati et al., 2001; Bagashev & Sawaya, 2013) • Stabilises Tat-TAR binding (Luo & Peterlin, 1993)

Domain IV and Domain V	48-72	Basic Arginine-rich region with RKKRRQRRR motif Glutamine-rich region (subject to highest degree of variability) (Jeang, Xiao & Rich, 1999)	• TAR RNA binding & interaction (Loret et al., 1992) • Nuclear localization signal (NLS) (Musinova et al., 2016) • Uptake of Tat by cells (Jeang, Xiao & Rich, 1999; Li et al., 2009) • Activation of nuclear factor kappa B (NF-κB) (Demarchi, Gutierrez & Giacca, 1999) • Induction of TNF-α (Philippon et al., 1994) • Upregulation of E-selectin (Cota-Gomez et al., 2002) • Angiogenic (Albini et al., 1996; Barillari et al., 1999; Vene et al., 2001) • Dendritic cell chemotaxis (Vene et al., 2001)
Chemotactic Domain	24-51	Mediates most chemoattractant functions (Cowley et al., 2011)	• Majority of Tat's effects on monocytes (Albini, Benelli, et al., 1998) • Mimics β-chemokines (Albini, Ferrini, et al., 1998) • Induction of IL-8 and vascular endothelial growth factor (VEGF) release from polymorphonuclear lymphocytes (PMNLs) (Benelli et al., 2000)
Neurotoxic Domain	31-61	Region primarily responsible for neurotoxic properties (Nath et al., 1996; Kruman, Nath & Mattson, 1998)	• Neuronal cytotoxicity via excitatory amino acid receptor activation & alteration of Ca^{2+} levels (Nath et al., 1996) • Neuronal oxidative stress, caspase activation & apoptosis (Kruman, Nath & Mattson, 1998)

2.6.4 The potential contribution of the Tat protein to endothelial dysfunction

2.6.4.1 Introduction to mechanisms by which Tat may promote endothelial dysfunction

HIV-associated endothelial dysfunction results from multiple, simultaneous pathogenic processes (see Figure 2.2). These include cytokine and chemokine release, upregulation of adhesion molecules, increased oxidative stress, alteration of vascular wall structure and integrity, and an imbalance between vasorelaxant and vasoconstricting factors.

The Tat protein's numerous non-transcriptional activities may contribute to all of these processes. The Tat protein, secreted from infected cells, has paracrine effects on bystander infected and uninfected cells (Ensoli et al., 1993). Endothelial cells are no exception. The Tat protein can cause a variety of pathogenic changes in endothelial cells, including endothelial cell apoptosis (Lafrenie et al., 1996; Park et al., 2001; Ma et al., 2016). Within the host cell, and independent of viral replication, Tat upregulates gap-junction expression. Pro-inflammatory molecules can then diffuse from infected cells to uninfected neighbouring cells (Berman et al., 2016). Once secreted, the Tat protein can cross cell membranes and modulate the expression of cellular genes and signalling pathways involved in inflammation (Toborek et al., 2005; Cota-Gomez et al., 2011; Musinova et al., 2016).

2.6.4.2 Expression of pro-inflammatory mediators

Tat is directly involved in the increased expression of pro-inflammatory molecules via NF-κB-dependent mechanisms (Nath et al., 1999; Weiss et al., 1999; Bennasser et al., 2002; Cota-Gomez et al., 2002; Lee et al., 2004; Chauhan et al., 2007; Ben Haij et al., 2015).

NF-κB is a potent inducer of TNF-α, interleukin-1 (IL-1), IL-6, MCP-1 and CD40. (Lafrenie et al., 1997; Park et al., 2001; Sui et al., 2007; Gresele et al., 2012) CD40 has a positive feedback effect on NF-κB, sustaining the upregulated response (Sui et al., 2007). MCP-1 induces recruitment and migration of monocytes across endothelial cells (Weiss et al., 1999) and mediates activation of CD16+ monocytes. CD16+ monocytes may be pro-atherogenic and an indicator of premature cardiovascular disease in younger HIV-infected patients (Parihar, Eubank & Doseff, 2010; Baker et al., 2014). In addition, Tat can directly activate interleukin-10 (IL-10) and stimulate VEGF receptors (Benelli et al., 2000; Gee et al., 2012).

2.6.4.3 Promotion of oxidative stress

The Tat protein promotes oxidative stress via several mechanisms. It represses manganese superoxide dismutase, increases nicotinamide adenine dinucleotide phosphate (NADPH) oxidation, upregulates various inflammatory molecules and promotes calcium influx into the cytoplasm (Kruman, Nath & Mattson, 1998; Kline & Sutliff, 2008; Cota-Gomez et al., 2011).

2.6.4.4 Upregulation of adhesion molecules

In endothelial cells, the Tat protein upregulates expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (Dhawan et al., 1997; Liu et al., 2005). It also induces E-selectin via a NF-κB-dependent mechanism (Cota-Gomez et al., 2002).

2.6.4.5 Impact on vascular tone and vessel wall remodelling

The Tat protein directly impacts vascular tone by decreasing the expression of endothelial nitric oxide synthase and upregulating expression of endothelin-1 (ET-1), a powerful vasoconstrictor (Paladugu et al., 2003; Chauhan et al., 2007). This imbalance between nitric oxide (the primary mediator of vasodilation) and ET-1, promotes vasoconstriction, which increases the risk of platelet aggregation and thrombosis. Additionally, in synergy with shear stress, the Tat protein may promote endothelial arterial wall remodelling (Parker et al., 2014).

2.6.4.6 The effect of Tat on cerebral vessels

HIV-infected individuals exhibit decreased cerebrovascular reactivity in comparison to HIV-uninfected controls (Chow et al., 2015). Tat, possibly via its effects on NO and ET-1, has been shown to decrease cerebrovascular reactivity, which can increase the risk of cerebral ischaemia (Blaser et al., 2002; Carrera et al., 2010; Silva et al., 2012). Tat also alters tight junction protein expression, increasing the permeability of the blood brain barrier (BBB) (András et al., 2003). Disruption of brain microvascular endothelial cells can activate platelets, promote thrombogenesis, and trigger cerebrovascular events (Yu et al., 2015).

Table 2.3. Biomarkers & proposed mechanisms of Tat-induced upregulation

Biomarker	Function	Proposed Mechanism of Upregulation by Tat
Interleukin 1 beta (IL-1β)	• Pyrogenic & pro-inflammatory • Proliferation & differentiation of immune cells • Induction of MAPKs and NF-κB (MAPKs phosphorylate and activate transcription factors for pro-inflammatory genes) and NF-κB (Turner et al., 2014) • Transcription of pro-inflammatory cytokines TNF-α, IL-6 & neutrophil recruiting chemokines • Increases BBB permeability & leukocyte invasion (Yang, Wu & Lu, 2010)	• Induces IL-1β independently of TNF-α, likely via NFκB (Nath et al., 1999) • Phospholipase C (PLC) / protein kinase C (PKC)-dependent regulation of MAPKs (Yang, Wu & Lu, 2010)
MCP-1	• Macrophage chemotaxis across vascular endothelium (Weiss et al., 1999) • Activation of pro-atherogenic CD16+ monocytes (Parihar, Eubank & Doseff, 2010; Baker et al., 2014)	• Likely via NFκB and Activator protein-1 (AP1) /MAPK pathway (Weiss et al., 1999; Toborek et al., 2002) • Initiates activation of the MAPK pathway and tyrosine kinase, stimulating production of TNF-α and then MCP-1 (Lee et al., 2011)

Endothelin-1	• Principal endothelium-derived contracting factor (Virdis, Ghiadoni & Taddei, 2010) • Potent vasoconstrictor (National Center for Biotechnology Information, 2017a) • ET_A receptor inhibition improves endothelial dysfunction and reduces atheroma formation (Barton et al., 1998)	• Regulates transcription of ET-1 via activation of NF-κB-responsive sites in ET-1 promoter (Chauhan et al., 2007)
IL-6	• Synthesis of acute phase proteins in liver • Leucocyte trafficking • T-cell activation, B-cell differentiation & antibody production (Turner et al., 2014)	• NFκB and/or MAPK pathway (Nookala & Kumar, 2014; Ben Haij et al., 2015) • Basic region of Tat needed for interaction with IL-6 promotor region (Ambrosino et al., 1997)
IL-10	• Inhibits cytokine production & mono-nuclear cell function • Anti-inflammatory (Turner et al., 2014)	• Tat induces IL-10, which is regulated by CREB-1 and specificity protein 1 (Sp-1) transcription factors, through the activation of MAPK (Gee et al., 2012) • Tat induces IL-1o via NF-κB, but activates NF-κB via two pathways: MAPK and PKC-dependent, involving CREB (Leghmari, Bennasser & Bahraoui, 2008) • TNF-α independent (Leghmari et al., 2008)

TNF-α	• Stimulates innate immune system: cytokine production, expression of adhesion molecules, phagocyte activation (Turner et al., 2014)	• N-terminal domain 1-55 essential for TNF-α production • Downstream activation of NFκB (Bennasser et al., 2002; Kiebala et al., 2010)
VEGF	• Increases vascular permeability • Angiogenesis • Chemoattractant (National Center for Biotechnology Information, 2017b)	• Interacts with VEGF receptors (Avraham et al., 2004) • Induces VEGF in PMNLs via chemoattractant region (Benelli et al, 2000)
E-selectin	• Leucocyte migration, tethering & rolling • Leucocyte cell-cell adhesion • Heterophilic cell-cell adhesion (National Center for Biotechnology Information, 2017c)	• NF-κB dependent mechanism via basic domain (Dhawan et al., 1997; Cota-Gomez et al., 2002)
V-CAM-1	• Leukocyte-endothelial cell adhesion & signal transduction • May play role in atherosclerosis development (National Center for Biotechnology Information, 2017d)	• NF-κB promotor and MAPK (Dhawan et al., 1997; Liu et al., 2005)
I-CAM-1	• Integrin binding • Leucocyte migration & adhesion • May be involved in atherosclerosis (National Center for Biotechnology Information, 2017e)	• NFκB-promoter and MAPK (Dhawan et al., 1997; Duan et al., 2013)

2.6.5 The Tat protein in the central nervous system

2.6.5.1 Entry of HIV into the CNS

HIV enters the CNS early in the course of infection, as a result of migration of infected monocytes and lymphocytes across the blood brain barrier. The brain then becomes a significant reservoir for the virus. Subsequent infection of neighbouring cells, predominantly perivascular macrophages and microglia, results in viral replication in the CNS (Bissel & Wiley, 2004; Yadav & Collman, 2009).

2.6.5.2 CNS escape

The sequestration of HIV-infected cells in the CNS is an important step in HIV-related morbidity. CNS inflammation and CSF viraemia can persist in individuals with a suppressed peripheral viral load. This phenomenon of “CNS escape” (Yilmaz et al., 2008; Canestri et al., 2010; Peluso et al., 2012), means that CNS viral replication, and hence viral protein production, may continue in individuals on effective antiretroviral therapy, who have suppressed peripheral viral loads.

2.6.5.3 Secretion of the Tat protein in the CNS

The secretion of Tat from infected CNS cells may not be suppressed by current antiretroviral therapy. The presence of ART does not eliminate Tat secretion (Mediouni et al., 2012; Bachani et al., 2013; Mousseau, Mediouni & Valente, 2015), and Tat is present in the brain and CSF of individuals with undetectable peripheral blood HIV-1 RNA (Johnson et al., 2013). Furthermore, the Tat protein may still be present in the CNS of an estimated 30-40% of patients in whom viral replication is suppressed in the CSF (Johnson & Nath, 2014). This may be explained by the fact that transcription or translation of some of the

regulatory and accessory genes, such as *tat*, can still take place in cells with non-productive infection (Bissel & Wiley, 2004). Concentrations of Tat needed to promote extra-cellular or non-transcriptional effects are much lower than those needed to transactivate the virus (Ensoli et al., 1993). The bystander effects of the Tat protein may therefore only need cellular hosts that are infected, but not necessarily producing new viral transcripts (Berman et al., 2016).

2.6.5.4 Tat-induced CNS IRIS

The presence of Tat in the CNS, even at low concentrations, may also provoke ischaemia via a unique form of IRIS. Recovery of the immune system after the initiation of antiretroviral therapy often provokes an inflammatory reaction to foreign antigens, commonly those from opportunistic infections. Johnson and Nath postulate that a reconstituted immune system may also respond to residual HIV in the CNS, either acutely, or in patients who have been established on ART for several years. Specifically, it may be high concentrations of the Tat protein that stimulate an inflammatory response. This response has several CNS manifestations, including ischaemia (Johnson et al., 2013; Johnson & Nath, 2014).

2.6.5.5 Risk to extra- and intra-cranial vessels

The non-transcriptional effects of the Tat protein on vascular endothelium could theoretically occur wherever the Tat protein is secreted by infected cells. The Tat protein freely crosses the blood brain barrier (Banks, Robinson & Nath, 2005). It is therefore conceivable that the presence of Tat in intracranial vessels could be either due to Tat secreted by infected cells in peripheral blood, or release of Tat from infected cells in the CNS. If the Tat protein is persistently released from the CNS, independent of peripheral viral replication, then it is possible that intra- and extra-cranial vessels may be at particular risk of endothelial injury.

Thus, the Tat protein has the potential to contribute to vascular injury, and the progression to stroke in both untreated and effectively-treated individuals.

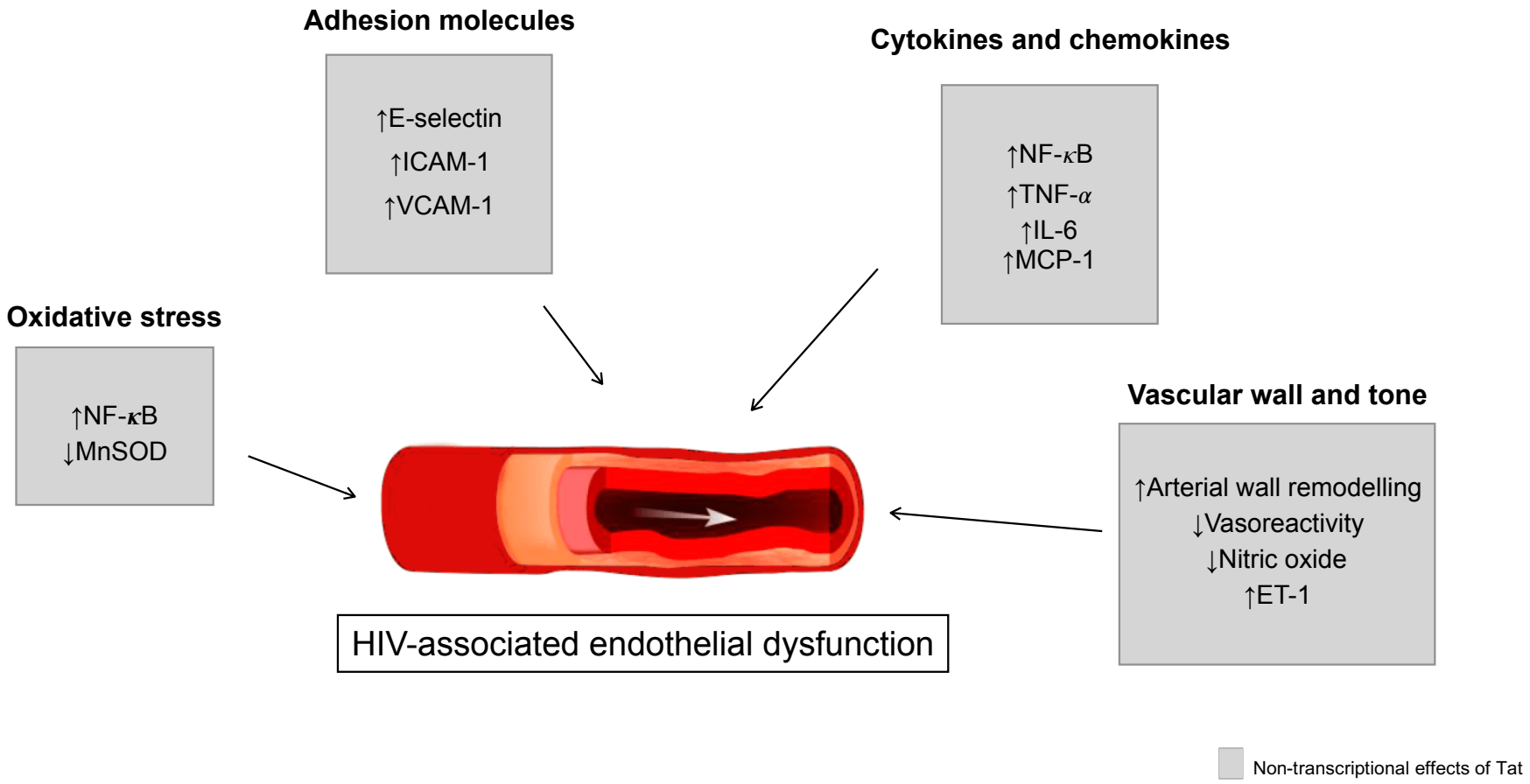


Figure 2.5. Potential mechanisms by which the Tat protein could contribute to HIV-associated endothelial dysfunction

2.7 HIV-1 genetic variability in disease pathogenesis

2.7.1 HIV-1 groups and subtypes

HIV-1 is divided into four genetically distinct groups. Group M (major) is responsible for the current HIV pandemic, whereas Groups N to P are found in selected regions. HIV-1 Group M is comprised of nine different subtypes or clades, with multiple circulating recombinant forms (CRFs) (Hemelaar, 2012). HIV-1 is genetically highly diverse. There may be as much as 25-35% genetic variation between subtypes. Even within subtypes, viral isolates may exhibit up to 20% variation (Santoro & Perno, 2013).

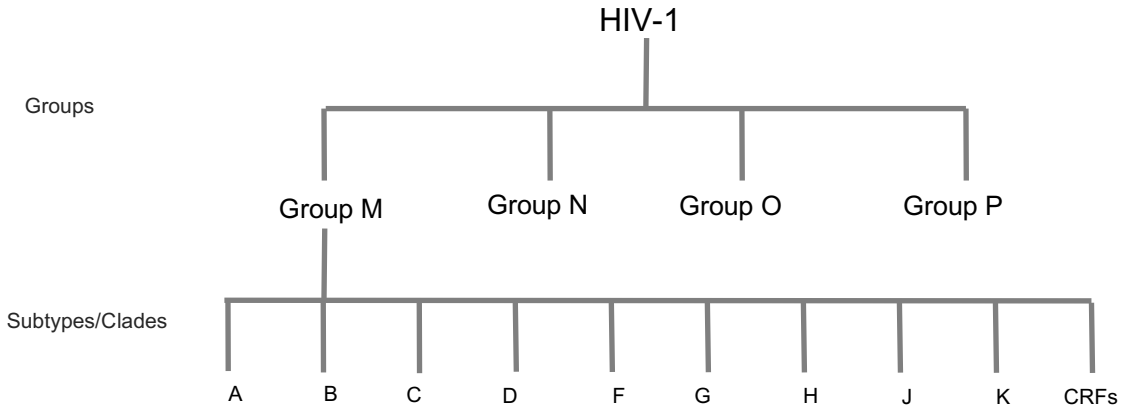


Figure 2.6. HIV-1 groups and subtypes. Adapted from (Hemelaar, 2012)

Subtype-C accounts for around 52% of all HIV infections worldwide, and predominates in sub-Saharan Africa (Ariën, Vanham & Arts, 2007). However, much of HIV-1 sequence research has historically been focused on HIV-1 Subtype-B (see Figure 2.7), which is most prevalent in Europe and North America (Bagashev & Sawaya, 2013; Santoro & Perno, 2013).

The pathogenesis of stroke in populations infected with the subtype that is specific to 52% of HIV-infected individuals needs further exploration, especially in a clinical, *in vivo* context.

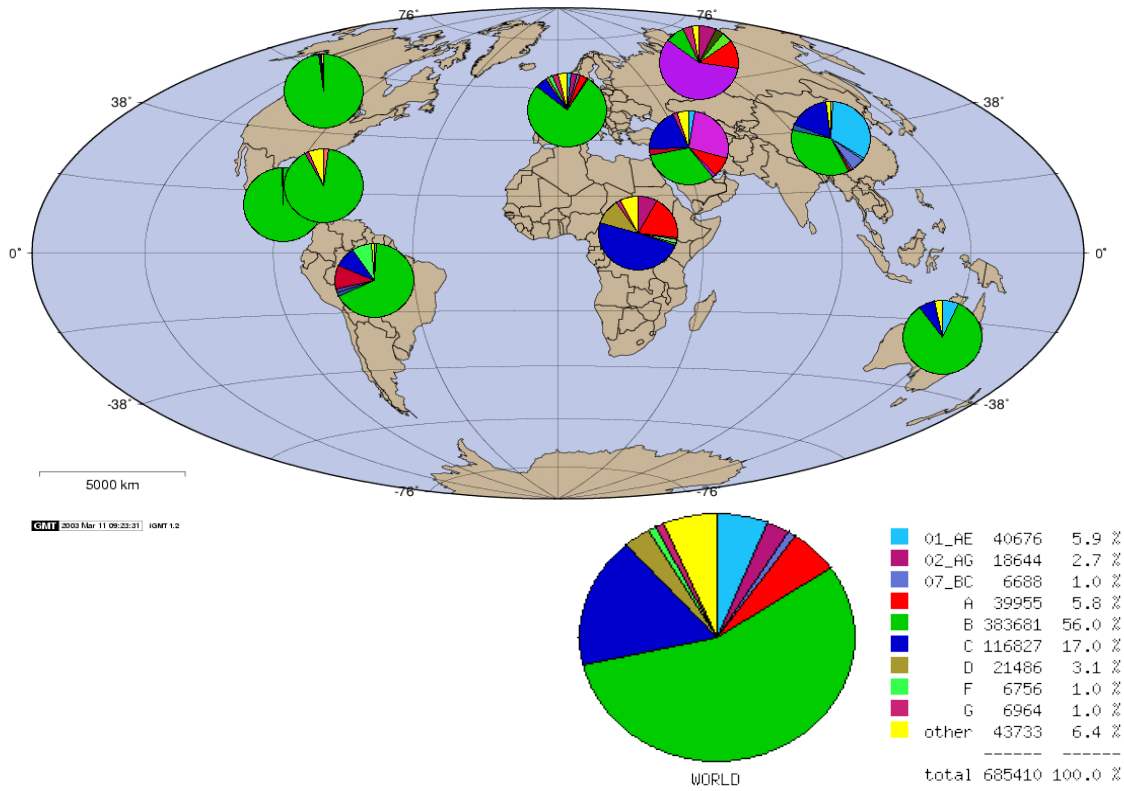


Figure 2.7. Global distribution of all HIV sequences obtained from research and uploaded to the HIV Sequence Database. The pie-chart reflects the large amount of research done on HIV-1 Subtype B in comparison to other subtypes. Image available from www.hiv.lanl.gov

2.7.2 Viral mutation in HIV-associated disease

Genetic variability and amino acid substitutions are considered to be an important mechanism for disease pathogenesis (Ng & Henikoff, 2006). Therefore, variation in the Tat protein may also attenuate or enhance its effects on vascular endothelium.

2.7.2.1 Mechanisms of viral mutation

The considerable genetic variation seen in HIV is partly due to a high replication rate, coupled with the notoriously error-prone reverse transcription enzyme. In addition, template-switching occurs between the two RNA strands

during reverse transcription. HIV-1 genetic mutations impact on disease progression, viral transmission and antiretroviral resistance patterns (Pant Pai, Shivkumar & Cajas, 2012; Santoro & Perno, 2013; Lloyd, Kent & Winnall, 2014; Palm et al., 2014).

Genetic variation is also influenced by selection pressure. Selection pressure for HIV-1 is determined by the environment in which the virus is present. Factors such as host-specific immunity and antiretroviral therapy will influence HIV's ability to replicate. Changes in amino acids will therefore assist or hinder the viral protein's ability to survive the environmental pressures imposed upon it. Variations in the amino acid sequence of a protein can be synonymous, meaning that they do not change the primary structure of a protein, or non-synonymous, in that the substitution alters the primary structure, and potentially the function of the protein. If a non-synonymous mutation (dN) at a particular site does not really affect the function and fitness of a viral protein, then the rate of non-synonymous compared to synonymous substitutions (dS) remains fairly balanced ($dS \approx dN$), and selection is neutral. However, if a non-synonymous substitution confers an evolutionary advantage, and improves viral fitness, then the rate of non-synonymous substitutions will increase relative to the rate of synonymous substitutions ($dN > dS$). This is the process of positive selection. Conversely, if the non-synonymous mutation is deleterious to viral function or fitness, then the rate of synonymous substitutions will exceed that of non-synonymous substitutions ($dS > dN$), indicating that negative selection is underway (Poon, Frost & Pond, n.d.).

2.7.2.2 Viral protein mutation in disease pathogenesis

Recent evidence suggests that sequence variations in HIV regulatory and accessory proteins may have a role to play in disease pathogenesis. Functional differences in regulatory or accessory proteins may also be subtype-specific. For example, the functional ability of Nef to downregulate CD4 and human leukocyte antigen (HLA) Class 1 receptors differs between subtypes A, B, C and D (Mann et al., 2013).

2.7.2.3 Viral protein mutation in HIV-associated neurocognitive disorders

Research into HIV-associated neurocognitive disorders (HAND) has shown that genetic diversity and variable expression of HIV-1 proteins may cause injury to, and loss of, neural cells, leading to clinically detectable neurological disease. Dual infection with different HIV-1 strains, resulting in increased viral diversity, has been associated with HAND, and may impact on neuropathogenesis (Wagner et al., 2016). On autopsy, specific viral polymorphisms can distinguish people with and without HAND (Power et al., 1994, 1998; Smit et al., 2001). Recent work on the HIV-1 viral protein R has shown that certain amino acids are associated with significant differences in neurocognitive performance in HIV-infected persons (Dampier et al., 2017). These findings in HAND suggest that viral diversity and sequence variations within HIV-1 proteins may influence disease risk.

2.7.2.4 Variation of the HIV-1 Tat protein in disease pathogenesis

The Tat protein itself is continually evolving, often due to host selection pressure (Allen et al., 2000). These multiple amino acid substitutions can be subtype-specific, and impact on functionality (Roy et al., 2015). The effect of Tat variants has been researched in different clinical contexts, but not in ischaemic stroke.

Mutations may affect the Tat protein's cytoplasmic concentration and release (Ensoli et al., 1993), as well as its neurotoxic and chemotactic properties in the CNS (Cowley et al., 2011). Functional differences also exist between subtypes. In one study, Subtype C (Tat-C) and Subtype-E (Tat-E) Tat proteins were more powerful transactivators, in comparison with Tat Subtype B (Tat-B) (Desfosses et al., 2005). Studies comparing Tat-B to a CRF showed that only Tat-B upregulated certain matrix metalloproteinases, as well as pro-inflammatory chemokines and components of complement (Woollard et al., 2014; Bhargavan & Kanmogne, 2017). Tat-B is also thought to be more neurotoxic than Tat-C isolates from India (Mishra et al., 2008).

There is still much to be clarified around the pathogenic potential of variations within Tat-C, especially in Sub-Saharan Africa. Southern-African Subtype-C is thought to be more neuro-virulent and have the potential to cause greater chemotaxis and neurocognitive impairment than Subtype-C isolates in South-East Asia, for example (Rao et al., 2013).

Despite *in vitro* evidence that the Tat protein has pro-inflammatory properties, with the potential to disrupt vascular endothelium, most of the exploration into Tat variants has been in relation to HAND and neuronal apoptosis. It is yet to be clarified whether there are any signature amino acid variations that are associated with stroke, or upregulation of endothelial biomarkers. Thus, sequencing and describing the Tat protein in our cohort of South-African participants may provide key insights into the pathogenesis of HIV-associated stroke in Tat-C infected individuals, as well as contributing to the knowledge-base for development of effective therapies to reduce the incidence of cerebrovascular disease in HIV.

2.8 Summary of the rationale for the study on the Tat protein in stroke

Ischaemic stroke and HIV contribute significantly to the disease burden in South Africa. HIV-infected individuals, treated and untreated, are at increased risk of ischaemic stroke compared to the HIV-uninfected population.

The pathogenesis of ischaemic stroke involves occlusion of vessels supplying the brain, either via embolism or in-situ thrombus development. Thrombotic vessel occlusion may occur when endothelial dysfunction reaches a critical threshold. Although the cause of ischaemic stroke in HIV is multifactorial, HIV itself is an independent risk factor, and the severity of HIV disease is positively correlated with endothelial dysfunction, a pro-thrombotic state, and stroke risk. In younger individuals, it may be the most important contributor to stroke pathogenesis.

Causes of stroke in HIV include opportunistic infections, cardio-embolism, coagulopathy and HIV-associated vasculopathy, which is thought to be a result of HIV-induced endothelial dysfunction.

Viral particles and proteins, infected monocytes and leucocytes, cytokine upregulation and immune reconstitution inflammatory syndrome are all postulated to contribute to the inflammation that drives HIV-associated endothelial dysfunction.

The HIV-1 Tat protein has numerous extracellular effects which could damage vascular endothelium. Secretion of the Tat protein can occur independently of viral replication and in the presence of antiretrovirals. Additionally, its paracrine effects occur at lower concentrations than those needed to transactivate the virus. These properties make it an ideal candidate for promoting HIV-associated endothelial dysfunction and stroke in both treated and ART-naïve individuals. Furthermore, amino acid variations in the Tat protein may influence disease progression and pathogenesis. Although the apoptotic properties of Tat have been explored in relation to HAND, there is a need for exploration into the pro-inflammatory and chemoattractant properties of Tat in the context of ischaemic stroke.

The Tat protein is one of multiple factors that may contribute to progression of endothelial dysfunction in HIV. The description of its genetic attributes and its effect on biomarkers of inflammation and endothelial dysfunction could further clarify stroke pathogenesis in HIV-infected individuals.

CHAPTER THREE: PLANNING THE RESEARCH

3.1 Research question

Do variations in the HIV-1 Tat protein have a role in HIV-associated endothelial dysfunction and stroke?

3.2 Hypotheses

3.2.1 There are significant differences in traditional cerebrovascular risk factors between young HIV-infected individuals with and without acute ischaemic stroke. Whilst HIV may be the single most important risk factor for stroke in individuals <45 years, traditional cerebrovascular risk factors may have a synergistic effect on stroke risk in HIV-infected individuals.

3.2.2 Recent CD4 count, CD4 nadir and viral load are significantly different between young HIV-infected individuals with acute ischaemic stroke, and those without acute ischaemic stroke. Low CD4 count and elevated viral load are indicators of immunocompromise, immune dysregulation and increased viral replication. These factors increase inflammation and therefore could influence endothelial dysfunction.

3.2.3 There are differences in Tat protein sequences between HIV-infected individuals with acute ischaemic stroke and non-stroke controls.

3.2.4 There are differences in Tat protein sequences between individuals with strokes due to HIV-associated vasculopathy and strokes due to alternative mechanisms.

3.2.5 Certain positions and amino acids in the Tat protein are specifically associated with biomarkers of endothelial activation and inflammation.

3.3 Aims and objectives

3.3.1 To compare the baseline demographic characteristics, cardiovascular risk factors and HIV-related factors between an HIV-infected young stroke group and HIV-infected non-stroke controls.

3.3.2 To describe the characteristics of acute ischaemic stroke in young HIV-infected individuals, in order to better understand the pathogenesis of young stroke in a South African population infected with HIV-1 Subtype C.

- 3.3.2.1 To describe the clinical phenotype, severity and aetiology of acute ischaemic stroke in young South African HIV-1 Subtype-C infected individuals
- 3.3.2.2 To separate the stroke group into two sub-groups: individuals with strokes due to HIV-associated vasculopathy, and individuals with strokes due to alternative mechanisms
- 3.3.2.3 To compare the CD4 count, treatment status and viral load between the sub-groups of individuals with acute ischaemic stroke

3.3.3 To describe and compare the amino acid composition of Subtype-C Tat exon 1 sequences between HIV-infected individuals with acute ischaemic stroke, and HIV-infected individuals without stroke.

- 3.3.3.1 To sequence the HIV-1 Tat exon 1 in HIV-infected individuals with and without stroke
- 3.3.3.2 To visualise and compare amino acid composition and variability between the stroke and control groups
- 3.3.3.3 To identify signature sites in the amino acid alignments that are distinctly representative of the stroke group relative to the control group
- 3.3.3.4 To investigate selection pressure on the stroke and control groups

3.3.4 To describe and compare the amino acid composition of HIV-1 Subtype-C Tat exon 1 sequences in HIV-infected individuals with strokes of differing aetiology

3.3.4.1 To separate the stroke group Tat protein sequences into two sub-groups: sequences representing strokes due to HIV-associated vasculopathy, and sequences representing strokes due to alternative mechanisms

3.3.4.2 To visualise and compare amino acid composition and variability between the strokes of differing aetiology

3.3.4.3 To identify signature amino acid differences between the two groups

3.3.5 To determine whether the signature residues from Tat exon 1 detected in this cohort are associated with markers of inflammation and endothelial dysfunction. These markers are: IL-1β, IL-6, IL-10, TNF-α, MCP-1, VEGF, endothelin-1, E-selectin, ICAM-1 and VCAM-1.

CHAPTER FOUR: METHODOLOGY

4.1 Introduction to methodology used in this study

This chapter describes the study design, sample size and study participants. I describe the study setting, recruitment of participants, modified inclusion and exclusion criteria, and final sample size obtained for the project. I describe the published case definitions used to classify stroke aetiology in the HIV-infected stroke group. I then explain the laboratory methods I used to sequence the *tat* exon 1: proviral DNA extraction, polymerase chain reaction (PCR), and Sanger sequencing. Details are provided about the bioinformatics software tools I used to sequence and analyse the *tat* exon 1 from the cohort's blood samples. This chapter also details the conventional statistical methods used to compare baseline characteristics between the groups, analyse the significance of the signature pattern analyses, and correlate the endothelial biomarkers with specific residues in *tat* exon 1.

4.2 Study design

This was a case-control study of individuals who were originally prospectively recruited for a larger study on HIV infection and stroke.

4.3 Study participants

This study was conducted as a sub-study of a larger research project entitled 'Stroke and HIV-infection: a study of markers of endothelial dysfunction and ultrasonographic vascular phenotypes'. This research, which commenced in 2010, aimed to investigate the association between HIV and stroke in young adults. This study enrolled a cohort of HIV-infected individuals presenting with acute ischaemic stroke between the ages of 18 and 45 years, who were then characterized clinically, biochemically, and radiologically in order to determine,

as accurately as possible, their stroke aetiology and to investigate mechanisms by which HIV confers stroke risk. It also had two control groups: HIV-infected non-stroke controls and HIV-uninfected acute stroke patients, all between the ages of 18 to 45 years, who then underwent the same data collection process.

4.3.1 Study setting

The stroke group was recruited from a tertiary hospital and its affiliated secondary hospitals in Cape Town, South Africa. Groote Schuur Hospital is one of two central hospitals in the Western Cape province, and serves the central metropole, as well as the southern and some of the western sub-districts. The HIV-infected non-stroke control group was recruited from two community health centres in Cape Town. Community health centres serve people in their local area. The two areas included Gugulethu, and Crossroads.

The Cape Town metropole is the second most populous urban area in South Africa. When the study participants were enrolled, the city had with an estimated population of 3.7 million people. In 2011, the unemployment rate was 23.9%. Informal dwellings comprised 21.6% of all households (Statistics South Africa, 2011a). The HIV-1 prevalence in the Cape Town metropole was 5.2% in 2012, and in 2011, HIV and cerebrovascular disease were the metropole's 4th and 5th leading causes of natural death, respectively (Shisana et al., 2014; Statistics South Africa, 2014).

4.3.2 Recruitment of the study cohort

For the original study, HIV-infected and uninfected stroke patients were recruited from Groote Schuur Hospital in Cape Town and its affiliated secondary-level hospitals in the surrounding area. Recruitment and enrolment of the HIV-infected and uninfected stroke patients took place between 1st August 2010 and 30th June 2013. These were individuals between the ages of 18 and 45 years, who presented with acute ischaemic stroke to the Stroke Service at Groote Schuur Hospital. Participants were enrolled at Groote

Schuur Hospital during the patient's admission, within 5 to 7 days of stroke onset. HIV-infected individuals without acute stroke were recruited as controls during the same time period from two community health centres in the Cape Town area. As far as possible the controls were matched for age, sex, and antiretroviral status (treated or untreated). Written informed consent was obtained from all participants, or their closest relative, if cognition or level of consciousness were impaired. The consent form for the original study, in English and isiXhosa, can be found in Appendix B.

To minimize the influence of conventional age-associated risk factors for stroke, the participants were all between 18 and 45 years of age.

4.3.3 Inclusion and exclusion criteria for this sub-study

This sub-study includes two of the three original groups: the HIV-infected stroke group, and HIV-infected non-stroke controls. Inclusion and exclusion criteria for these two groups were modified for the purpose of this study.

Table 4.1. Modified inclusion and exclusion criteria

	Modified Inclusion Criteria	**Modified Exclusion Criteria**
Stroke cases	• HIV-infected • Age >18 years and <45 years • Ischaemic Stroke • Available blood specimens for laboratory analysis* • Written consent obtained	• HIV-uninfected • Age <18 years or >45 years • Haemorrhagic stroke, subarachnoid, subdural or epidural haemorrhage
Controls	• HIV-infected • Age > 18 years and <45 years • Available blood specimens for laboratory analysis* • Written consent obtained	

**Additional criteria for the purpose of this study*

Of the original 63 HIV-infected young strokes, 62 met the above criteria. All 99 of the original HIV-infected control group met the criteria for inclusion in this study.

4.3.4 Final sample size for the Tat protein research project

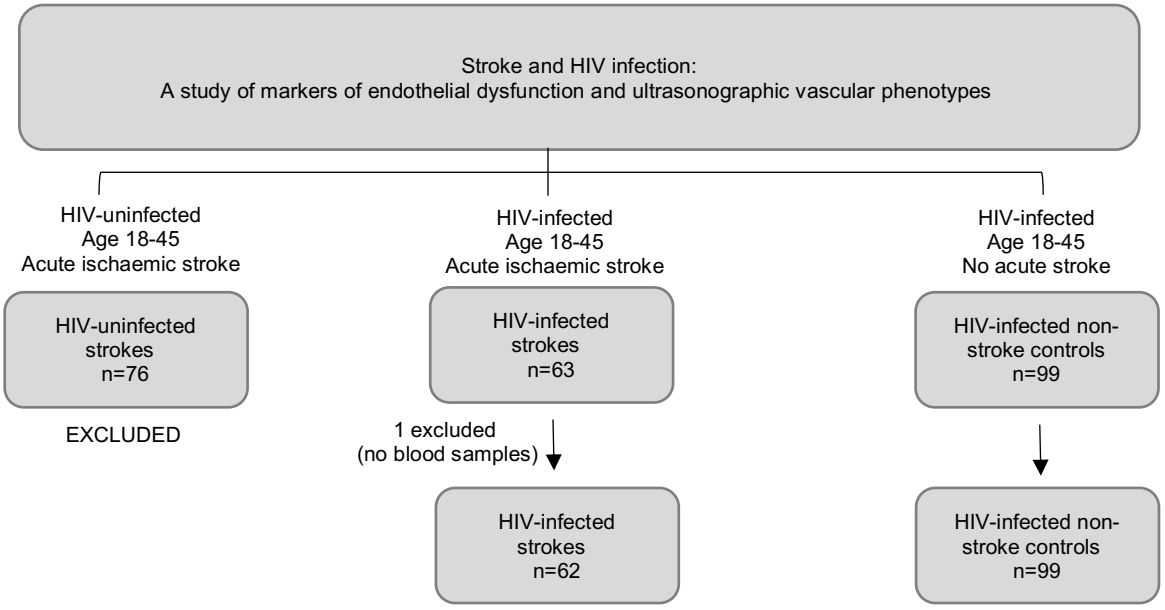


Figure 4.1. Attainment of final sample size for the study on the Tat protein

4.4 Specimens and imaging

4.4.1 Blood tests and imaging performed between 2010 and 2013

All participants had 30 millilitres of whole blood taken on enrolment into the original study. For the stroke participants, blood samples were taken within the first five days of stroke onset. The samples were separated into serum, Ethylenediaminetetraacetic acid (EDTA) plasma and buffy coats and stored at -80°C. Initial blood tests included chemistry, haematology, coagulation tests and serology. Biomarkers of endothelial activation and inflammation were performed with cytokine assays. These included IL-1β, IL-6, IL-10, TNF-α, MCP-1, VEGF, Endothelin-1, E-selectin, ICAM-1 and VCAM-1.

54/58 (93.1%) individuals in the stroke group and 3/71 (4.2%) of the controls had CSF taken by lumbar puncture for analysis. Tests done on CSF included cell count, chemistry, microscopy, tuberculous and fungal culture, cryptococcal latex antigen test (CLAT), India Ink stain, and a viral panel screen: varicella zoster virus (VZV), herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), Epstein-Barr virus (EBV) and cytomegalovirus (CMV). VZV screening was done via polymerase chain reaction (PCR), as VZV-Immunoglobulin G (Ig-G) testing was unavailable. Laboratory technicians analysing blood and CSF samples were blinded to whether the sample was from the stroke or control group. 48/58 (82.8%) of individuals with stroke and 49/71 (69%) of controls had duplex Doppler imaging of the carotid arteries to determine the intima-media thickness. All of the stroke subjects had computerized tomography and/or magnetic resonance imaging of the brain, as well as electrocardiogram, echocardiogram, bubble studies or cardiac magnetic resonance imaging to determine stroke aetiology. Angiography of cerebral vessels was also performed if clinically indicated.

4.4.2 Analysis of the Tat protein performed in this study

I used the stored EDTA buffy coats for the DNA procurement, PCR and Sanger sequencing of the Tat protein in this study.

4.5 Classification of stroke aetiology

The aetiology of HIV-associated strokes is complex, and the classification is therefore not straightforward. The categories defined by the traditional Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification (Adams et al., 1993) may not adequately cover the multiple causes commonly seen in HIV infection. A 2016 consensus paper has classified the different causes of HIV-associated stroke, in order to clarify and standardize definitions of stroke aetiology for future research into ischaemic stroke in HIV-infected individuals. The published algorithm uses a prescribed battery of tests and assists

researchers in determining the aetiology of HIV-associated ischaemic stroke. The main causes include opportunistic infection, cardio-thromboembolism, vasculitis, accelerated atherosclerotic vasculopathy, non-atherosclerotic vasculopathy, coagulopathy and small vessel disease. Ischaemic strokes which do not fit into any of the above categories may either have evidence of multiple aetiologies, or are classified as cryptogenic stroke (Benjamin, Bryer, et al., 2016). This algorithm was used to determine the aetiology for all stroke cases in this study (see Appendix C).

4.6 Comparison of clinical characteristics

4.6.1 Summary of statistical analyses performed in this study

Statistical analysis was performed on the clinical data for the cohort using Stata® (StataCorp LLC, Texas, USA). The shape of data distributions was evaluated with Kernel Density Plots. Continuous measurements were reported with mean and standard deviation, where the data were normally distributed, and comparisons between the groups were done with a two-sample t-test with unequal variances. Where the data were obviously skewed on Kernel Density Plot, continuous variables were reported with median and interquartile range (IQR), and comparisons were made with the two-sample Wilcoxon rank-sum (Mann-Whitney) test. Counts (No.), and percentages (%), were used for nominal variables, and compared using the Pearson Chi-square test of Independence or Fisher's exact test, where appropriate. An alpha value of 0.05 was used as the cut-off for significance in the analyses. A binary logistic regression model was fitted to evaluate carotid intima-media thickness, controlling for age and waist circumference. Pair-wise deletion was used to handle missing data for certain clinical and laboratory variables, and the valid percentages for nominal variables reported. Where there was a large number of missing values, the actual number of values analysed was reported in the results tables (see Chapter 5, Tables 5.1-5.4). Graphs were constructed using Microsoft Excel and GraphPad Prism 7.0c. Bioinformatic analysis of the Tat protein was done once the sequences were obtained (see section 4.8).

4.6.2 A note on the use of p-values

There has been much debate in recent years about the use of a p-value to determine the meaning of a single result (Goodman, 1999). A recent guide on interpretation of the results of statistical tests acknowledged that any conclusion drawn about the likelihood of a hypothesis being true or not, cannot rely solely on statistical methods (Greenland et al., 2016). The results from the statistical tests that I performed in this study were part of a variety of considerations taken into account. Where possible, inferences I made about the clinical and scientific significance of my findings were not based on a single p-value, but rather on the totality of evidence obtained in this cohort.

Sequencing of *tat* exon 1

4.6.3 Summary of sequencing methods

Proviral DNA was extracted from the stored peripheral blood samples of the study participants. We then performed pre-nested and nested polymerase chain reactions to isolate the *tat* exon 1 fragment. The PCR product for each participant was then sequenced with Sanger sequencing.

Table 4.2. Kits and chemical products used for sequencing

Product or Kit	Manufacturer
Machery-Nagel NucleoSpin® Blood Kit	Machery-Nagel GmbH & Co. KG, Düren, Germany
Nuclease Free Water	Promega, Madison, Wisconsin (WI), United States of America (USA)]
GoTaq® Flexi DNA Polymerase	Promega, Madison, WI, USA
5X Colorless GoTaq® Flexi Buffer	Promega, Madison, WI, USA
Magnesium Chloride Solution, 25mM	Promega, Madison, WI, USA
Deoxynucleotide Triphosphates (dNTPs)	Promega, Madison, WI, USA
SeaKem® LE Agarose	Whitehead Scientific (Pty) Ltd, Cape Town, SA
Tris Acetate EDTA buffer	Qiagen, Hilden, Germany
GR Green Nucleic Acid Stain	Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, SA
Promega 6 x Blue/Orange Loading Dye	Promega, Madison, WI, USA
Promega 1kb DNA ladder	Promega, Madison, WI, USA
Parafilm®	Bemis Company Inc., Neenah, WI, USA
Macherey-Nagel Nucleospin® Gel and PCR Clean-up Kit	Machery-Nagel GmbH & Co. KG, Düren, Germany
Applied Biosystems BigDye® Terminator	Applied Biosystems Inc., CA, USA
Applied Biosystems BigDye® XTerminator™ Purification Kit	Applied Biosystems Inc., CA, USA

Table 4.3. Equipment used for sequencing

Machine or Equipment	Manufacturer
Applied Biosystems Veriti™ 96 Well Thermal Cycler	Applied Biosystems Inc., California (CA), USA]
Applied Biosystems® GeneAmp® PCR System 9700	Applied Biosystems Inc., CA, USA
UVIprochemi II D-77 LS-26M gel documentation system	UVitech, Cambridge, UK
Nanodrop™ ND 1000	NanoDrop Products, Delaware (DE), USA
Applied Biosystems 3130xl Genetic Analyzer	Applied Biosystems Inc., CA, USA

4.6.4 Proviral DNA extractions

DNA was isolated from 200µl of prepared buffy coat using the Macherey-Nagel NucleoSpin® Blood Kit for extraction of genomic DNA from blood.
The kit protocol (Macherey-Nagel, 2016) was followed and proviral DNA was eluted in 100 microlitres (µl) of pre-heated buffer solution. The DNA concentration of the samples was then checked with the NanoDrop™ ND 1000 Spectrophotometer to ensure that the eluted DNA concentration would be adequate for downstream reactions.

4.6.5 Polymerase chain reaction

For the PCR, the Promega GoTaq® Flexi Kit was used, according to manufacturer's instructions (Promega Corporation, 2013). We elected to amplify *vpr* (HXB2 5559-5850) and *tat* exon 1 (HXB2 5831-6045) as a single fragment. The *vpr* gene lies in close proximity to, and slightly overlaps, *tat* exon 1. Due to the availability of primers, and to simplify the PCR process, we initially elected to amplify the two genes as a single piece, to be separated after sequencing.

The Applied Biosystems Veriti™ 96 Well Thermal Cycler and the Applied Biosystems GeneAmp® PCR System 9700 were used for the PCR, according to the user guides (Applied Biosystems, 2008, 2010a).

The primers and protocol used are summarized below.

4.6.5.1 Primers and protocol for PCR of *tat* exon 1 and *vpr*

Table 4.4. Primers used for pre-nested PCR of *tat* exon 1 & *vpr*

	Primer	Oligonucleotide Sequence	Tm in °C	HXB2 Position
Forward Primer	Vif-1	5'-GGGTTTATTACAGGGACAGCAGAG-3'	67	4900→4923
Reverse Primer	CATH-4R	5'-GTACCCCATAATAGACTGTGACC-3'	64	6329←6351
Expected Size	1451 base pairs			

Table 4.5. Cycling conditions for pre-nested PCR of *tat* exon 1 & *vpr*

Step	Cycles	Temperature (°C)	Time
Initial Denaturation	1	94	2 min
Denaturation	40	94	30 sec
Annealing		60	30 sec
Elongation		68	2 min
Final Elongation	1	68	10 min
Holding	1	4	Indefinite

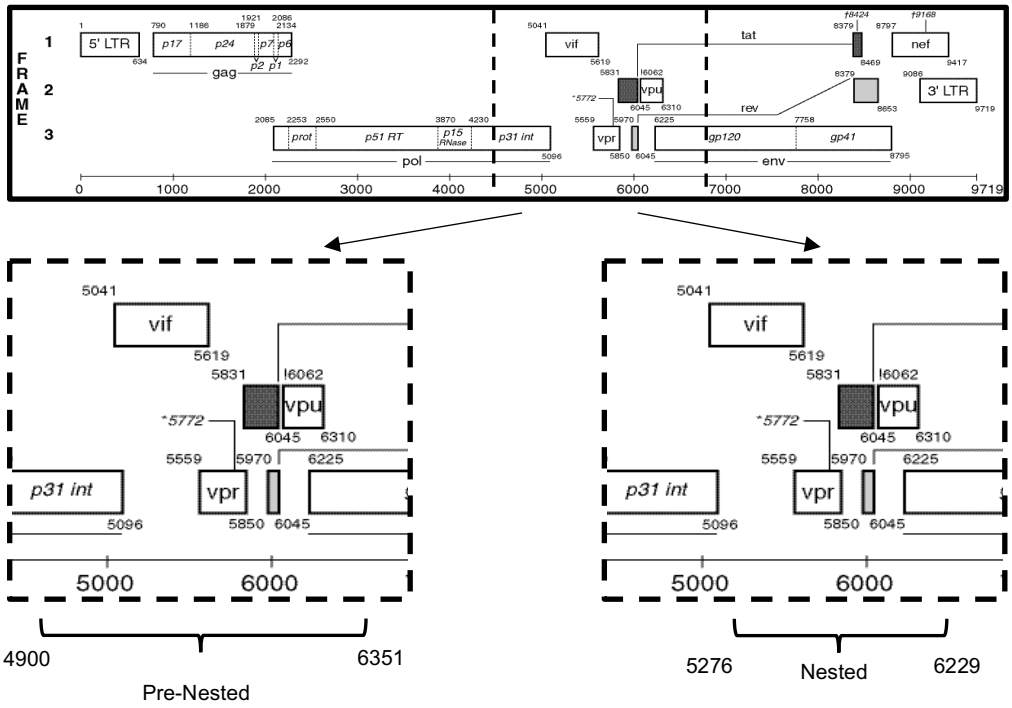


Figure 4.2. Approximate locations of the pre-nested & nested PCR fragments for *tat* exon 1 & *vpr*. Adapted from HIV Genome Map (www.hiv.lanl.gov)

Table 4.6. Primers used for nested PCR of *tat* exon 1 & *vpr*

	Primer	Oligonucleotide Sequence	Tm in °C	HXB2 Position
Forward Primer	Vif-1F	5'-GGAATTTGGGTCATGGAGTCTCCATA-3'	68	5276→5301
Reverse Primer	Tat-1_OR	5'-CTCATTGCCACTGTCTTCTGC-3'	64	6209←6229
Expected Size	953 base pairs			

Table 4.7. Cycling conditions for nested PCR of *tat* exon 1 & *vpr*

Step	Cycles	Temperature (°C)	Time
Initial Denaturation	1	94	2 min
Denaturation	40	94	30 sec
Annealing		60	30 sec
Elongation		68	1 min
Final Elongation	1	68	10 min
Holding	1	4	Indefinite

There were 15 samples which did not amplify using the *vpr* and *tat* primers. These samples underwent a repeat PCR with alternative primers that were specific for the *tat* exon 1 region only. The primers and protocol used are summarized below.

4.6.5.2 Primers and protocol for PCR of *tat* exon 1 only

Table 4.8. Primers used for pre-nested PCR of tat exon 1

	Primer	Oligonucleotide Sequence	Tm in °C	HXB2 Position
Forward Primer	Tat-1_OF	5'-AAAGCCACCTYTGCCTAG-3'	57	5517→5534
Reverse Primer	Tat-1_OR	5'-CTCATTGCCACTGTCTTCTGC-3'	64	6209←6229
Expected Size	712 base pairs			

Table 4.9. Cycling conditions for pre-nested PCR of *tat* exon 1

Step	Cycles	Temperature (°C)	Time
Initial Denaturation	1	94	2 min
Denaturation	40	94	30 sec
Annealing		55	30 sec
Elongation		68	1 min
Final Elongation	1	68	10 min
Holding	1	4	Indefinite

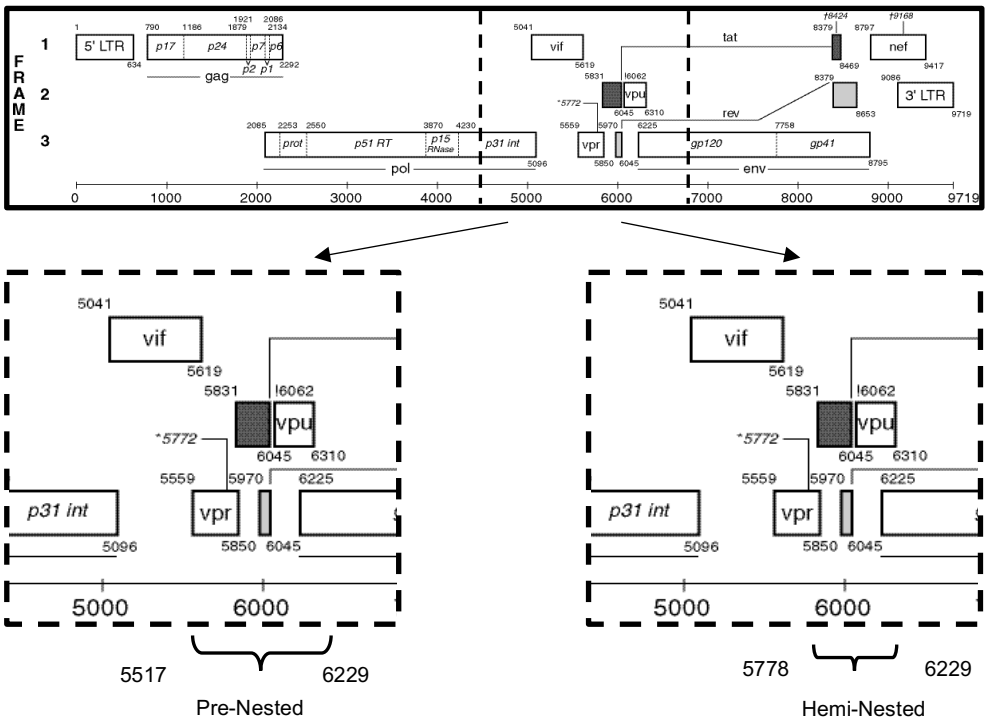


Figure 4.3. Approximate locations of the pre-nested & hemi-nested PCR fragments for *tat* exon 1. Adapted from the HIV Genome Map (www.hiv.lanl.gov)

Table 4.10. Primers used for hemi-nested PCR of *tat* exon 1

	Primer	Oligonucleotide Sequence	Tm in °C	HXB2 Position
Forward Primer	Tatx-1F	5'-AATTGGGTGCCAGCATAGC-3'	62	5778→5796
Reverse Primer	Tat-1_OR	5'-CTCATTGCCACTGTCTTCTGC-3'	64	6209←6229
Expected Size	451 base pairs			

Table 4.11. Cycling conditions for hemi-nested PCR of *tat* exon 1

Step	Cycles	Temperature (°C)	Time
Initial Denaturation	1	94	2 min
Denaturation	40	94	30 sec
Annealing		55	30 sec
Elongation		68	1 min
Final Elongation	1	68	10 min
Holding	1	4	Indefinite

4.6.6 Gel electrophoresis and visualisation

The gene fragments were separated by 0.8% agarose gel electrophoresis. The agarose gels were prepared with agarose powder and 1 x Tris-Acetate-EDTA (TAE) buffer. GR Green Nucleic Acid Stain was added to the agarose gel solution at a ratio of 1µl:10ml. The samples were loaded with Promega 6 x Blue/Orange Loading Dye. Promega 1kb DNA ladder was used as a marker. Each gel contained one negative control. The electrophoresis was run at 80V and 400mA for 35 minutes.

The UVIprochemi II D-77 LS-26M gel documentation system was used for fluorescence and image acquisition of the proviral DNA fragments.

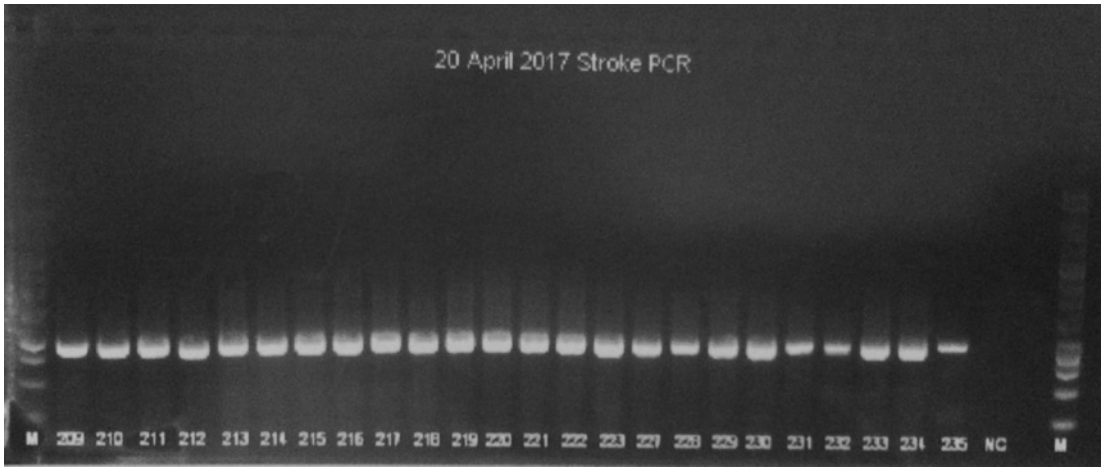


Figure 4.4. An example of an image acquired after gel electrophoresis and fluorescence

4.6.7 PCR purification

The PCR purifications were performed with the Nucleospin® Gel and PCR Clean-up kit, according to manufacturer instructions (Machery-Nagel, 2017). The DNA concentration of the purified PCR products was then measured with the Nanodrop Spectrophotometer to calculate dilutions for sequencing.

4.6.8 DNA Sanger sequencing

Whilst there is an enormous biodiversity of HIV-1 within individuals, a number of dominant sequence clusters exist (Yin et al., 2012). The predominant sequence is determined by selection pressures such as antiretroviral therapy, host immune response and environmental factors (Dampier et al., 2016). Sanger sequencing identifies the consensus sequence, and will not detect minor variants in an individual (Dampier et al., 2017).

The sequencing of the purified PCR products was done using the Applied Biosystems BigDye® Terminator v3.1 Cycle Sequencing Kit, according to the user guide (ThermoFisher Scientific, 2016). The DNA was diluted to a concentration of 15-25 ng/μl. The primers, master mixes and cycling conditions used are detailed in the tables below.

Table 4.12. Primers for cycle sequencing of *tat* exon 1 & *vpr*

	Primer	Oligonucleotide Sequence	HXB2 Position
Forward Primer	Vif-1F	5'-GGAATTTGGGTCATGGAGTCTCCATA-3'	5276→5301
Reverse Primer	Tat-1_OR	5'-CTCATTGCCACTGTCTTCTGC-3'	6209←6229

Table 4.13. Primers for cycle sequencing of *tat* exon 1

	Primer	Oligonucleotide Sequence	HXB2 Position
Forward Primer	Tatx-1F	5'-AATTGGGTGCCAGCATAGC-3'	5276→5301
Reverse Primer	Tat-1_OR	5'-CTCATTGCCACTGTCTTCTGC-3'	6209←6229

Table 4.14. Master mix for sequencing reactions

Reagent	1X (μL)
Nuclease Free Water	4.5
5 x Reaction Buffer	3
XTerminator Reaction Mix	0.5
Primer (5pM/μL)	1
Total aliquot	**9**
DNA Sample (15-25ng/μL)	1

Table 4.15. Conditions for cycle sequencing of *tat* exon 1 & *vpr*

	Temperature	Time	Number of Cycles
Denaturation	96° C	10 seconds	25
Annealing	58° C	5 seconds	
Extension	60° C	5 minutes	

Table 4.16 Conditions for cycle sequencing of tat exon 1

	Temperature	Time	Number of Cycles
Denaturation	96° C	10 seconds	25
Annealing	55° C	5 seconds	
Extension	60° C	4 minutes	

After cycle sequencing, the fragments were then purified with the BigDye® XTerminator™ Purification Kit, according to the manufacturer's protocol (Applied Biosystems, 2007).

Table 4.17. Master mix for purification of cycle sequencing products

Reagent	1X (µL)
SAM™ Solution	4.5
XTerminator™ Solution	0.5
Total aliquot	**55 (µL**

The sequences were then converted into raw data files with the Applied Biosystems 3130xl Genetic Analyzer, according to the user guide (Applied Biosystems, 2010b). Of the cohort of 161 participants, we obtained 58/62 and 76/99 readable nucleotide sequences for the stroke and control groups respectively. We used these for further analysis.

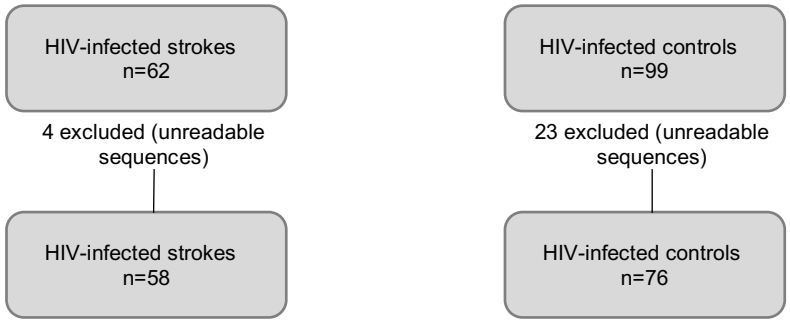


Figure 4.5. Number of nucleotide sequences suitable for further analysis

4.7 Sequence analysis

4.7.1 Introduction to bioinformatics for sequence analysis

I used bioinformatics software tools to organise and analyse the sequences. I converted the raw data files into fasta format, which is the file type used by most software for sequence analysis. The nucleotide sequences were translated into amino acid sequences and I separated the cohort into stroke and control groups. The sequences underwent quality control assessment and subtyping. 5/76 (6.6%) of the control sequences were excluded after subtyping, to create a cohort that was composed entirely of Subtype-C sequences. This was to minimise the influence of greater genetic variability seen between subtypes. I then created consensus sequences for the groups, visualised dataset similarity between groups, and performed signature pattern analysis. Additionally, I looked for evidence of positive selection pressure in the stroke and control groups.

Table 4.18. Bioinformatics software used for sequence analysis

Software	Company/Source	Function/Description
AnalyzeAlign	www.hiv.lanl.gov	• Consensus sequence generation • Weblogo generation • Calculates frequency by position & finds variants within the alignment
CLC Sequence Viewer 7.8.1	www.qiagenbioinformatics.com	• Sequence visualization, editing and analysis • Sequence alignment
Consensus Maker	www.hiv.lanl.gov	• Consensus sequence generation
Datamonkey	www.datamonkey.org	• Analysis of selection pressure
Entropy	www.hiv.lanl.gov	• Quantifies uncertainty in a dataset • Consensus sequence generation

Geneious R11	www.geneious.com (Biomatters Limited, 2017)	• Bioinformatics Software Platform
HIV Mutation Browser	www.hivmut.org (Davey et al., 2014) Collaboration between Briggs Group (European Molecular Biology Laboratory, Heidelberg) and Schneider Group (Luxembourg Centre for Systems Biomedicine, Luxembourg)	• HIV-1 mutation data from all available scientific literature
Jumping profile Hidden Markov Model	www.jphmm.gobics.de (Zhang et al., 2006)	• Subtyping and detection of recombinants in HIV-1
Los Alamos HIV Sequence Database	www.hiv.lanl.gov	• Database of HIV genetic sequences • Access to tools to analyse and visualize data
MAFFT	http://mafft.cbrc.jp/alignment/software	• Multiple sequence alignment software
REGA HIV-1 Subtyping Tool Version 3.0	Stanford University HIV Drug Resistance Database(De Oliveira et al., 2014)	• Determination of HIV-1 subtype
SeqPublish	www.hiv.lanl.gov	• Sequence Alignment Publishing Tool
Sequencher® version 5.2.4	www.genecodes.com (Gene Codes Corporation, 2014)	• DNA Sequence Analysis and contig assembly
Shannon Entropy Two		• Determination of site-specific variability
Quality Control	www.hiv.lanl.gov	• Prepares sequences for submission to GenBank • Subtyping • Finds similar database sequence • Constructs phylogenetic trees • Detection of number of stop codons and frameshifts • Detection of hypermutations
Viral Epidemiology Signature Pattern Analysis	www.hiv.lanl.gov/content/sequence/VESPA/vespa.html (Korber & Myers, 1992)	• Signature patterns in query sequences relative to background sequences
WebLogo	www.weblogo.threeplusone.com (Crooks et al., 2004)	• Sequence logo generation

4.7.2 Acquisition and preparation of final sequences for analysis

The HXB2 reference sequence was downloaded from the Los Alamos National Laboratory (LANL) HIV Database. The HXB2 reference sequence is the most commonly used reference strain for functional studies. This internationally-accepted reference sequence facilitates the uniform identification of a position number and precise location of interest in HIV-1 DNA or proteins (Korber et al., 1999). The HXB2 *tat* exon 1 was imported and used as the reference to assemble the sequence contigs in Sequencher version 5.2.4. (Gene Codes Corporation, 2014). Each Sequencher-generated contig was manually checked, then exported in fasta format into Geneious version R11 (Biomatters Limited, 2017). Of the 161 samples, I obtained 134 readable sequences.

I created a multiple alignment of the entire cohort using Multiple Alignment using Fast Fourier Transform (MAFFT) within Geneious. The sequences were then codon-aligned and manually checked. One of the study cohort sequences (1/134; 0.75%) had a single amino acid insertion at position 52, resulting in a multiple alignment that was 73 amino acids in length. Most functional studies of amino acid substitutions and activities of various Tat domains have utilized the HXB2 reference sequence, which is 72 amino acid residues in length. Furthermore, analysis of the sequences required the use of the HIV Mutation Browser, which annotates proteins according to the HXB2 reference sequence. I therefore decided to gap-strip the sequence with the insertion, to obtain a 72-amino acid sequence alignment of the entire cohort. This allowed for analysis of mutations at positions which corresponded with the scientific literature on the Tat protein. As there was only one sequence with an insertion, gap-stripping was unlikely to significantly compromise the validity of the results in this cohort.

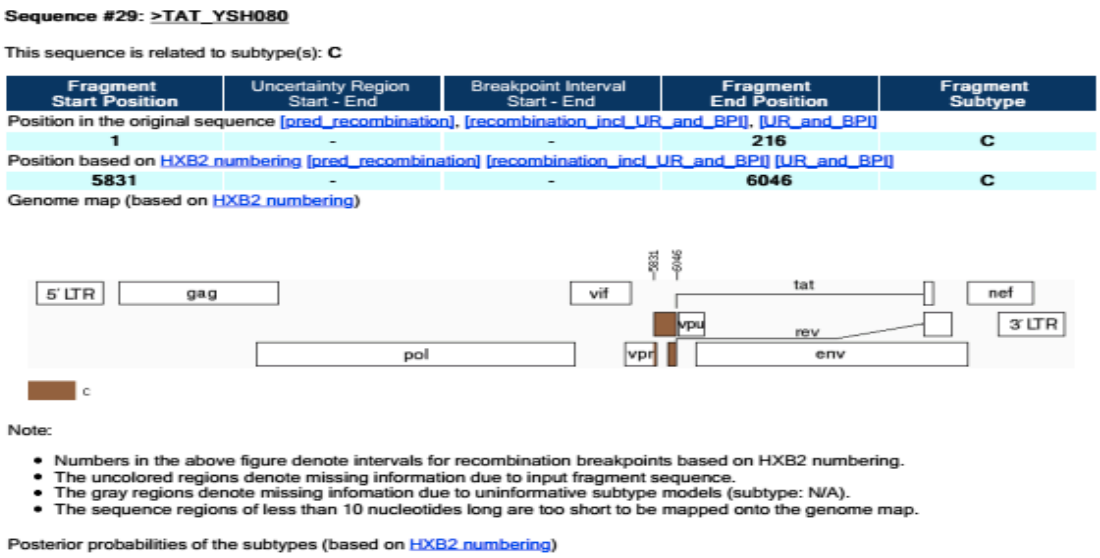
The sequences were then separated into stroke and control groups, and realigned. All nucleotide sequences were then translated into amino acids, realigned and manually re-checked to confirm the alignments.

4.7.3 Quality Control

I performed sequence quality analysis on all sequences using the Quality Control Tool. This tool examines nucleotide sequences for common problems, and prepares sequence sets for submission to GenBank.

4.7.4 Subtyping of the study cohort

The study cohort was subtyped using the jumping profile Hidden Markov Model (Zhang et al., 2006). This was cross-checked with REGA HIV-1 Subtyping tool (De Oliveira et al., 2014). The results of the subtyping showed that 129/134 (96.3%) of all study cohort sequences were Subtype-C, which is the predominant subtype both globally and in Sub-Saharan Africa. The other subtypes, all in the control group, were excluded from the subsequent analyses, as the significant genetic variation between subtypes could influence the amino acid signature patterns in this study. The excluded subtypes were A1 (n=3), B (n=1) and G (n=1).



Sequence #29: >TAT_YSH080

This sequence is related to subtype(s): **C**

Fragment Start Position	Uncertainty Region Start - End	Breakpoint Interval Start - End	Fragment End Position	Fragment Subtype
Position in the original sequence [pred_recombination], [recombination_incl_UR_and_BPI], [UR_and_BPI]				
1	-	-	**216**	**C**
Position based on HXB2 numbering [pred_recombination] [recombination_incl_UR_and_BPI] [UR_and_BPI]				
5831	-	-	**6046**	**C**

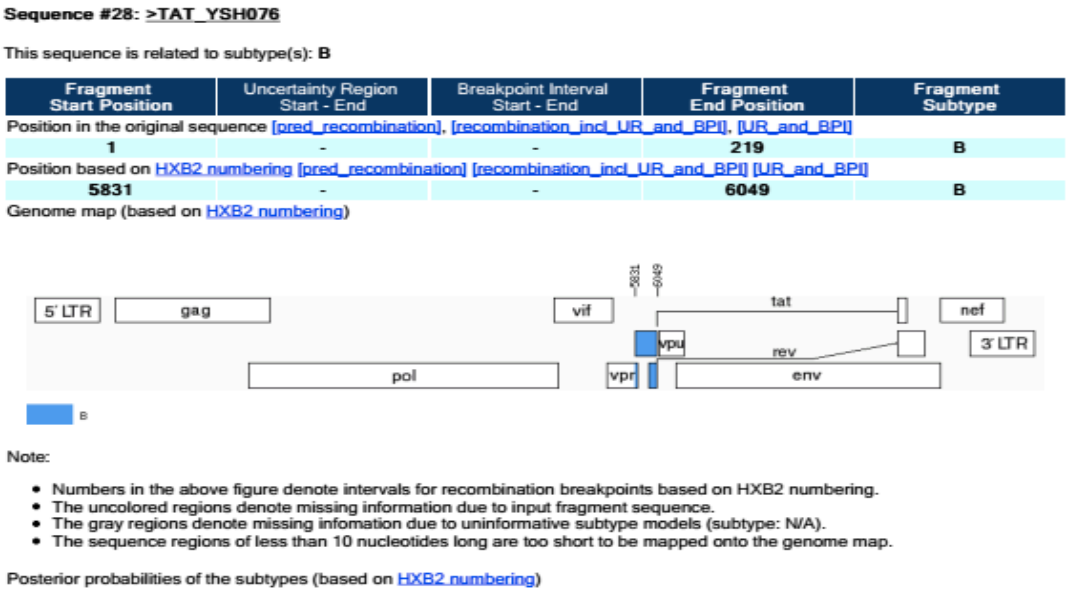
Genome map (based on HXB2 numbering)

Note:

- Numbers in the above figure denote intervals for recombination breakpoints based on HXB2 numbering.
- The uncolored regions denote missing information due to input fragment sequence.
- The gray regions denote missing infomation due to uninformative subtype models (subtype: N/A).
- The sequence regions of less than 10 nucleotides long are too short to be mapped onto the genome map.

Posterior probabilities of the subtypes (based on HXB2 numbering)

Figure 4.6. Example of subtyping results from the jumping profile Hidden Markov Model. The participant identified as YSH080 was Tat Subtype-C, and was included in the analysis.



Sequence #28: >TAT_YSH076

This sequence is related to subtype(s): **B**

Fragment Start Position	Uncertainty Region Start - End	Breakpoint Interval Start - End	Fragment End Position	Fragment Subtype
Position in the original sequence [pred_recombination], [recombination_incl_UR_and_BPI], [UR_and_BPI]				
1	-	-	**219**	**B**
Position based on HXB2 numbering [pred_recombination] [recombination_incl_UR_and_BPI] [UR_and_BPI]				
5831	-	-	**6049**	**B**

Genome map (based on HXB2 numbering)

Note:

- Numbers in the above figure denote intervals for recombination breakpoints based on HXB2 numbering.
- The uncolored regions denote missing information due to input fragment sequence.
- The gray regions denote missing infomation due to uninformative subtype models (subtype: N/A).
- The sequence regions of less than 10 nucleotides long are too short to be mapped onto the genome map.

Posterior probabilities of the subtypes (based on HXB2 numbering)

Figure 4.7. Example of subtyping results from the jumping profile Hidden Markov Model. The participant identified as YSH076 was Tat Subtype-B, and was excluded from the analysis.

4.7.5 Consensus sequences

Consensus sequences for the stroke and control groups were created with four separate online tools, in order to cross-check accuracy. Consensus sequences were created using the Advanced Consensus Maker, SeqPublish, AnalyzeAlign and Entropy tools, all four of which showed 100% agreement on the derived consensus for each group. The consensus sequences were used to visualize dataset similarity.

4.7.6 Visualization of dataset similarity

SeqPublish was used to identify similarity between the stroke and control datasets. SeqPublish is a sequence alignment publisher tool. It aligns a dataset to a consensus sequence and represents identical residues at each position with dashes. The stroke group sequences were aligned to the consensus sequence for the control group.

4.7.7 Visualization and analysis of site-specific variability

The CLC Sequence Viewer (Qiagen Bioinformatics, 2017) was used to visualize the variability of amino acid residues at each position in relation to a consensus of the dataset. The software creates a consensus sequence for the cohort, then generates a histogram depicting the conservation of the consensus residue at each position.

4.7.8 Signature pattern analysis

Signature pattern analysis identifies positions in a sequence at which the most common amino acid differs between a query sequence alignment and a background sequence alignment. The comparison detects an amino acid signature that is unique to the query group. It detects amino acid substitutions that may be unique to the query group relative to the background group.

Signature pattern analysis was done with Viral Epidemiology Signature Pattern Analysis (VESPA), which calculates the frequency of all amino acids for the query and background groups at each position in the alignments. It then selects the positions for which the most common character in the query group differs from the background group. The analysis highlights amino acids that characterize the unique differences between two groups of sequences. The specific amino acid signature is obtained by looking for the set of amino acids that is conserved within each group, but differs between the two groups (Korber & Myers, 1992). Signature pattern analysis purposefully uses the most common amino acid at a position, rather than merely describing positions that exhibit differences in amino acid distributions (Huang et al., 2012).

The VESPA amino acid frequency calculations for all analyses were cross-checked with AnalyzeAlign. There was 100% agreement in the frequency calculations across all groups between the two software tools.

Signature pattern analysis was also performed for the stroke group, which was separated into two subgroups by aetiology.

It is important to note that 11 sequences in total (8 in the control group, and 3 in the stroke group) were short, and did not reach the full 72 amino acid length on Sanger sequencing. These were included in the analysis, but I was aware that from position 31 onwards, at least 1 of the 11 short sequences had missing data, which may have affected signature pattern analysis.

I used sequence logos to depict the signature amino acid differences between the groups. Sequence logos present an alternative visual overview of the sequence characteristics unique to each group. A sequence logo graphically represents the conservation of amino acids in a set of aligned sequences. The logo depicts the consensus sequence as well as amino acid diversity at each position. I created sequence logos for the regions around the signature amino acids detected by signature pattern analysis. The sequence logo for the Tat protein consists of a stack of single-letter amino acid abbreviations at each position on the x-axis. The height of the symbols on the y-axis represents the relative frequency of each amino acid at that position. Sequence logos were created using Weblogo 3.0. (Crooks et al., 2004) and cross-checked with AnalyzeAlign.

4.7.9 Separation of the stroke group by stroke cause

The participants in the stroke group had their stroke aetiology classified according to published case definitions (Benjamin et al., 2012; Benjamin, Bryer, et al., 2016). The first was those strokes due to HIV-associated vasculopathy. I included cryptogenic stroke in this group. Cryptogenic strokes are those strokes for which, after exhaustive investigations, no cause has been found. In these individuals, HIV infection itself is possibly the only major risk factor that remains. Cryptogenic stroke may represent undiagnosed HIV-associated vasculopathy (Benjamin et al., 2012, 2017). The background group included all strokes that had a confirmed or probable cause that was not directly due to HIV-associated vasculopathy. Six patients were excluded from the signature pattern analysis because of incomplete evaluation.

Table 4.19. Stroke sequences classified by aetiology

Strokes due to HIV-associated vasculopathy (n=25)	Strokes due to alternative mechanisms (n=27)
• Non-atherosclerotic vasculopathy (n=14) • HIV-associated vasculitis (n=4) • Accelerated atherosclerotic vasculopathy (n=3) • Cryptogenic (n=3) • Small vessel disease without hypertension (n=1)	• Opportunistic infections (n=13) • Cardioembolism (n=9) • Other determined cause (n=3) • Small vessel disease with hypertension (n=2)

This separation of the strokes into two groups was to explore whether an amino acid signature pattern could be detected between the strokes with a confirmed or probable alternative cause, in relation to the strokes due to HIV-associated vasculopathy. The paracrine effects of the Tat protein may result in the direct endothelial damage that is postulated to contribute to HIV-associated vasculopathy. Amino acid mutations in the Tat protein could alter these paracrine effects. An attenuation or enhancement of the chronic persistent inflammatory process or viral replication may determine the extent of endothelial dysfunction directly caused by HIV versus endothelial dysfunction from other mechanisms.

4.7.10 Signature amino acid mutation search

The substitutions at the amino acid positions identified with signature pattern analysis were then explored with the HIV Mutation Browser. This database contains mutation data collated from all HIV-related scientific literature. The data are “identified and catalogued using computational text-mining methods” (Davey et al., 2014), and are frequently updated. The HIV Mutation Browser can be used to find literature describing the mutation phenotype, as well as any functional effect of the substitution. It has four options for data review. The feature view enables visualisation of the region in which the mutation resides, and its relationship with any important functional areas. The sequence view shows the amino acid sequence of the protein, and data for each residue can

be accessed by clicking on the residue of interest. The table view displays the most common amino acid at each position, as well as its conservation at that site, the secondary structure and number of known mutations at that position. The residue view displays links to scientific articles related to the residue, as well as a residue information panel which displays structural and conservation information (Davey et al., 2014).

Figure 4.8. HIV Mutation Browser home page. www.hivmut.org

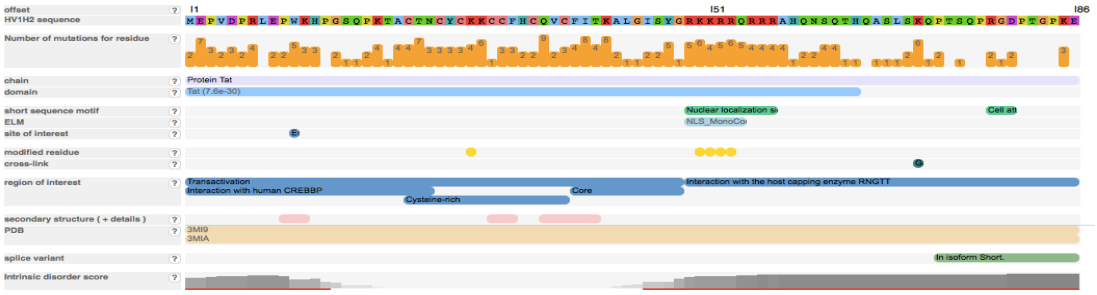


Figure 4.9. HIV Mutation Browser Feature View. www.hivmut.org

4.7.11 Significance of signature pattern analyses

Fisher's exact test was used to determine the significance of the amino acid signature patterns identified by VESPA. P-values were determined for signature amino acids in the stroke group relative to the control group. P-values were also determined for the signature pattern identified for the strokes that were separated by aetiology. A p-value of ≤0.05 was used to represent statistical significance of an amino acid substitution at that position.

4.7.12 Determination of positive selection in the stroke and control groups

I assessed positive selection via Datamonkey (http://classic.datamonkey.org), a selection analysis website that employs various selection pressure models to analyse selection pressure on a set of uploaded sequences.

I used Single-Likelihood Ancestor Counting (SLAC) to analyse positive selection pressure in the cohort. SLAC is recommended for data sets with more than 40 sequences (Kosakovsky Pond & Frost, 2005) and has sufficient power to detect positive and negative pervasive selection. This model assumes that “selection pressure for each site is constant along the entire phylogeny” (HyPhy, 2017). SLAC is a conservative method, with a type 1 error rate less than the nominal p-value. The default significance level is therefore set at 0.1. “SLAC uses a combination of maximum-likelihood (ML) and counting approaches to infer nonsynonymous (dN) and synonymous (dS) substitution rates on a per-site basis for a given coding alignment and corresponding phylogeny” (HyPhy, 2017). I used the most general time-reversible nucleotide substitution model (REV) to evaluate the phylogenetic tree of the alignment and estimate nucleotide substitution bias rates (Poon, Frost & Pond, n.d.).

4.8 Correlation of signature positions with endothelial biomarkers

4.8.1 Rationale for correlation of signature positions with biomarkers

The scope of this project did not include cell-culture studies to assess the functional impact of the viral isolates in this cohort. I was nevertheless interested as to whether any of the signature positions identified had a correlation with the endothelial biomarkers measured in these individuals. I elected to do an experimental statistical analysis as a preliminary step, with the view that cell-culture studies can be done in future work. I decided to follow

the example of Dampier *et al*. (Dampier et al., 2017), who correlated specific amino acids in HIV-1 Vpr with neurocognitive status using statistical models.

4.8.2 Model construction process

I took a slightly different approach to that followed by Dampier *et al*., due to the fact that we were working with ten outcome variables (the biomarkers), rather than the two they used to look at neurocognitive status. I also wanted to avoid having to do Bonferroni adjustment, as it assumes a universal null hypothesis and may increase Type II error rates (Perneger, 1998).

I chose to control for the effect of age, CD4 count, stroke or non-stroke and ART status (treated or untreated) on the biomarkers. I then introduced the three signature positions identified by VESPA into the models. At each position, I chose the most common amino acids for that position and coded them into categories. For position 21, proline was the indicator variable, with alanine and other amino acids as a reference category. At position 29, histidine, lysine and arginine were the indicator variables, with other amino acids as the reference category. At position 58, alanine was the indicator variable, with threonine as the reference category.

Linear regression models were fitted for all 10 biomarkers and then the residual analysis plots were inspected to assess normality and variance for each model. On inspection, E-selectin had little deviation from normality, and displayed constant variance. VCAM-1, ICAM-1, VEGF, TNF-α, Endothelin-1 and MCP-1 had slight deviation from normality, and were natural log transformed to stabilise variance.

Many of the values for IL-1β, IL-6, and IL-10 were 0.00, and had serious deviation from normality with non-constant variance. IL-1β was excluded from analysis because only four values were above 0.00, and complete separation occurred with the stroke variable and position 29. IL-6 and IL-10 were treated as binary variables, and logistic regression models were fitted for these two biomarkers. On inspection of the standardised Pearson and deviance

residuals, no large values to indicate a poorly-fitting model were observed for IL-6 or IL-10.

The significance level was set at 0.05, to evaluate whether the signature amino acids identified by VESPA had a positive or negative effect on the value of the biomarker in the cohort.

Overall, the models were a reasonable fit, given that this was intended as a preliminary exploration of correlation of the signature positions with the biomarkers. Limitations included problems with non-constant variance with some of the biomarker models. There were also a few outliers on some of the models, mostly with CD4 count, and I anticipate that the models can be improved with regards to this variable. A non-linear relationship with CD4 count and endothelial biomarker activation was noted, which may be physiological. The models were re-run with the square-root of the CD4, but this did not make a marked difference.

CHAPTER FIVE: RESULTS

5.1 Introduction to results

In this chapter, I present the results of the analyses conducted on the case and control groups. Firstly, a comparison of the two groups at baseline is presented, looking at demographics, traditional cardiovascular risk factors, laboratory values and imaging as well as HIV-related factors. In the group with acute arterial ischaemic stroke, I describe in detail the overall profile of HIV-associated stroke in this cohort. The clinical stroke phenotype, stroke severity and stroke aetiology are reported, in order to better understand the type of arterial ischaemic stroke commonly encountered in HIV-1 Subtype-C infected South African individuals, and the factors that contribute to the progression of endothelial dysfunction to ischaemic stroke.

Thereafter the results of the sequence analysis are presented, looking at the consensus sequences of the two groups, visualisation of dataset similarity and variability, signature pattern analysis and selection pressure.

5.2 Demographics of the study cohort

Table 5.1. Demographics of study cohort

	Strokes n=58	Controls n=71	P value[a]
Mean age, years (SD)	33.0 (5.8)	33.3 (6.1)	0.7924
Gender			0.239
Male	22 (37.9)	20 (28.2)	
Female	36 (62.1)	51 (71.8)	
Ethnicity			**0.007**
Black African	52 (89.7)	71 (100.0)	
Mixed Ancestry	6 (10.3)	0	
Caucasian/other	0	0	
Unemployed*	28 (49.1)	41 (60)	0.288

Abbreviations: SD, standard deviation
Values are depicted as No. (%), unless otherwise indicated
*Missing data; pairwise deletion used in the analysis
[a]P value was calculated for categorical variables using Fisher's 2-sided exact test or Pearson's Chi-square test. P-values for continuous variables were derived with the t-test or two-sample Wilcoxon rank-sum (Mann-Whitney) test.

The two groups were similar with regards to age and gender, with a mean age of 33.0 years and 33.3 years in the stroke and control groups respectively, and a predominance of females (62.1%; 71.8%). The ethnicity profile was different between the two groups (p=0.007). The stroke group was comprised of 89.7% Black Africans, and 10.3% were individuals of Mixed Ancestry. The control group was comprised entirely of individuals of Black African ancestry. Unemployment, as a reflection of socioeconomic status, was similar between groups.

5.3 Risk factor profile

Table 5.2. Risk factor profile

	Strokes n=58	Controls n=71	P value[a]
Hypertension	11 (19)	8 (11.3)	0.220
Mean SBP, mmHg (SD)	120.8 (16.1)	120.4 (21.0)	0.9121
Diabetes	4 (6.9)	0 (0.0)	**0.039**
Mean waist circumference, cm (SD)	86.9 (13.2)	90.4 (15.7)	0.1895
Dyslipidaemia	0 (0.0)	0 (0.0)	
Smoker*	17 (29.8)	21 (29.6)	0.976
Median pack years (IQR)	7.0 (7.0)	2.5 (3.0)	**0.0111**
Stratified pack years[b]			**0.004**
<5 pack years	5 (29.4)	18 (85.7)	
5-10 pack years	7 (41.2)	1 (4.8)	
>10 pack years	5 (29.4)	2 (9.5)	
Alcohol use*	16 (28.1)	32 (45.1)	**0.048**
Median units/wk (IQR)	8.0 (24.0)	9.0 (17.5)	0.1695
Substance use	4 (6.9)	3 (4.2)	0.700
History of previous TIA	5 (8.6)	0 (0.0)	
History of previous stroke	3 (5.2)	1 (1.4)	0.3258
Known cardiac disease	2 (3.4)	0 (0.0)	0.200
History of recent infection <3mo	13 (22.4)	10 (14.1)	0.219
Type of recent infection[c]			**0.009**
Chickenpox/shingles	2 (15.4)	0 (0.0)	
Respiratory tract	7 (53.8)	2 (20)	
Tuberculosis	2 (15.4)	0 (0.0)	
Genital	0	5 (50)	
Unknown	2 (15.4)	3 (30)	
Chickenpox/shingles (<6mo)	7 (12.1)	3 (4.2)	0.112
Enrolment during pregnancy/ puerperium	1 (2.8)	0 (0.0)	
Oral contraception	1 (2.8)	2 (3.9)	

Abbreviations: cm, centimetres; mmHg, millimetres of mercury; mo, months; SBP, systolic blood pressure; SD, standard deviation; TIA, transient ischaemic attack; wk, week
Values are depicted as No. (%), unless otherwise indicated.
*Missing data; pairwise-deletion used in analysis
[a]P value was calculated for categorical variables using Fisher's 2-sided exact test or Pearson's Chi-square test. P-values for continuous variables were derived with the t-test or two-sample Wilcoxon rank-sum (Mann-Whitney) test.
[b] Expressed as a No. (%) of those who were current smokers
[c]Expressed as a No. (%) of those who reported recent infection

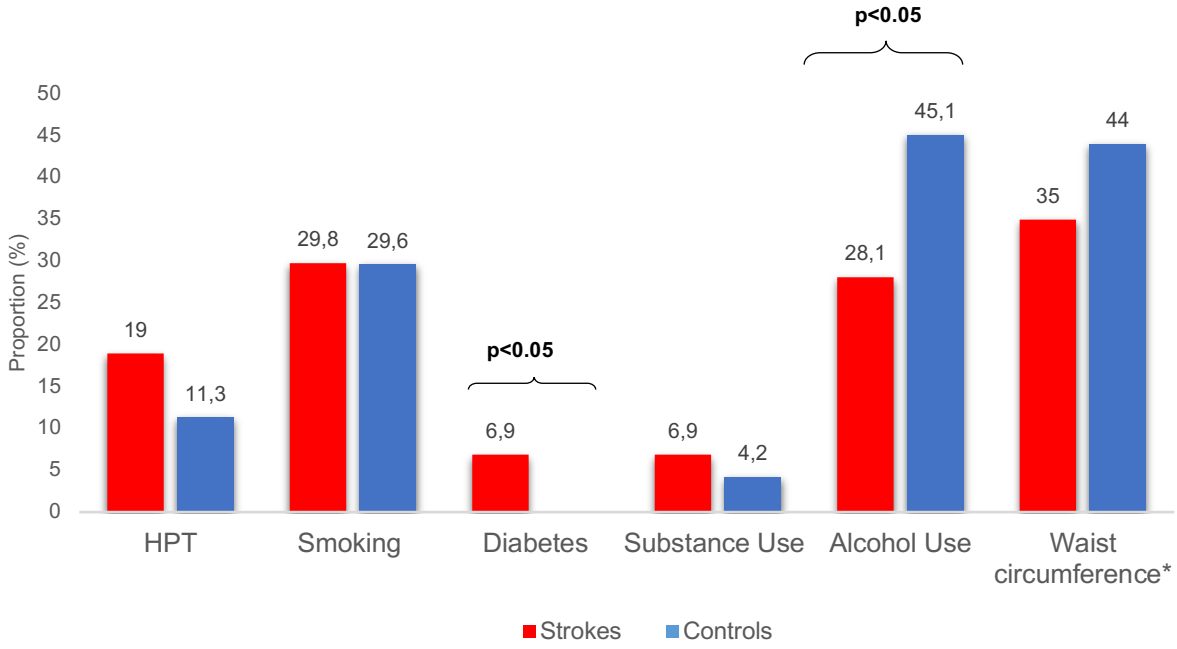


Figure 5.1. Comparison of traditional cardiovascular risk factors

Abbreviations: HPT, hypertension
**A waist circumference predictive of metabolic syndrome*

Of the known traditional risk factors for cardiovascular disease, there were no differences in hypertension (19% vs 11.3%), mean systolic blood pressure (120.8mmHg; 120.4mmHg), mean waist circumference (86.9cm vs 90.4cm), current smokers (29.8% vs 29.6%), substance use (6.9% vs 4.2%) or known cardiac disease (3.4% vs 0%) between the stroke and control groups. A waist circumference predictive of metabolic syndrome (Motala et al., 2011) was present in 38.7% of female and 28.6% of male stroke subjects, and in 49% of females and 30% of males in the control group.

The stroke group had a higher prevalence of diabetes (6.9% vs 0.0%, p=0.039) and more pack-years of smoking in comparison to the controls (41.2% and 29.4% of stroke subjects had a >5 and >10 pack-year history of smoking respectively, compared with 4.8% and 9.5% of controls, p=0.004). Alcohol use was more prominent in the control group, with 45.1% of controls reporting regular alcohol use, in comparison to 28.1% of the stroke group (p=0.048). Although 5.2% of the stroke group and 1.4% of the control group reported a previous stroke, this difference was not statistically significant.

There was no difference in the number of participants reporting chicken pox or shingles in the 6 months preceding enrolment (12.1% of the stroke group;

4.2% of controls). However, the two groups reported different types of infection in the 3 months preceding enrolment (p=0.009). Of those who reported a recent infection, respiratory tract infection (53.8%), chicken pox or shingles (15.4%) and tuberculosis (15.4%) were more common in the stroke group, whilst genital infections (50%), unknown infections (30%) and respiratory tract infections (20%) predominated in the controls.

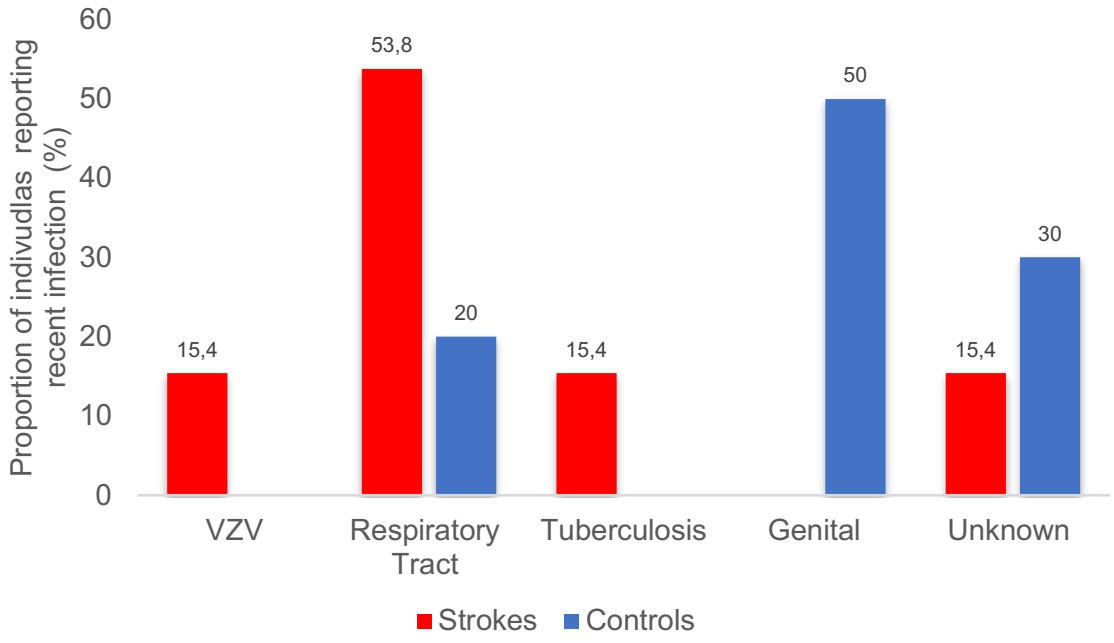


Figure 5.2. Type of infection reported in the three months preceding enrolment

5.4 Laboratory values and imaging

Table 5.3. Laboratory values and imaging

	Strokes n=58	Controls n=71	P-value[a]
Fasting lipogram*	**n=50**	**n=16**	
Mean total cholesterol, mmol/L (SD)	4.15 (1.47)	3.18 (0.56)	**0.0004**
Mean LDL, mmol/L (SD)	2.58 (1.39)	1.80 (0.53)	**0.0045**
Mean Trigs, mmol/L (SD)	1.25 (0.56)	0.69 (0.34)	**0.0001**
Mean HDL, mmol/L (SD)	0.98 (0.36)	1.01 (0.50)	0.7657
Serum RPR/TPHA positive	4 (6.9)	0 (0.0)	**0.034**
APLA screen positive	8 (13.8)	0 (0.0)	
Autoimmune vasculitis screen positive	4 (6.9)	0 (0.0)	
Carotid Intima-Media Thickness*	**n=48**	**n=49**	
Mean CIMT, mm (SD)	0.50 (0.9)	0.56 (0.9)	**<0.001**
Cerebrospinal fluid analysis	**n=54**	**n=3**	
Mean CSF polymorphs, cells/mm^3 (SD)	0.81 (5.0)	0.0 (0.0)	
Mean CSF lymphocytes, cells/mm^3 (SD)	23.4 (82.9)	17.7 (20.9)	
Mean CSF glucose, mmol/L	2.97 (0.5)	3.1 (0.4)	
Mean CSF protein, g/dL	0.49 (0.3)	0.55 (0.3)	
CSF FTA positive	4 (7.4)	0.0 (0.0)	
CSF VZV PCR positive	6 (11.1)	0.0 (0.0)	
Full Blood Count*			
Mean Hb, g/dL (SD)	11.7 (2.1)	12.4 (1.8)	0.0670
Mean WCC, cell x 10^9/L (SD)	6.35 (3.39)	5.06 (1.62)	**0.0117**
Mean Platelets, cells x 10^9/L (SD)	317.5 (128)	263.9 (82.5)	**0.0085**

Abbreviations: APLA, antiphospholipid antibody; BP, blood pressure; CIMT, carotid intima-media thickness; CSF, cerebrospinal fluid; Hb, haemoglobin; HDL, high density lipoprotein; LDL, low density lipoprotein; RPR, rapid plasma reagin; TB, tuberculosis, TIA, transient ischaemic attack; Trigs, triglycerides; WCC, white cell count
Values are depicted as No. (%), unless otherwise indicated.
*Missing data: pairwise deletion used in analysis
[a]*P value was calculated for categorical variables using Fisher's 2-sided exact test or Pearson's Chi-square test. P-values for continuous variables were derived with the t-test or two-sample Wilcoxon rank-sum (Mann-Whitney) test.*
.

Laboratory values and carotid duplex Doppler for assessment of cardiovascular risk differed between the two groups. Mean total cholesterol (4.15 mmol/l vs 3.18 mmol/l, p=0.0004), LDL (2.58 mmol/l vs 1.80 mmol/l, p=0.0045) and triglycerides (1.25 mmol/l vs 0.69 mmol/l, p=0.0001) were higher in the stroke group compared with the control group. 6.9% of the stroke group and none of the controls tested positive for syphilis (p=0.034). Screening for antiphospholipid antibody syndrome and vasculitis was positive in 13.8% and 6.9% of the stroke group respectively, whilst none of the control group had a positive screening test for these conditions. Mean carotid intima-media thickness was greater in the control group (0.56mm vs 0.50mm, p<0.001). When a binary logistic regression model was fitted to evaluate CIMT, controlled for age and waist circumference, results showed that for every unit increase in CIMT, the odds of stroke are expected to decrease by a factor of 0.224 (77.6%; SE=0.085, p<0.001).

Lumbar puncture was performed in 54/58 individuals in the stroke group, and 3/71 individuals in the control group. 7.4% and 11.1% of the stroke group were positive for neurosyphilis and varicella zoster virus PCR, respectively. The small number of individuals in the control group precluded any meaningful comparisons between the CSF variables. There were differences in the white cell and platelet counts between the two groups, but neither of these was outside of the local laboratory's normal reference ranges.

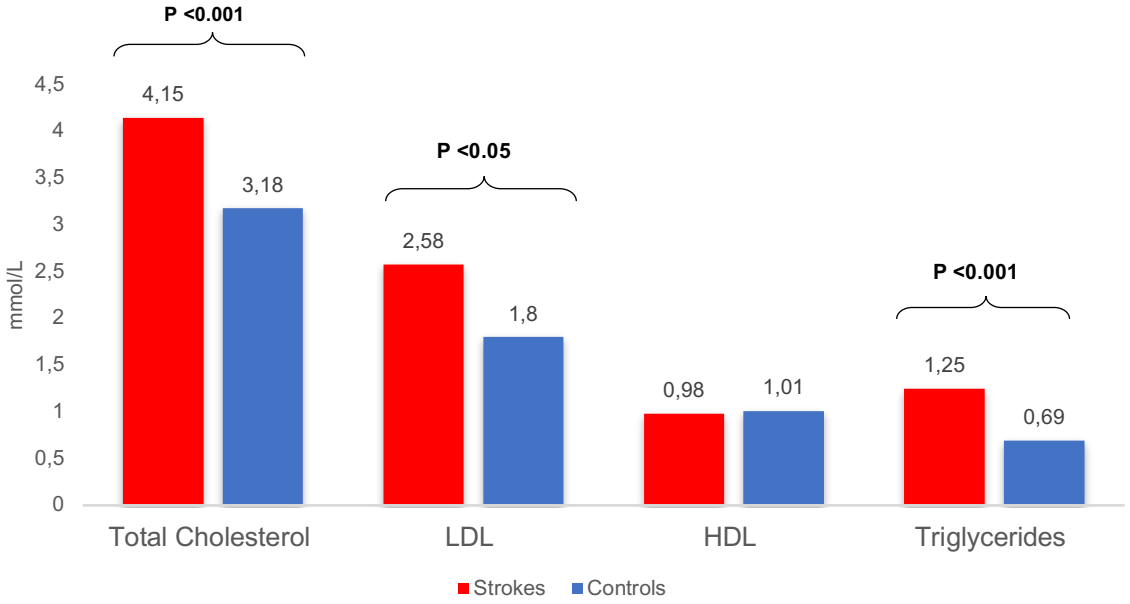


Figure 5.3. Fasting lipogram values

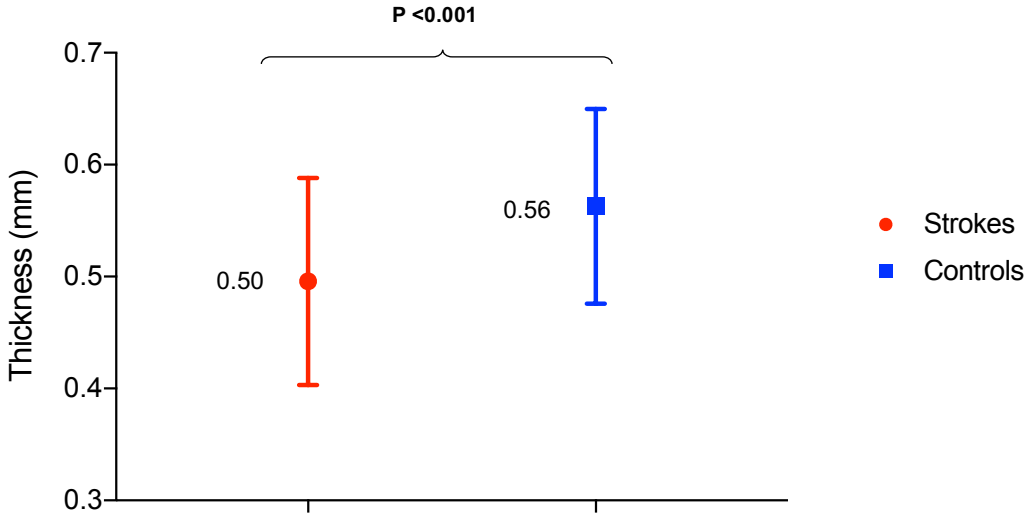


Figure 5.4. Carotid intima-media thickness, mean with SD

5.5 HIV-related factors

Table 5.4. HIV-related factors

	Strokes n=58	Controls n = 71	P-value[a]
Antiretroviral therapy			
Prior ART	27 (46.6)	36 (50.7)	0.639
Median duration ART, months (IQR)	12.5 (37)	27 (11.5)	0.2024
Default ART[b]	7/27 (25.9)	0 (0.0)	**0.003**
Viral load			
Median viral load, log_{10} copies/ml (range)	4.58 (0-6.38)	4.13 (0-4.84)	0.28
Viral suppression on ART (<20 copies/ml)	10/20 (50)	19/36 (52.8)	
CD4+ T-lymphocyte count			
Median CD4 count, cells/μl (IQR)*	208.5 (106-382)	322.5 (182.5-483.8)	**0.0121**
Median CD4 nadir, cells/μl (IQR)	112 (34-270.8)	179 (130-304)	**0.0068**
Median CD4 on treatment, cells/μl	288.5	305	0.3359
Median CD4 count off treatment, cells/μl	207	327.5	**0.0402**
Documented CD4 count < 6 mo prior to stroke	18/58 (31)		
Drop in CD4 count >10cells/μl post-stroke	5/18 (27.8)		

Abbreviations: ART, antiretroviral therapy
Values are depicted as No. (%), unless otherwise indicated
*Missing data; pairwise deletion used in analysis
[a]*P value was calculated for categorical variables using Fisher's 2-sided exact test or Pearson's Chi-square test. P-values for continuous variables were derived with the t-test or two-sample Wilcoxon rank-sum (Mann-Whitney) test.*
[b]*Expressed as No. (%) of those previously on ART*

A similar proportion of the stroke and control groups (46.6%; 50.7%) had been exposed to ART prior to enrolment. Treatment duration for the controls was more than twice that of the stroke group (median of 27 months vs 12.5 months, p=0.2024). In the stroke group, 25.9% of those who had been on prior ART had defaulted treatment, whilst none of the controls had interrupted therapy (p=0.003).

The stroke group had a lower CD4 count compared with the controls (median 208.5 cells/μl vs 322.5 cells/μl, p=0.012). Individuals with stroke also had a lower CD4 nadir than the controls (median 112 cells/μl vs 179 cells/μl, p=0.008). Untreated individuals in the stroke group had a lower CD4 count than untreated controls (median 207 cells/μl, vs 327.5 cells/μl, p=0.04). 31% of stroke patients had a CD4 count documented at their local clinic within 6 months prior to their stroke. Of those, 27.8% had a drop in their CD4 count >10

cells/μl after their stroke. In the stroke group, median viral load was 4.58 log_{10} copies/ml and 50% of those on ART were virally suppressed. Amongst the controls, median viral load was 4.13 log_{10} copies/ml, with effective viral suppression in 52.8% of those on ART.

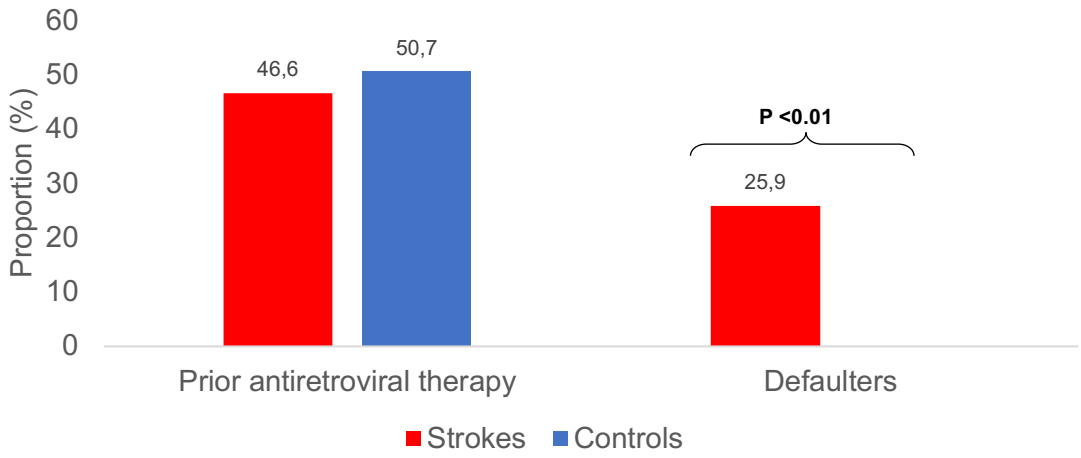


Figure 5.5. Treatment status.

Defaulters are expressed as the proportion of those who had been on prior ART, who had interrupted treatment at the time of enrolment.

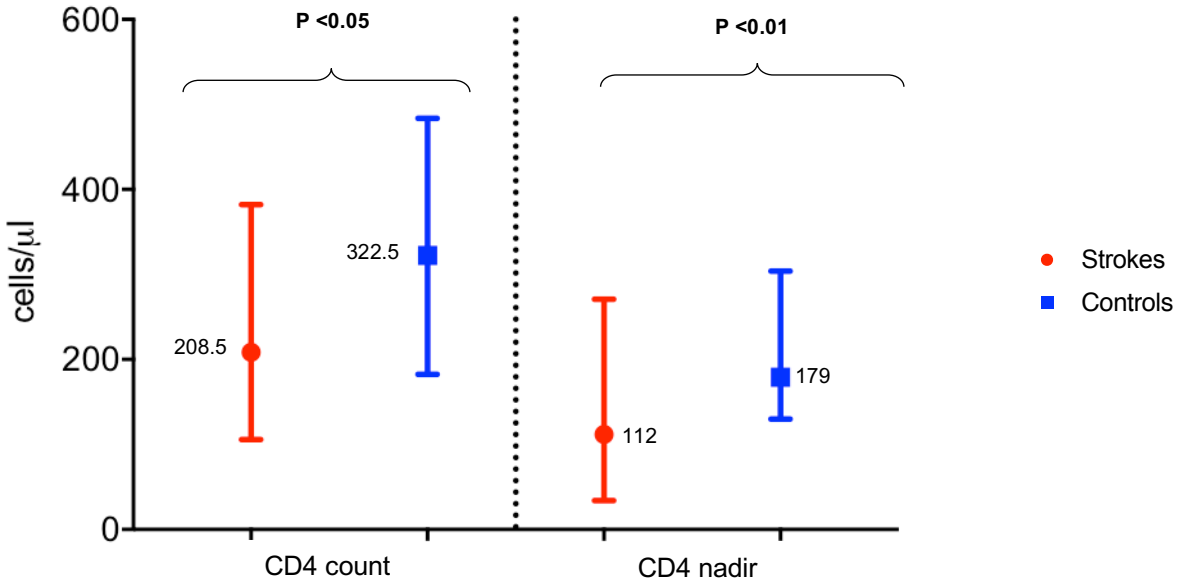


Figure 5.6. CD4 count and CD4 nadir, median with IQR

5.6 Biomarkers of endothelial activation and inflammation

The stroke group had higher median VCAM-1, TNF-α, VEGF, MCP-1, IL-6 and IL-10 in comparison to the controls. Median E-selectin, Endothelin-1, ICAM-1 and IL-β were not different between the groups.

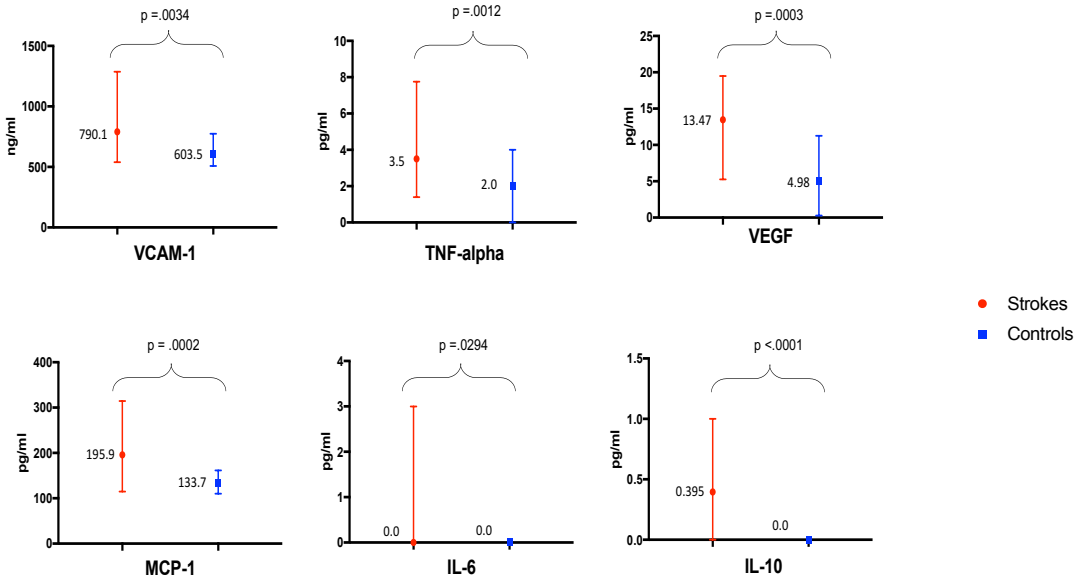


Figure 5.7. Endothelial biomarkers that were statistically significantly different between the stroke and control groups, median with IQR

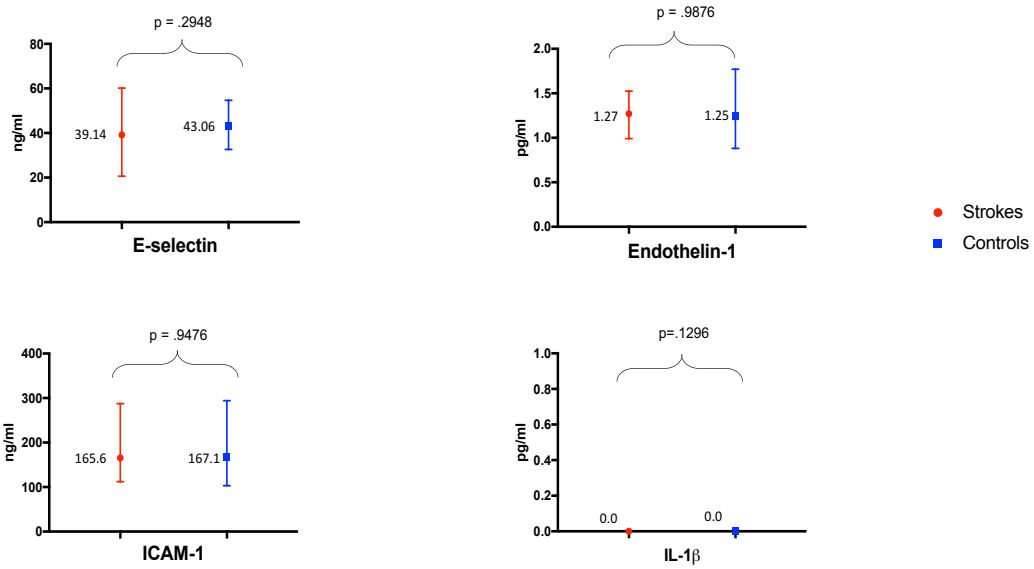


Figure 5.8. Endothelial biomarkers that were not statistically significantly different between the stroke and control groups, median with IQR

5.7 Stroke characteristics

Table 5.5. Type, severity and aetiology of HIV-associated-stroke

	Strokes n= 58
Oxfordshire Community Stroke Project Classification	
PACI	40 (69)
TACI	10 (17.2)
LACI	3 (5.2)
POCI	5 (8.6)
Stroke Severity Scores	
Mean NIHSS (SD)	10.5 (7.1)
Modified Rankin Scale (mRS)	
mRS 0	1 (1.7)
mRS 1	5 (8.6)
mRS 2	9 (15.5)
mRS 3	10 (17.2)
mRS 4	24 (41.4)
mRS 5	9 (15.5)
mRS 6	0
Died	3 (5.2)
Cause of Stroke	
Non-atherosclerotic vasculopathy	14 (24.1)
Opportunistic infections	
VZV	9 (15.5)
TB	0 (0.0)
Cryptococcal meningitis	0 (0.0)
Syphilis	4 (6.9)
Cardioembolism	9 (15.5)
HIV-associated vasculitis	4 (6.9)
Small vessel disease	3 (5.2)
Accelerated atherosclerotic vasculopathy	3 (5.2)
Cryptogenic	3 (5.2)
Incomplete evaluation	6 (10.3)
Other determined cause	3 (5.2)
Confirmed cause	40 (68.97)
Probable Cause	9 (15.5)
Unknown cause	9 (15.5)

Abbreviations: LACI, lacunar infarction; mRS, modified Rankin Scale; NIHSS, National Institutes of Health Stroke Scale; PACI, partial anterior circulation infarction; POCI, posterior circulation infarction; TACI, total anterior circulation infarction; TB, tuberculosis; VZV, varicella zoster virus;
Cause of stroke determined using the algorithm for arterial ischemic stroke in HIV (Benjamin, Bryer, et al., 2016)
Values are depicted as No. (%), unless otherwise indicated

By Oxfordshire Community Stroke Project Classification, the clinical features of which were verified by brain imaging, 69% of the stroke group had features of partial anterior circulation infarction. The most common modified Rankin Scale Score was 4 (41.4% of individuals). 5.2% of stroke participants died.

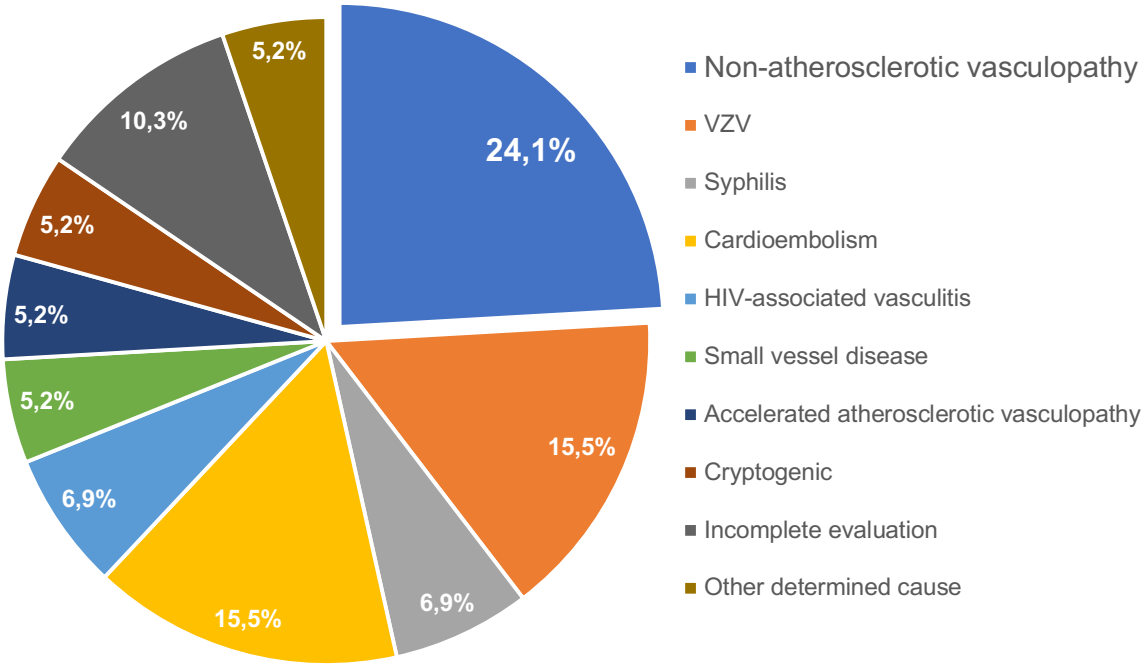


Figure 5.9. Stroke aetiology in the 58 HIV-infected participants with acute arterial ischaemic stroke. Non-atherosclerotic vasculopathy was the most frequent cause of stroke.

The most frequent cause of stroke was non-atherosclerotic vasculopathy (24.1%), followed by VZV (15.5%) and cardio-embolism (15.5%). Of the stroke cases that underwent a complete evaluation, 48.1% were due to a form of HIV-associated vasculopathy or cryptogenic stroke. Strokes due to other mechanisms accounted for 51.9% of those who had a complete evaluation.

Table 5.6. Stroke participants separated by aetiology

Strokes due to HIV-associated vasculopathy (n=25)	Strokes due to alternative mechanisms (n=27)
• Non-atherosclerotic vasculopathy (n=14) • HIV-associated vasculitis (n=4) • Accelerated atherosclerotic vasculopathy (n=3) • Cryptogenic (n=3) • Small vessel disease without hypertension (n=1)	• Opportunistic infections (n=13) • Cardio-embolism (n=9) • Other determined cause (n=3) • Small vessel disease with hypertension (n=2)

Table 5.7. HIV-related factors in stroke participants separated by aetiology

	HIV-associated vasculopathy n=25	Alternative mechanisms n =27	P-value[a]
Antiretroviral therapy			
Prior ART	14 (56.0)	11 (40.7)	0.4051
Defaulted ART[b]	5/14 (35.0)	1/11 (9.1)	0.1804
Viral load			
Median viral load, log_{10} copies/ml (range)	3.61 (0-6.22)	4.79 (0-5.49)	0.3206
CD4+ T-lymphocyte count			
Median CD4 count, cells/μl (range)	207 (6-711)	271 (6-926)	0.0623

Abbreviations: ART, combined antiretroviral therapy
Values are depicted as No. (%), unless otherwise indicated
[a]P value was calculated for categorical variables using Fisher's 2-sided exact test or Pearson's Chi-square test. P-values for continuous variables were analysed with the t-test or two-sample Wilcoxon rank-sum (Mann-Whitney) test.
[b]Expressed as No. (%) of those previously on ART

There was no statistically significant difference in the proportion of patients on ART, median viral load or median CD4 counts between the stroke groups separated by aetiology. The observed trend was towards a lower CD4 count, with more ART defaulters in the individuals with stroke due to HIV-associated vasculopathy.

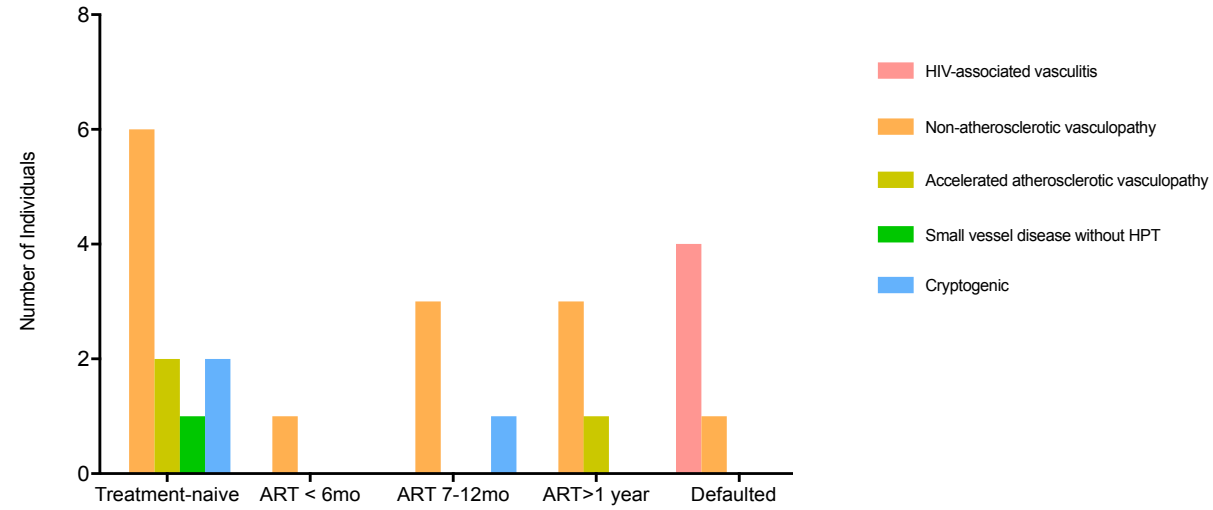


Figure 5.10. Treatment status of participants with stroke due to HIV-associated vasculopathy

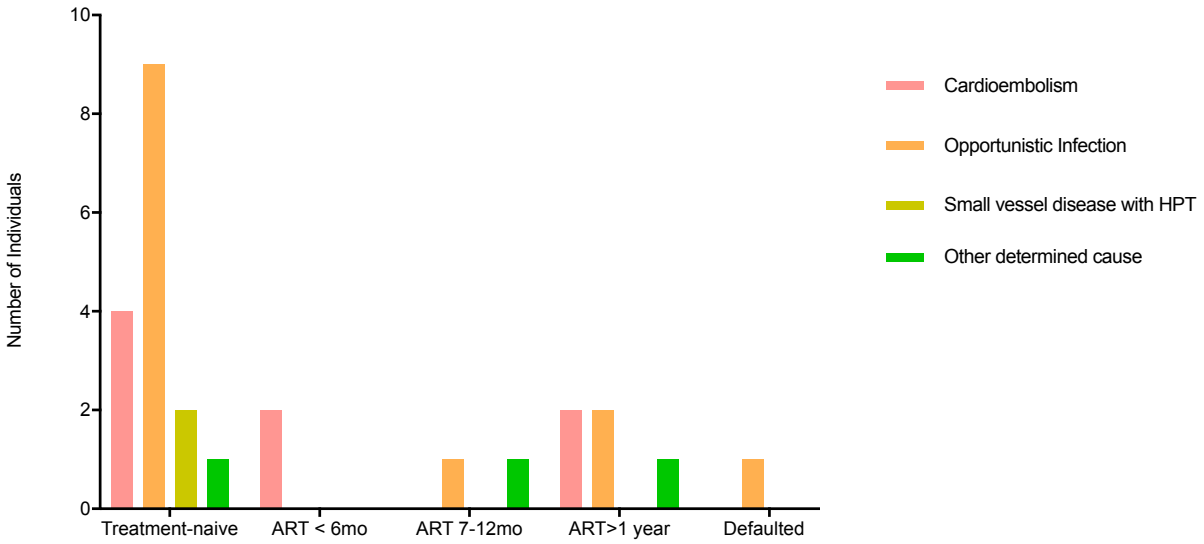


Figure 5.11. Treatment status of participants with stroke due to alternative mechanisms

In the HIV-associated vasculopathy group, most participants were treatment-naïve. 42.9% of those with strokes due to non-atherosclerotic vasculopathy were treatment-naïve, with an additional 42.9% on treatment for 7-12 months or >1 year. All of those with strokes due to HIV-associated vasculitis had defaulted treatment. In the group with strokes due to alternative mechanisms,

69.2% of those with opportunistic infections were treatment-naïve, with none having started ART in the prior 6 months.

Figure 5.12 demonstrates that the strokes due to non-atherosclerotic vasculopathy and HIV-associated vasculitis, and opportunistic infections, comprising 31% and 22.4% of all strokes respectively, occurred at lower median CD4 counts compared with other stroke causes (p=0.0458*).

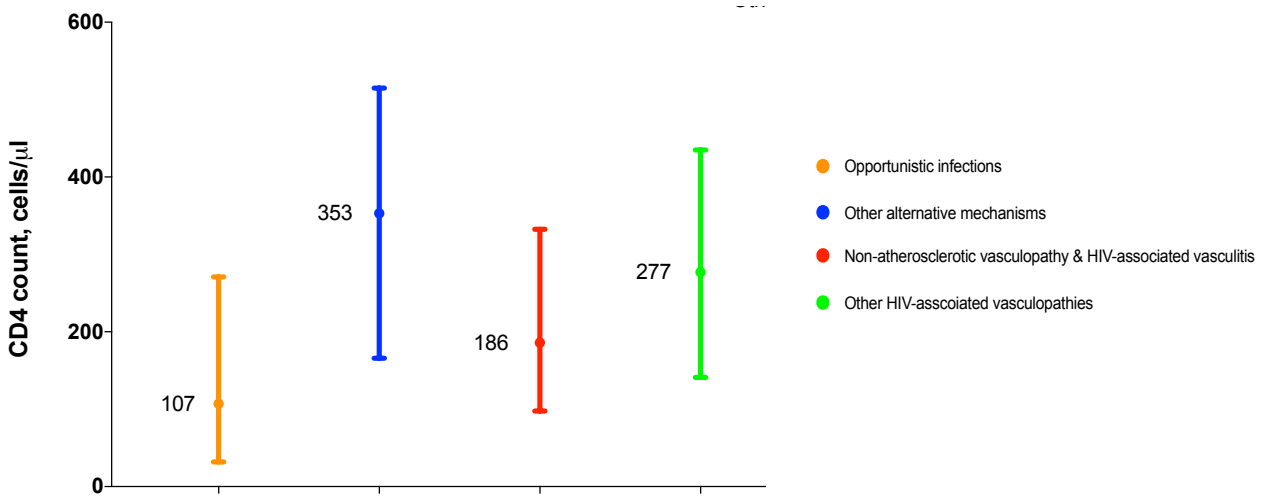


Figure 5.12. Median CD4 counts of strokes by aetiology
*Kruskal-Wallis test, approximate p-value

5.8 Consensus sequences and dataset similarity

5.8.1 Consensus sequence comparisons for the stroke and control groups

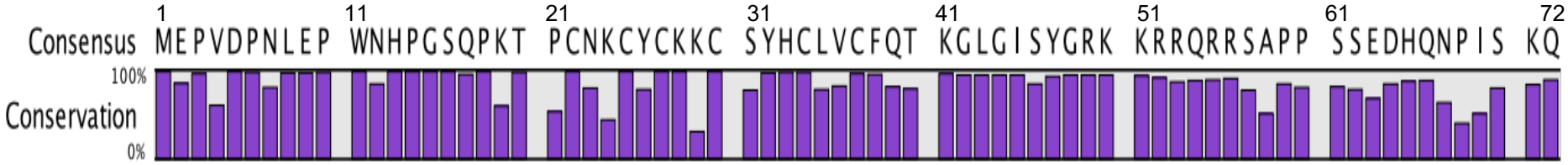


Figure 5.13. Consensus sequence of all participants with the histogram depicting amino acid variability at each position. Positions 24, 29 and 68 show more than 50% variability.

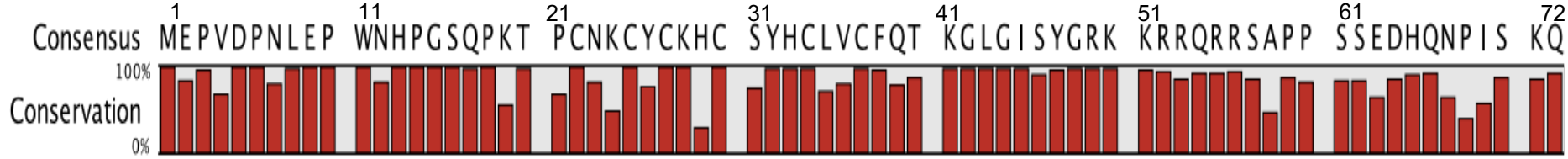


Figure 5.14. Consensus sequence of stroke group with the histogram depicting amino acid variability at each position. Positions 24, 29, 58 and 68 show more than 50% variability.

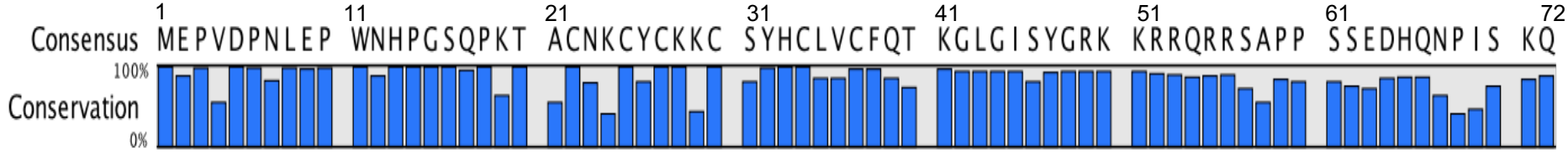


Figure 5.15. Consensus sequence of control group with the histogram depicting amino acid variability at each position. Positions 24, 29, 68 and 69 show more than 50% variability.

5.8.2 Consensus sequence comparisons for the strokes divided by aetiology

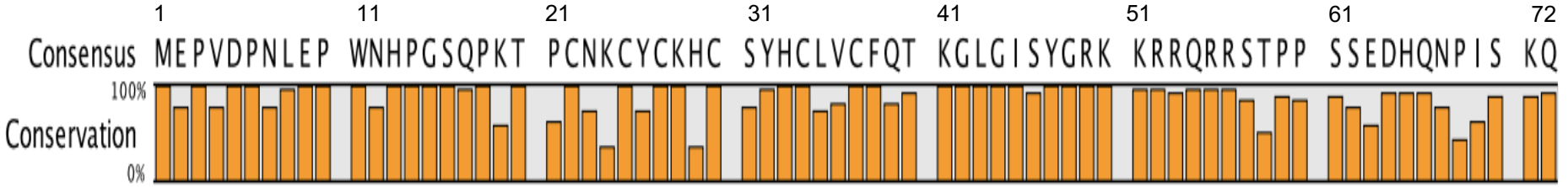


Figure 5.16. Consensus sequences of strokes due to alternative mechanisms, with the histogram depicting amino acid variability at each position. Positions 24, 29, 58 and 68 show more than 50% variability.

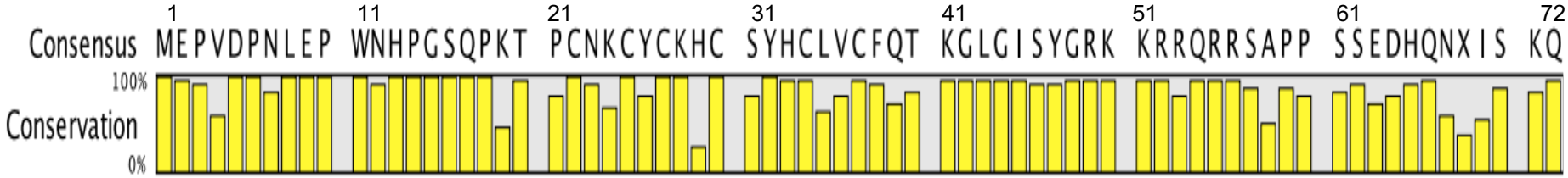


Figure 5.17. Consensus sequence of strokes due to HIV-associated vasculopathy, with the histogram depicting amino acid variability at each position. Positions 19, 29, 58 and 68 show more than 50% variability. X indicates an ambiguous symbol where no majority amino acid can be found.

5.8.3 Visualisation of stroke sequences relative to control consensus

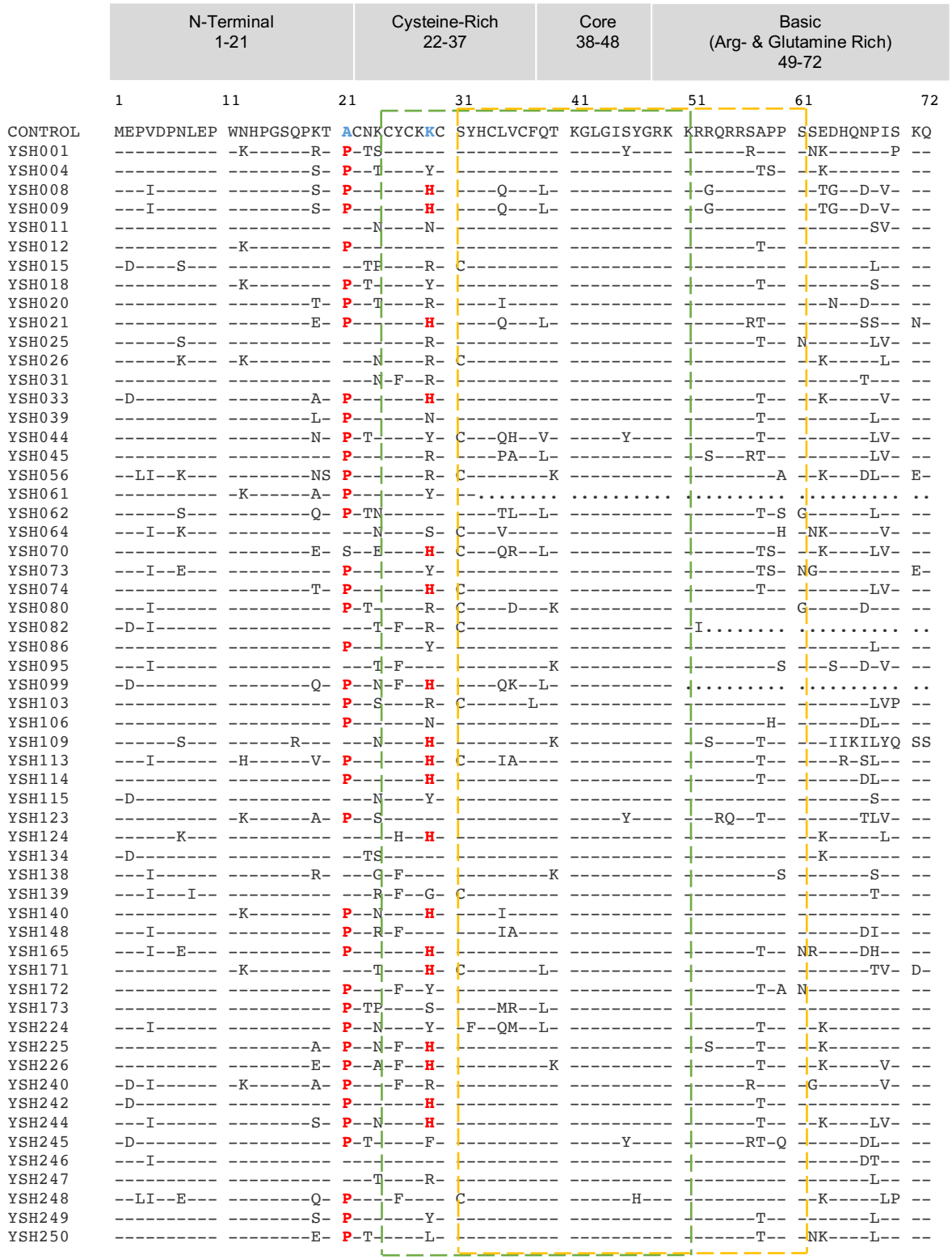


Figure 5.18. SeqPublish alignment of the stroke sequences (each participant identified by YSH…) in relation to the consensus sequence of the control dataset at the top of the figure. Identical residues are represented with dashes. The red highlighted letters indicate the signature amino acids for the stroke group. The green boxed area represents the chemotactic domain. The yellow boxed area represents the neurotoxic domain.

5.9 Signature pattern analysis

5.9.1 Signature pattern analysis of stroke and control groups

Signature pattern analysis identified two signature amino acids in the stroke group relative to the controls. At position 21, the signature amino acid for the stroke group was proline. The most common amino acid at this position in the control group was alanine (p=0.003). At position 29, the signature amino acid for the stroke group was histidine. The most common amino acid at this position in the control group was lysine (p=0.0002).

Table 5.8. Signature amino acids identified by VESPA for the stroke group relative to the control group. The p-value was derived with Fisher's exact test

Signature amino acids for stroke group	**P**	**H**
Frequency among stroke set	0.690	0.310
Frequency among control set	0.437	0.113
Control amino acids	**A**	**K**
Frequency among stroke set	0.293	0.172
Frequency among control set	0.563	0.451
Alignment position	**21**	**29**
Mutation	**A21P**	**K29H**
P-value	0.0030	0.0002

5.9.2 Signature pattern analysis of strokes separated by aetiology

Signature pattern analysis identified one signature amino acid that was unique to the strokes due to alternative mechanisms, relative to the strokes due to HIV-associated vasculopathy. At position 58, threonine was the signature amino acid for the strokes due to alternative mechanisms, whilst alanine was the most common amino acid at this position in the strokes due to HIV-associated vasculopathy (p=0.571).

Table 5.9. Signature amino acid residue identified by VESPA for the strokes due to alternative mechanisms relative to the strokes due to HIV-associated vasculopathy. The p-value was derived with Fisher's exact test.

Signature amino acids for strokes of alternative mechanisms	**T**
Frequency among alternative mechanisms set	0.538
Frequency among HIV-associated vasculopathy set	0.458
HIV-associated vasculopathy amino acids	**A**
Frequency among alternative mechanisms set	0.462
Frequency among HIV-associated vasculopathy set	0.542
Alignment position	**58**
Mutation	**A58T**
P-value	0.571

5.9.3 Visualisation of signature amino acids identified by VESPA

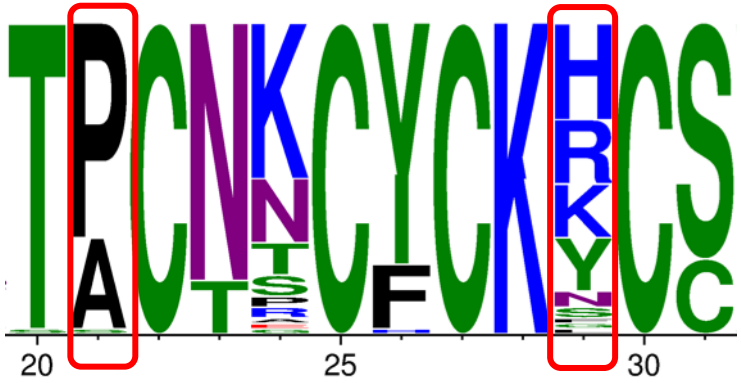


Figure 5.19. Weblogo depicting amino acids representing stroke group sequences from position 20 to 31. The signature positions are highlighted with a red box.

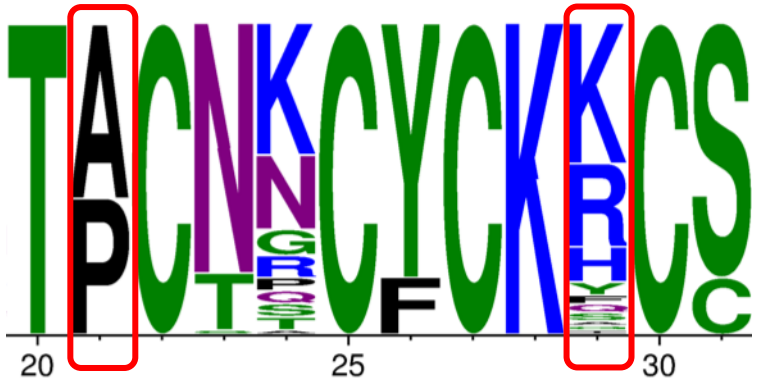


Figure 5.20. Weblogo depicting amino acids representing control group sequences from position 20 to 31. The signature positions are highlighted with a red box.

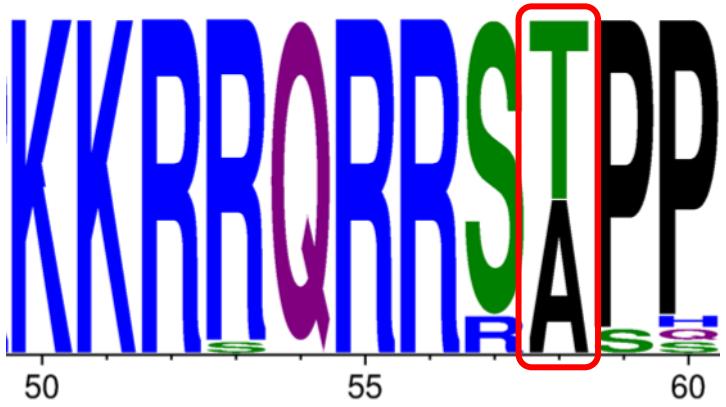


Figure 5.21. Weblogo depicting amino acids representing strokes due to alternative mechanisms from position 50 to 60. The signature position is highlighted with a red box.

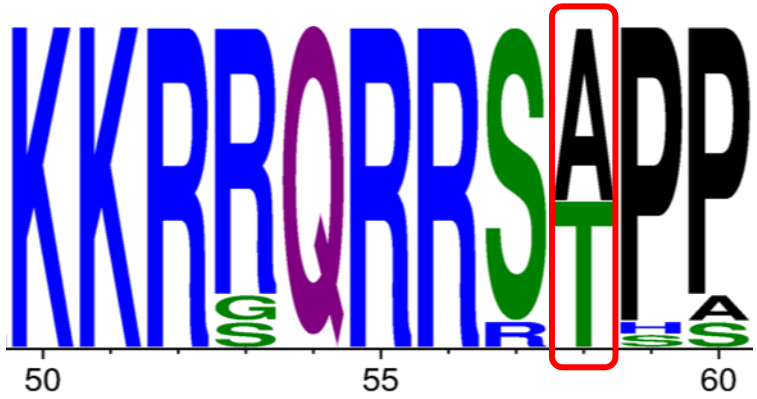


Figure 5.22. Weblogo depicting amino acids representing strokes due to HIV-associated vasculopathy from position 50 to 60. The signature position is highlighted with a red box.

5.10 Selection pressure on the stroke and control groups

5.10.1 Selection pressure on the stroke group

SLAC (Job ID upload.331776652031340.1) found 8 positively-selected sites in the stroke group. Of the signature residues, position 21 was a neutral site in the stroke group, with no significant difference in rates of non-synonymous and synonymous substitutions at that position. Positions 29 and 58 were under positive selection in the stroke group.

At position 29, most substitutions were between histidine and tyrosine, with the assumption that histidine was replaced by tyrosine. However, the phylogenetic tree produced by the SLAC analysis is unrooted, so the directionality of the substitutions is uncertain.

At position 58, all non-synonymous substitutions were between alanine and threonine, with 62.5% of transitions away from threonine towards alanine, but once again, the unrooted phylogenetic tree means that reversed directionality is also possible.

Table 5.10. Positions in the stroke group under positive selection pressure

Position under positive selection pressure	Synonymous Changes	Non-synonymous changes	P-value[a]
2	0	8	0.08
4	0	7	0.09
19	3	33	0.07
29*	5.33	41.67	0.07
36	1	13	0.03
58**	0	8	0.04
68	0	25	0.00
69	0	15	0.03

[a] Probability of observing as many or fewer synonymous changes, computed using an extended binomial distribution. Interpreted as the p-value for positively selected sites.

*Signature residue identified on signature pattern analysis between strokes and controls.

**Signature residue identified on signature pattern analysis within the stroke group.

5.10.2 Selection pressure on the control group

SLAC (Job ID upload.113973080424211.1) found 7 positively-selected sites in the stroke group. Of the signature residues between the strokes and controls, position 21 was under positive selection, whilst position 29 was a neutral site in the control group, with no significant difference in rates of non-synonymous and synonymous substitutions (see section 2.8.2.1).

At position 21, all non-synonymous substitutions were between alanine and proline, with the majority of transitions estimated to be from alanine to proline. However, the unrooted phylogenetic tree produced by the SLAC analysis is unrooted, so the directionality of the substitutions is uncertain.

Table 5.11. Positions in the control group under positive selection pressure

Position under positive selection pressure	Synonymous Changes	Non-synonymous changes	P-value[a]
4	0	15	0.01
21*	1	11	0.05
58	0	10	0.02
67	0	14	0.02
68	0	19	0.00
69	0	22	0.00
70	1	8	0.08

[a] Probability of observing as many or fewer synonymous changes, computed using an extended binomial distribution. Interpreted as the p-value for positively selected sites.

*Signature residue identified on signature pattern analysis between strokes and controls.

5.11 Correlation of signature positions with biomarkers of endothelial activation and inflammation

Results of the linear and logistic regression models are presented below. The models controlled for stroke, age, current CD4 count and treatment status.

Proline at position 21 had a positive association with IL-6, relative to the other amino acids at that position (p=0.04). Proline was the signature amino acid for the stroke group at position 21. Lysine at position 29 had a negative effect on MCP-1, relative to the other amino acids at that position (p=0.05). Lysine was the most common amino acid in the control group at this position.

Table 5.12. Correlation of signature positions with E-selectin

Position	Coefficient	P-value (P>|t|)	Model Parameters
21P	-0.2941257	0.952	F (9, 106) = 0.75
29H	-3.031126	0.624	
29K	-4.078011	0.496	Prob > F = 0.6634
29R	-0.9486931	0.876	
58A	-6.188187	0.197	R^2 = 0.0598

Table 5.13 Correlation of signature positions with VCAM-1

Position	Coefficient	P-value (P>|t|)	Model Parameters
21P	0.0015189	0.987	F (9, 106) = 2.28
29H	0.0519273	0.667	
29K	-0.078646	0.501	Prob > F = 0.0225
29R	-0.1256439	0.292	
58A	-0.0737015	0.431	R^2 = 0.1620

Table 5.14. Correlation of signature positions with ICAM-1

Position	Coefficient	P-value (P>|t|)	Model Parameters
21P	0.1147528	0.519	F (9, 106) = 0.70
29H	-0.101676	0.648	
29K	-0.0867685	0.688	Prob > F = 0.7082
29R	0.017948	0.935	
58A	0.0403682	0.815	R^2 = 0.0560

Table 5.15. Correlation of signature positions with TNF-α

Position	Coefficient	P-value (P>|t|)	Model Parameters
21P	-0.047877	0.831	F (9, 74) = 2.23
29H	-0.0196558	0.942	
29K	0.1575572	0.551	Prob > F = 0.0294
29R	-0.0179	0.947	
58A	-0.1904476	0.368	R^2 = 0.2131

Table 5.16. Correlation of signature positions with MCP-1

Position	Coefficient	P-value (P>|t|)	Model Parameters
21P	0.1261866	0.276	F (9, 105) = 3.21
29H	-0.1241808	0.393	
29K	-0.2772934	0.051	Prob > F = 0.0018
29R	-0.1560142	0.278	
58A	0.0261245	0.815	R^2 = 0.2155

Table 5.17. Correlation of signature positions with Endothelin-1

Position	Coefficient	P-value (P>|t|)	Model Parameters
21P	-0.0575325	0.647	F (9, 106) = 1.57
29H	0.1943985	0.219	
29K	0.0219021	0.886	Prob > F = 0.1349
29R	0.0084944	0.956	
58A	-0.063859	0.601	R^2 = 0.1174

Table 5.18. Correlation of signature positions with VEGF

Position	Coefficient	P-value (P>|t|)	Model Parameters
21P	-0.1414024	0.560	F (9, 83) = 1.88
29H	0.4054982	0.148	
29K	0.2856484	0.324	Prob > F = 0.0662
29R	0.1498331	0.608	
58A	0.0717865	0.753	R^2 = 0.1693

Table 5.19. Correlation of signature positions with IL-6

Position	Coefficient	P-value (P>|t|)	Model Parameters
21P	1.179238	0.035	LR chi^2 (9) = 8.82
29H	-0.0686666	0.912	
29K	-0.0955874	0.883	Prob > chi^2 = 0.4542
29R	-0.0272182	0.966	
58A	0.2524991	0.616	

The Hosmer-Lemeshow test showed that this model was a good fit. (p =0.6796)

Table 5.20. Correlation of signature positions with IL-10

Position	Coefficient	P-value (P>|t|)	Model Parameters
21P	-0.0082811	0.987	LR chi^2 (9) = 19.14
29H	-0.6145017	0.323	
29K	-0.5116046	0.412	Prob > chi^2 = 0.0240
29R	-0.5305942	0.397	
58A	-0.4788905	0.332	

The Hosmer-Lemeshow test showed that this model was a good fit. (p =0.8825)

5.12 Summary of results

5.12.1 Cohort demographics

The HIV-infected stroke group and HIV-infected non-stroke controls were well-matched with regards to age and gender. The ethnic profile differed between the groups, with the presence of individuals of Mixed Ancestry in the stroke group.

5.12.2 Traditional cardiovascular risk factors

The stroke group had a higher prevalence of diabetes, higher fasting lipid values, and more smoking pack-years than the control group. The control group had a greater carotid intima-media thickness than the stroke group, and a higher prevalence of ethanol use. The groups were otherwise comparable with regards to classical cardiovascular risk factors.

5.12.3 HIV-related factors

Individuals in the stroke group were statistically significantly more immunocompromised than their non-stroke counterparts, with a history of treatment interruption. There was no difference in the proportion of those on antiretroviral therapy, or the duration of therapy between the groups.

5.12.4 Biomarkers of endothelial dysfunction and inflammation

The stroke group had higher median VCAM-1, TNF-α, VEGF, MCP-1, IL-6 and IL-10 than the controls. Median E-selectin, Endothelin-1, ICAM-1 and IL-1β did not differ between the groups.

5.12.5 Characteristics of ischaemic stroke in South African Subtype-C infected individuals

Ischaemic stroke in this cohort of Subtype-C HIV-infected individuals were moderately severe, and predominantly a result of medium- to large-vessel disease. HIV-associated vasculopathy and opportunistic infections were the

most common causes of stroke and occurred in individuals who were mostly treatment-naïve, had defaulted treatment, or were on ART for >1 year.

5.12.6 Signature patterns in stroke and control groups

Two signature amino acids were unique to the stroke group relative to the controls. Proline was the signature residue at position 21 in the stroke group, whilst alanine was the most common amino acid at that position in the controls. Histidine was the signature residue for the stroke group at position 29, whilst lysine was the most common amino acid in the control group at the same position.

5.12.7 Signature patterns in strokes of different aetiologies

Threonine at position 58 was the signature amino acid for the individuals with strokes due to mechanisms other than HIV-associated vasculopathy. Those with strokes due to HIV-associated vasculopathy had alanine as the most common amino acid at this position.

5.12.8 Positive selection pressure

Positive selection pressure was detected at 8 sites in the stroke group, and 7 sites in the control group. Positions 29 and 58 were under positive selection in the stroke group, whilst position 21 was under positive selection in the controls.

5.12.9 Effect of Tat signature residues on biomarkers of endothelial dysfunction and inflammation

Proline at position 21 was associated with a relative increase in IL-6 compared with alanine and other amino acids at this position. Lysine at position 29 was associated with a relative decrease in MCP-1 compared with histidine and other amino acids at that position.

CHAPTER SIX: DISCUSSION

6.1 Introduction to discussion

This study investigated the potential role of the HIV-1 Tat protein in HIV-associated endothelial dysfunction and ischaemic stroke in a cohort of young HIV-1 Subtype-C-infected individuals in Cape Town, South Africa. Primarily, it sought to describe the amino acid profile of the Tat protein in a group of young HIV-infected individuals with and without acute ischaemic stroke. Furthermore, it sought to characterize the amino acid profile of the Tat protein in strokes of differing aetiology, as well as to establish whether certain positions and amino acid residues in the Tat protein exon 1 were significantly associated with biomarkers of endothelial dysfunction and inflammation measured in the participants.

The following sections review the demographic profile of the two groups at baseline, before discussing the results in relation to the study hypotheses.

6.2 Demographics of the cohort

The two groups were well-matched in terms of age and gender but differed in ethnicity. The absence of individuals of other ethnicities in the control group reflected the geographically-limited sampling. Most areas of Cape Town still have spatial segregation of ethnic groups (Statistics South Africa, 2011b; van Rooyen & Barros, 2017). The control group was recruited from two community health centres, which, although serving a large proportion of the city's population, are confined to selected areas of Cape Town. Groote Schuur Hospital serves a much wider geographic area, stretching from the central sub-district, to the southern and western sub-districts.

6.3 Traditional cerebrovascular risk factors

Hypothesis 3.2.1: There are significant differences in traditional cerebrovascular risk factors between young HIV-infected individuals with and without acute ischaemic stroke. Whilst HIV may be the single most important risk factor for stroke in individuals <45 years, traditional cerebrovascular risk factors may have a synergistic effect on stroke risk in HIV-infected individuals.

6.3.1 Traditional cerebrovascular risk factors

The results supported the hypothesis that, in a young HIV-infected population, there are differences in conventional cerebrovascular risk factors between those with and without acute ischaemic stroke.

The stroke group had a higher prevalence of diabetes, and their fasting lipogram values were higher than controls. Although these values were within the internationally-defined optimal range (Expert Panel on Detection, Evaluation, 2001; Alberti, Zimmet & Shaw, 2006), this may be explained by the fact that lipid values in predominantly Black African populations are lower than those of other ethnic groups (Steyn et al., 2005). However, interpretation of the lipogram comparison was limited by the large number of missing values in the control group. Although there was a similar proportion of current smokers within each group, a statistically significant higher number of individuals in the stroke group had a more than 10 pack-year smoking history. This may have increased their stroke risk relative to the controls.

The groups were similar in many other respects. There were no statistically significant differences in hypertension, systolic blood pressure, current smoking, substance use, known cardiac disease, previous stroke, and waist circumference between the groups. These results were unexpected. The INTERSTROKE study demonstrated that hypertension, smoking, cardiac

disease are associated with ischaemic stroke in African populations (O'Donnell et al., 2016), and the INTERHEART study showed that waist circumference was a strong predictor of cardiovascular disease in Black Africans (Yusuf et al., 2005).

Our findings therefore support the suggestion that whilst traditional cardiovascular risk factors may play a role in ischaemic stroke in HIV, their contribution is less influential than that of HIV itself (see 2.3.4.3 and 2.3.4.4).

6.3.2 Carotid intima-media thickness

The results of the carotid-intima media thickness (CIMT) measurements in this cohort confirmed that CIMT in HIV is a difficult topic to elucidate. In HIV-uninfected individuals, greater CIMT is an independent predictor of cardiovascular disease and is associated with increased stroke risk in individuals <50 years (O'Leary et al., 1999; Lorenz et al., 2006; Li et al., 2008). In HIV-infected individuals however, the association is not so clear. There is no current consensus on the impact of HIV on CIMT, as well as its usefulness in predicting stroke in HIV-infected individuals.

In this cohort, CIMT was not associated with ischaemic stroke in HIV. Unadjusted analysis showed that the control group had significantly greater mean CIMT measurements compared with the stroke group. The subsequent binomial logistic regression model demonstrated that for two individuals of the same age and same waist circumference and one unit difference in CIMT, the one with a higher CIMT has a 0.224 times lower odds of having a stroke, than the one with the lower CIMT. There were limitations to the analysis. Firstly, this was a cross-sectional analysis, and we couldn't draw any conclusions about future stroke risk in these individuals. Additionally, the conclusions were based on incomplete CIMT data. Whilst 82.8% of the stroke group had carotid duplex Doppler done, only 69% of the controls had carotid imaging performed. Our cohort's age-range may also limit any conclusions around the effect of HIV on

CIMT. A recent pooled analysis demonstrated that, in individuals aged 30-49, HIV itself may not affect CIMT (Hanna et al., 2016).

The mean CIMT may not have been sufficient to impact stroke risk in either group. In a large prospective study, the minimum thickness that increased the hazard risk ratio for stroke, when adjusted for age, sex and other risk factors was 0.63mm (Lorenz et al., 2006). In this cohort of HIV-infected individuals, the mean CIMT did not reach this significant threshold. Thus, although the control group had a significantly greater CIMT, it may not have been sufficient to increase their stroke risk.

Although it has been reported that traditional cardiovascular risk factors are the most important determinant of greater CIMT in HIV-infected individuals (Stein et al., 2013; Ssinabulya et al., 2014; Pacheco et al., 2015, 2016; Schoffelen et al., 2015), this was not evident in this cohort. The groups were in many respects similar in terms of cardiovascular risk profile, and the stroke group, in fact, had an increased prevalence of diabetes, higher fasting lipid values, and a greater pack-year smoking history. Logically, the stroke group should have had a greater mean CIMT than the controls, when in fact the inverse was true in this study.

Perhaps it was the severity of HIV disease that trumped cardiovascular risk factors in influencing CIMT in this study. CIMT has been shown to have an inverse relationship with viral load (Hanna et al., 2016), and thus, the more immunocompromised stroke group (see section 6.4.1) could plausibly have had a lower CIMT than their non-stroke counterparts.

The greater CIMT in the control group may also be explained by the differences in exposure to, and duration of, antiretroviral therapy between the groups. Although not significant, the control group had more than twice the median duration of therapy compared to the strokes, and more of the control participants were actually on treatment at the time of enrolment (50.7% of controls vs 34.5% of strokes). Several studies have shown that treated individuals, as well as their duration of treatment, may have an association

with increased CIMT and sub-clinical atherosclerosis in HIV-infected individuals (Kwiatkowska et al., 2011; Papiţă et al., 2011; Lorenz et al., 2012; Krikke et al., 2017).

The significance of carotid intima-media thickness in HIV remains unclear. More studies are needed to explore the role of CIMT as a predictor of stroke in HIV-infected individuals. Large prospective studies, with individuals in different age categories, are needed to evaluate the impact of HIV on CIMT, and its usefulness as a predictor of stroke in HIV-infected individuals.

6.3.3 History of recent infection

A history of recent infection, particularly respiratory tract infection, is common in stroke patients worldwide (Syrjänen et al., 1988; Grau et al., 1995; Bova, Bornstein & Korczyn, 1996; Das et al., 2011). VZV, manifesting either as chicken-pox or shingles, is also a risk factor for stroke (Gilden et al., 2000, 2009).

Both groups were asked to report any infection they had had within the three months preceding enrolment. The most commonly reported infections in the stroke group were a respiratory tract infection, VZV (in the form of chicken pox or shingles) and TB. The control group reported other types of infections that are not linked to stroke risk. In the stroke group, VZV rash was the second most frequently reported infection, and VZV vasculopathy was a relatively common cause of stroke in this cohort (15.4% of individuals). Interestingly, not all the individuals who reported VZV rash had VZV vasculopathy as the cause of their stroke, and not all individuals with VZV vasculopathy had reported a rash prior to their stroke. This highlights the diagnostic difficulty with VZV-vasculopathy: the fact that there is often a long interval between the clinical syndrome and stroke, and also that a visible rash does not always precede VZV vasculopathy (Nagel et al., 2007). Although the same number of individuals reported a history of tuberculosis, none had CNS TB, and no strokes were due to tuberculous meningitis.

Our findings indicate that a history of certain opportunistic infections in HIV-infected people should alert the clinician to an increased risk of stroke in these individuals. These are infections that have a propensity for vascular injury, such as VZV, syphilis and disseminated tuberculosis.

Traditional cerebrovascular risk factors in young HIV-infected individuals

The traditional cardiovascular risk profile in young Subtype-C HIV-infected individuals with acute ischaemic stroke was similar in many respects in comparison with age- and gender-matched HIV-infected individuals without acute stroke. However, diabetes, higher fasting lipid values and a greater pack-year history of smoking distinguished those with stroke from controls. Traditional cardiovascular risk factors may synergistically enhance the risk of stroke in HIV-infected individuals younger than 45 years.

6.4 HIV-related factors

Hypothesis 3.2.2*: Recent CD4 count, CD4 nadir and viral load are significantly different between young HIV-infected individuals with acute ischaemic stroke, and those without acute ischaemic stroke. Low CD4 count and elevated viral load are indicators of immunocompromise, immune dysregulation and increased viral replication. These factors increase inflammation and therefore could impact on endothelial dysfunction.*

6.4.1 CD4 count and CD4 nadir

Our results support the hypothesis that recent CD4 count and CD4 nadir have an inverse association with ischaemic stroke risk.

Immune dysregulation, from infection and loss of CD4+ T-lymphocytes, is an important contributor to HIV-associated endothelial dysfunction (see section 2.3.5.2) and increases the risk of opportunistic infections. Both endothelial dysfunction and certain opportunistic infections are risk factors for stroke in HIV-infected individuals (Benjamin, Bryer, et al., 2016). Recent CD4 count has been shown to be more strongly associated with ischaemic stroke risk than CD4 nadir or viral load (Marcus et al., 2014). A low CD4 nadir is also predictive of long-term mortality in HIV-infected individuals (Mills et al., 2012).

One might argue that it is possible that the CD4 count measured at enrolment had dropped acutely in the individuals with ischaemic stroke, thereby producing a false difference in the median CD4 counts between the groups. However, a pilot analysis to test this theory was performed. Of 18 individuals who had a CD4 count documented in the 6 months prior to their enrolment, 72.2% did not have a relative decrease in CD4 count post-stroke. Furthermore, the stroke group also had a lower CD4 nadir, and higher rates of treatment interruption than the controls. Untreated individuals in the stroke group also

had a lower CD4 count than untreated controls.

These factors indicate that the stroke group was more immunocompromised than the control group before enrolment. This may have put them at greater risk of stroke from HIV-associated endothelial dysfunction, opportunistic infections and other mechanisms related to immune dysfunction.

6.4.2 Viral load

Whilst the observed trend was towards a higher viral load in the stroke group, the results did not support the hypothesis that viral load was significantly different between young HIV-infected individuals with acute stroke, and young HIV-infected non-stroke controls.

Exposure of the vessel wall to viral particles is postulated to be part of the mechanism of HIV-associated endothelial dysfunction (Benjamin et al., 2012). Indeed, clinical studies have demonstrated that individuals with high viral loads are at increased risk of stroke compared to those with viral suppression (Siedner, in press; The Data Collection on Adverse Events of Anti-HIV Drugs (DAD) Study Group, 2003; Lichtenstein et al., 2010; Chow et al., 2012; Marcus et al., 2014; Sico et al., 2015). Furthermore, the severity of endothelial dysfunction is positively correlated with high viraemia (Blum et al., 2005).

In this cohort, only 21.1% of stroke patients were virally suppressed, and the median viral load was 4.58 $\log_{10}$ copies/ml. This would indicate that these individuals were at high risk of severe endothelial dysfunction and ischaemic stroke.

The controls also had evidence of uncontrolled viral replication. Only 26.7% of the control group was virally-suppressed, and the median viral load was 4.13 $\log_{10}$ copies/ml. One could argue that if viraemia was present in individuals without stroke, then it is either not a significant contributor to endothelial dysfunction, nor a good indicator of stroke risk.

However, the controls also showed evidence of endothelial dysfunction. Despite not suffering an ischaemic event, the controls had similar levels of adhesion and vasoconstrictor molecules compared with the stroke group. Furthermore, the pathogenesis and progression of endothelial dysfunction to stroke in HIV is complex. As suggested by Maggi *et al.* (Maggi, Ingrassia & D'Annunzio, 2008), stroke in HIV may be the result of HIV-associated endothelial dysfunction in conjunction with other risk factors. Whilst viraemia is associated with increased severity of endothelial dysfunction, it may not be sufficient to produce the degree of endothelial dysfunction needed to result in thrombosis and occlusion in all individuals.

6.4.3 Antiretroviral therapy

Antiretroviral therapy may improve vascular endothelial dysfunction by reducing viral replication rate and the incidence of opportunistic infections. However, recent initiation of ART may also increase the risk of stroke. Reconstitution of the immune system upregulates the pro-inflammatory milieu. This study provided further evidence to support the idea that untreated HIV, as well as interruption of treatment, infer greater risk for cardiovascular disease than antiretroviral therapy itself (The Strategies for Management of Antiretroviral Therapy (SMART) Study Group, 2006; Corral et al., 2009; Chow et al., 2012).

Newer antiretroviral agents are considered safer in terms of cardiovascular risk (Worm et al., 2010). With the inclusion of treatment defaulters, only 34.5% of the stroke group was on treatment at enrolment, compared to 50.7% of the controls. The controls were also established on ART for a median of 27 months, compared with a median of 11 months in the stroke group. This supports evidence that treatment of HIV, and ART duration, have an inverse relationship with stroke risk (The Strategies for Management of Antiretroviral Therapy (SMART) Study Group, 2006; Corral et al., 2009; Chow et al., 2012).

The median duration of treatment for those with stroke was 11 months, which may have put them at higher risk of endothelial dysfunction secondary to IRIS. However, further examination of ART duration in the stroke group, as discussed in section 6.5, shows that IRIS was less likely to be a significant mechanism for stroke in this cohort.

Immune status, antiretroviral therapy and ischaemic stroke

Young HIV-infected individuals with acute ischaemic stroke are more immunocompromised and have higher rates of treatment interruption than HIV-infected individuals without acute stroke. Multiple aspects of HIV and its treatment have a cumulative effect on the severity of endothelial dysfunction and risk of progression to stroke. These include low CD4 nadir, low recent CD4 count, high viral load and treatment interruption. This supports evidence that treatment interruption and HIV disease severity may increase risk of endothelial dysfunction and cardiovascular disease, even in young HIV-infected individuals.

6.5 Stroke characteristics

I described the clinical phenotype, severity and aetiology of ischaemic stroke in this study. I did so in order to further the understanding of acute ischaemic stroke in our unique context. The stroke profile of South African Subtype-C infected individuals at the epicentre of the HIV epidemic is distinct from ischaemic stroke described in first-world cohorts.

6.5.1 Stroke severity

Most strokes were moderately severe, where affected individuals were unable to attend to their own bodily needs or walk unassisted (van Swieten et al., 1988). 5.2% of individuals died following their stroke.

6.5.2 Cause of stroke

Medium- to large-vessel disease predominated, with strokes due to non-atherosclerotic vasculopathy being the most common, followed by VZV vasculopathy.

The spectrum of HIV-associated endothelial dysfunction is represented by cryptogenic stroke, small vessel disease without hypertension, non-atherosclerotic vasculopathy, accelerated atherosclerotic vasculopathy and HIV-associated vasculitis. This range of phenotypes, under the umbrella-term of HIV-associated vasculopathy, accounted for 43.1% of all strokes in this cohort. Opportunistic infections were also a prominent cause of stroke: VZV and neurosyphilis together accounted for 22.4% of all strokes.

6.5.3 HIV-associated vasculopathy and alternative mechanisms of stroke in HIV

Further analysis of the clinical characteristics of the two stroke groups divided by aetiology did not detect any substantial differences in the CD4 counts or viral loads between the two groups. Figure 5.12 highlights the possible reason for the findings observed. Opportunistic infections, which comprised the largest proportion of strokes due to alternative mechanisms, occurred in individuals with a median CD4 <200 cells/μl.

Similarly, non-atherosclerotic vasculopathy and HIV-associated vasculitis, comprising the largest proportion of strokes due to HIV-associated vasculopathy, occurred in individuals with a median CD4 <200 cells/μl. These observations support previous findings that strokes due to opportunistic infections occur at relatively low CD4 counts. Similarly, non-atherosclerotic vasculopathy and HIV-associated vasculitis also occurred in more immunocompromised individuals, supporting the idea that most HIV-associated endothelial dysfunction occurs with more advanced HIV disease.

However, the CD4 counts of those with HIV-associated vasculopathy covered a wide range, from 6 to 711 cells/μl, indicating that HIV-associated endothelial dysfunction is a complex phenomenon. One explanation is that the Tat protein has the potential to contribute to inflammation and endothelial injury at any CD4 count. HIV-infected cells are never fully eradicated, even when the immune system is restored by ART (Chun et al., 1999). Latently-infected cells continue to secrete Tat (see section 2.7.5.3), which may continue to contribute to endothelial injury in individuals with higher CD4 counts.

At a lower CD4 count, with more productively infected cells, the extra-cellular release of viral particles and Tat may be more extensive. Thus, non-atherosclerotic vasculopathy and HIV-associated vasculitis may not need additional factors to reach a threshold of thrombosis and clinical ischaemia. However, accelerated atherosclerotic vasculopathy occurred at higher CD4

counts in this cohort, suggesting that HIV-induced inflammation in these individuals needed the synergistic effect of traditional cardiovascular risk factors to reach a threshold of thrombosis and occlusion (see the “two-step hypothesis”, section 2.3.4.3).

6.5.4 The role of IRIS in HIV-associated vasculopathy

A recent study on HIV-associated ischaemic stroke in Malawi postulated that most strokes due to HIV-associated vasculitis and non-atherosclerotic vasculopathy may have been due to IRIS, with many individuals having recently initiated ART (Benjamin et al., 2017). In contrast, all our individuals with strokes due to HIV-associated vasculitis had defaulted treatment, and in those with non-atherosclerotic vasculopathy, 71.4% were either treatment-naïve, had defaulted, or were on treatment for more than a year. Furthermore, 69.2% of individuals with strokes due to opportunistic infections were also treatment-naïve. This suggests that the virus itself, in combination with other factors, rather than IRIS, contributed to endothelial dysfunction and stroke in this cohort. CSF analysis of Tat levels in the stroke group would have been needed to explore the theory that ischaemia could also be a form of chronic CNS IRIS, in response to ongoing levels of Tat secretion (see section 2.7.5.4) (Johnson & Nath, 2014).

Ischaemic stroke in young South African individuals infected with HIV-1 Subtype C

Ischaemic stroke in young Subtype-C HIV-infected individuals in South Africa differs from that in the developed world. The most common cause of stroke in this cohort was HIV-associated vasculopathy, followed by opportunistic infections. The pathogenesis of these two main causes was more likely due to untreated or inadequately-treated HIV infection than an IRIS phenomenon. HIV-associated vasculopathy as a whole is a complex phenomenon, with the various phenotypes occurring at different CD4 counts, suggesting that they represent a spectrum of HIV-associated endothelial dysfunction. In populations of young-HIV infected individuals with a paucity of traditional cardiovascular risk factors, emphasis should be placed on the prevention of opportunistic infections and ensuring adequate antiretroviral coverage to maintain good CD4 counts.

6.6 Signature pattern analysis of the Tat protein between strokes and controls

Hypothesis 3.2.3: *There are differences in Tat protein sequences between HIV-infected individuals with acute ischaemic stroke and non-stroke controls.*

The function of HIV-1 Tat may be affected by amino acid variations in its first exon. Amino acid substitutions, detected by signature pattern analysis, could result in increased endothelial damage by increasing viral replication rate, facilitating chemoattraction, or inducing the expression of pro-inflammatory cytokines. Our findings supported our hypothesis that there are signature differences in the amino acid composition of the Tat protein between HIV-infected individuals with and without acute ischaemic stroke.

6.6.1 Visual comparison of datasets

The consensus sequences depicted by the CLC Sequence viewer (see section 5.8.1 and 5.8.2, figures 5.13-5.17) allow for easy identification of the signature amino acids between the groups, as well as the amino acid variability in the sequences. The consensus sequences for the stroke and control groups are identical except at positions 21 and 29, and the consensus sequences for the divided stroke groups are identical at all residues except at position 58.

The CLC histograms show that within the cohort of Tat-C, sequences have >50% site-specific variation at only four positions. The relative conservation of amino acids at positions 1-20 and 30-55 in this cohort probably reflects the importance of the N-terminal, cysteine-rich and basic domains in the Tat protein's primary function. The critical role of these regions in transactivation, which dramatically enhances viral replication, could functionally constrain the degree of amino acid variability at these sites. The high degree of variability at positions 24, 29, 58 and 68 in the stroke group, and positions 24, 29, 68 and 69 in the control group may reflect a lesser degree of functional restraint (Yamaguchi-Kabata & Gojobori, 2000). A similar picture was seen in the consensus sequences and histograms of the strokes separated by aetiology.

Both SeqPublish and the Weblogos (see sections 5.8.3 and 5.93, respectively) of the different groups clearly demonstrate the dominance of proline at position 21 and histidine at position 29 in the stroke group, relative to the alanine and lysine at those positions in the control group. The Weblogos of the stroke group divided by aetiology also provides a visual confirmation of the difference in residues at position 58.

6.6.2 A21P: Proline is a signature amino acid at position 21 in HIV-infected individuals with acute ischaemic stroke

The A21P substitution lies in the N-terminal domain of the Tat protein, which contributes to the initiation of transcription in the host cell (Siderovski et al., 1992). It also mediates binding to the CREB (CBP/p300) complex, which is involved in promotion of initial transcription of viral RNA (Deng et al., 2000; Bagashev & Sawaya, 2013; Davey et al., 2014; Musinova et al., 2016). It may also assist with altering the response of T-lymphocytes to viral antigens, by suppressing antigen-induced activation (Mitola et al., 2000). Amino acid substitutions at this position may therefore influence HIV-1 replication by altering transcription or the T-lymphocyte response to infected cells.

The A21P substitution is one of four substitutions described at position 21 in the HIV-1 Tat protein. A21P has been noted in several circulating recombinant forms in Cameroon (Teto et al., 2016). A21P is also present in two highly virulent strains from central Africa, Tat Mal and Tat Eli. In a functional study looking at six Tat protein isolates, Tat Mal and Tat Eli had the highest transactivation activity compared to viral isolates from Europe, America and other African regions. However, the study concluded that it was position 22 rather than 21 that had the greatest impact on transactivation ability. A viral isolate from a long-term non-progressor, with a complete inability to transactivate, differed from all other strains with a mutation to serine at position 22. Substitutions at position 21, whilst possibly altering the structure of the

protein, do not seem to have a significant impact on transactivation ability (Peloponese et al., 1999).

A study looking at Tat clones from three epidemiologically linked individuals, agreed with this conclusion. Whilst A21P was present in one of the individuals, other substitutions in the core and basic regions were more likely to have been responsible for the effect on transactivation ability, which was measured with a luciferase reporter assay (Sivakumaran et al., 2007).

In summary, although the A21P mutation has been described in two highly virulent isolates, it was not found to be functionally significant with regards to altering transactivation. Whilst there are few functional studies to date, available research seems to indicate that it is less likely that A21P could enhance endothelial dysfunction by increasing viral replication rate.

6.6.3 K29H: Histidine is a signature amino acid at position 29 in the stroke group

The K29H mutation is one of six mutations described at position 29. K29H has been described in Tat-D isolates in Cameroon (Teto et al., 2016) but not in Tat-C isolates elsewhere in the literature.

Position 29 is in the cysteine-rich and chemotactic regions. The chemotactic region, between residues 24 and 51, may be responsible for a significant proportion of monocyte and PMNL chemoattraction, as well as induction of VEGF and IL-8 release by affected cells (Albini, Benelli, et al., 1998; Albini, Ferrini, et al., 1998; Benelli et al., 2000). The cysteine-rich region is involved in leucocyte chemoattraction, and supports transactivation (Vene et al., 2001; Campbell et al., 2004). It has been suggested that amino acid substitutions in this region are detrimental to transactivational ability (Li et al., 2009). Although most studies show that the basic domain is sufficient for transactivation, Tat Oyi, a strain isolated from a seropositive long-term non-progressor in Gabon, is unable to transactivate, and this loss of function has been attributed to a

C22S mutation in the cysteine-rich region (Peloponese et al., 1999; Campbell & Loret, 2009).

Lysine, the most common amino acid at position 29 in the control group, may play an important role in the physical and functional properties of the Tat protein. Lysine-associated hydrogen bonds are important for protein stability, and lysine residues at certain positions in the cysteine-rich, core, and basic regions could enhance Tat activity. Whilst reduced transactivation has been attributed to loss of lysine at positions 28, 41, 50, and 51, it has been suggested that lysine at other positions may only play a minor role in transactivation ability (Desfosses et al., 2005). However, in other small studies, mutation of lysine to another amino acid at position 29 reduced transactivational activity, and may affect HIV-1 RNA splicing (Berro et al., 2006; Yukl et al., 2009). Whether the mutation of lysine to histidine, as seen in this cohort, may have the same effect, needs further exploration.

In a recent study on HAND, looking at Subtype-C Tat sequences in an Indian cohort, the presence of lysine at position 29 appeared to have a clinically protective role. Lysine at position 29 was more common among participants without cognitive impairment, and arginine at this position was a signature residue in those with higher Global Deficit Scores. This substitution changed the secondary structure of the dicysteine motif, which may have altered Tat's neurotoxic potential (Tilghman et al., 2014).

The difficulty of drawing conclusions from functional analysis of mutations in the cysteine-rich region is illustrated by the recent debate with regards to the dicysteine motif at positions 30 and 31. Functional analysis of mutations *in vitro* may not reflect the clinical, *in vivo* effects of substitutions at these residues (de Almeida et al., 2013; Paul et al., 2014, 2017). The lack of clear clinical implications of the laboratory findings around the C30C31S mutation implies that functional studies are not straightforward, and other genetic variations may be responsible for the observed effects *in vitro*.

In summary, any mutation in the cysteine-rich and chemotactic regions may have an impact on the transactivational and/or chemoattractant properties of Tat, potentially altering its effects on viral replication and endothelial dysfunction. The study on Tat-C in HAND has presented the possibility that the loss of lysine at position 29 may alter the pathogenic potential of the Tat protein. Thus, the loss of lysine at position 29 in this cohort may have rendered the stroke group more vulnerable to endothelial dysfunction, by altering the chemoattractant abilities of the Tat protein.

Specific amino acids in the HIV-1 Tat protein are significantly associated with ischaemic stroke in young HIV-infected individuals

Two signature amino acids in Tat exon 1 are significantly associated with acute ischaemic stroke in young Subtype-C HIV-infected individuals in South Africa. These signature residues are located in regions important for the initiation of transactivation and for chemoattraction. Mutations in these domains may alter HIV-1 replication and exposure of vascular endothelium to inflammatory cells. This could affect the degree of HIV-associated endothelial dysfunction and stroke risk. Functional studies are needed to identify the effect of Tat isolates with these signature residues on endothelial cells in vitro.

6.7 Signature pattern analysis of the Tat protein between strokes of differing aetiologies

Hypothesis 3.2.4: *There are differences in Tat protein sequences between individuals with strokes due to HIV-associated vasculopathy and strokes due to alternative mechanisms.*

Individuals with strokes due to HIV-associated vasculopathy may have a greater degree of direct HIV-associated endothelial dysfunction than those with strokes due to other mechanisms, such as opportunistic infections. Therefore, I wondered whether the extent of endothelial dysfunction may be altered by differences in the amino acid composition of the Tat protein in these individuals. Although there was a signature amino acid difference between the two groups, it lacked significance and the hypothesis could not be fully supported.

6.7.1 A58T: Threonine is a signature amino acid at position 58 in strokes due to alternative mechanisms

The A58T substitution is in the basic domain, and within the neurotoxic region defined by Albini *et al*.

The basic domain is important for transactivation, TAR-binding, and the uptake of Tat into cells via a nuclear localisation signal (Loret et al., 1992). Mutations in this region, with the loss of residues key to translocation, may result in a non-functional Tat protein in the cytoplasm (Hauber, Malim & Cullen, 1989; Vives, Brodin & Lebleu, 1997). Although residues 49-57 may be sufficient for binding to TAR, the adjacent core and glutamine-rich regions synergistically optimise TAR interaction (Luo & Peterlin, 1993; Jeang, Xiao & Rich, 1999). The glutamine-rich region may facilitate transactivation by stabilizing the Tat-TAR complex, and augments viral replication (Loret et al., 1992; Li et al., 2012). The basic region cooperates with the cysteine-rich region to mediate lymphocyte adhesion and migration across the endothelium. The two regions

facilitate the formation of homodimers that mediate heparin-sulphate proteoglycan-dependent lymphocyte adhesion to endothelial cells (Urbinati et al., 2009). The basic domain is also chemotactic for dendritic cells (Vene et al., 2001).

Importantly, the basic domain is primarily responsible for the activation of MAPK (Rusnati et al., 2001), NF-κB and TNF-α, the downstream effects of which activate many other pro-inflammatory molecules, including E-selectin, IL-1β (Nath et al., 1999), MCP-1 (Weiss et al., 1999; Toborek et al., 2002), and IL-6 (Philippon et al., 1994; Demarchi, Gutierrez & Giacca, 1999; Cota-Gomez et al., 2002; Nookala & Kumar, 2014).

A58T has been described in a circulating recombinant form in Cameroon, and threonine at position 58 is thought to be a phosphorylation site, allowing for post-translational modification of the Tat protein (Teto et al., 2016). The A58T mutation was first detected in a fast-replicating viral isolate with increased transactivation ability (Leguern et al., 1993). Analyses with real time quantitative PCR and ELISA has shown that viral isolates with the A58T mutation are potent transactivators when compared to wild-type viral isolates. However, the A58T mutation displays reduced modulation of cellular genes similar to those induced by interferon (IFN) (Kukkonen et al., 2014). Viral isolates with the Tat A58T mutation have impaired ability to up- or down-regulate interferon-stimulated genes (ISGs), which are part of the innate immune response to viruses.

There are various theories around the clinical effects of ISG modulation. On the one hand, upregulation of ISGs can reduce viral replication by activating anti-viral factors. In some cell types, this may result in viral latency, and allow the virus to escape immune surveillance. However, direct stimulation of innate immunity by ISGs may also activate macrophages, which produce cytokines. A strong innate immune response to the virus plays an important role in the persistent immune activation seen in HIV infection, which is hypothesized to contribute to endothelial dysfunction (Feinstein et al., 2016).

Conversely, whilst reduced modulation of ISGs may allow viral replication to occur, it may also attenuate stimulation of innate immunity. This would theoretically reduce chronic persistent inflammation and bystander injury to endothelial cells (Kukkonen et al., 2014). This hypothesis comes from studies done on natural hosts of the simian immunodeficiency virus (SIV). Despite high levels of viral replication, these animals have a limited anti-viral immune response, which reduces the generalised immune activation and bystander injury seen in non-natural hosts of SIV. One of the proposed mechanisms for the attenuation in chronic persistent inflammation is down-regulation of IFN-stimulated pathways (Silvestri et al., 2003; Bosinger et al., 2009; Jacquelin et al., 2009).

The A58T variant seen in the group with strokes due to alternative mechanisms could reduce Tat's ability to modulate ISGs. In these individuals, reduced provocation of innate immunity may have slowed the rate of chronic persistent inflammation and thus the progression of HIV-associated endothelial dysfunction. It is possible that other mechanisms in this group, such as opportunistic infections, may have superseded HIV-associated vasculopathy as the cause of vascular injury, thrombosis and occlusion.

A small study comparing Tat protein variability in HIV-infected individuals with different rates of disease progression found the A58T substitution in 25% of long-term survivors. However, although A58T has been associated with differences in replication rates, no functional differences could be detected using the transfection-expression method *in vitro* (Quinones-Mateu et al., 1998).

Thus, the A58T mutation could have less of an impact on HIV-associated endothelial dysfunction and HIV-associated vasculopathy by attenuating chronic persistent inflammation. However, the evidence for reduced chronic persistent inflammation in HIV-infected individuals with the A58T mutation is far from robust, and other studies have found that A58T is not a more potent transactivator than other isolates. Furthermore, although A58T was identified as a signature amino acid in this study, subsequent analysis with the Fisher's exact test showed that it was not statistically significant.

A signature amino acid in the HIV-1 Tat protein distinguishes stroke pathogenesis in young HIV-infected individuals with acute ischaemic stroke

A signature amino acid in the basic region of Tat exon 1 distinguishes stroke due to HIV-associated vasculopathy from stroke due to alternative mechanisms. The impact of the A58T mutation in strokes due to alternative mechanisms is unclear, but may alter modulation of innate immunity and chronic persistent inflammation, as well as the induction of pro-inflammatory molecules. Further signature pattern analysis with a larger number of patients is needed to confirm whether this signature pattern persists. Functional studies are also needed to assess the impact of A58T viral isolates on endothelial cells in vitro.

6.8 Selection pressure in the stroke and control groups

The Tat protein is continually evolving due to host selection pressures such as host immune response, antiretroviral therapy and concomitant infections. Positive selection occurs when a non-synonymous substitution, which may alter the structure and/or function of a protein, confers an evolutionary advantage to the virus. It may increase viral fitness by improving viral replication rate, evading the host immune response or developing resistance to an antiretroviral medication. I was interested to see whether positive selection was present at any of the signature sites identified by VESPA, as well as to determine which other positions were important for improving viral fitness in this cohort.

Whilst I can be reasonably confident that the SLAC analysis accurately identifies sites under positive selection, I can only comment on the directionality of the amino acid substitutions with caution. This is because SLAC produces an unrooted phylogenetic tree, which means that reversed directionality is also possible. The SLAC method identified 8 amino acid sites in the stroke group and 7 sites in the control group that were under positive selection pressure. The majority of the sites under positive selection in both groups were in the N-terminal or basic domains, which suggests that positive selection was greatest in the regions that would improve viral replication via enhancement of transcription.

6.8.1 Position 21

Position 21 was under positive selection in the control group but evolving neutrally in the stroke group. In the control group, the majority of transitions were from alanine to proline, suggesting that proline (the signature amino acid present in the stroke group), was more advantageous to viral fitness in the control group.

6.8.2 Position 29

Position 29 was under positive selection pressure in the stroke group but not the control group. With the majority of transitions between histidine and tyrosine, it suggests that either tyrosine or histidine is a favourable mutation for viral fitness at position 29 in the stroke group. Lysine, the most common amino acid in the control group, was only selected for in 17.9% or 3.6% of non-synonymous substitutions in the stroke group, depending on the directionality of the transition. Tilghman *et al.* detected positive selection pressure at position 29 in Tat-C in participants with normal cognition, with the presumed transition away from the signature amino acid associated with neurocognitive impairment (Tilghman et al., 2014). The signature pattern analyses of our participants, as well as those of Tilghman *et al.*, show that lysine at position 29 is more likely to be present in individuals without stroke or neurocognitive impairment, hinting at a possible protective role in disease pathogenesis. However, lysine may not confer an advantage to viral fitness: in both studies, lysine was not noted to be predominantly selected for in those positions under positive selection pressure.

6.8.3 Position 58

Position 58, a signature residue detected in the comparison between the strokes of differing aetiologies, was also under positive selection pressure in the stroke group. 62.5% of transitions were presumed to be from threonine to alanine, which is the signature amino acid at position 58 in strokes due to HIV-associated vasculopathy. The transition away from threonine at position 58 may have to do with the fact that it is a phosphorylation site, which could alter the post-translational modification of the Tat protein, perhaps to the detriment of viral fitness. Positive selection analysis was not done on the stroke group separated by aetiology, which would have given a better indication of whether the amino acid transitions were for, or against, the signature amino acids identified in HIV-associated vasculopathy versus alternative mechanisms. This is an avenue for further exploration.

Specific amino acids in the HIV-1 Tat protein associated with stroke and endothelial dysfunction are under positive selection in a cohort of young HIV-infected individuals

Analysis of selection pressure in this cohort showed that all signature amino acids associated with stroke and HIV-associated vasculopathy were under positive selection. These findings indicate that the stroke group's signature amino acids may have been more advantageous to viral fitness than those of the controls. Alanine at position 58 was also selected for in the stroke group, indicating that the amino acid common to the group with stroke due to HIV-associated vasculopathy, may have a role in improving viral fitness.

6.9. Serum biomarker analysis

Hypothesis 3.2.5: *Certain positions and amino acids in the Tat protein are specifically associated with biomarkers of endothelial activation and inflammation.*

6.9.1 Evaluation of biomarkers between the groups

I had access to 10 biomarkers of endothelial activation and inflammation, measured in all participants on enrolment into the study. My expectations were that all 10 biomarkers would be significantly higher in the stroke group, due to the recent ischaemic insult.

Only 6 of the 10 biomarkers were statistically significantly higher in the stroke group. Median E-selectin, Endothelin-1, ICAM-1 and IL-β were not significantly different between strokes and controls. This highlights the likelihood that most HIV-infected individuals have a degree of underlying endothelial dysfunction, with a persistent elevation of vasoconstrictors and adhesion molecules.

6.9.2 Correlation of signature residues with biomarkers of endothelial activation and inflammation

Due to limited resources, I was unable to assess the effect of the signature mutations with *in vitro* studies. I therefore attempted to explore the effect of the Tat protein signature residues on endothelial dysfunction with statistical methods. I controlled for age, stroke, treatment status and CD4 count in an attempt to isolate the effect of the Tat protein signature residues on activation of endothelial biomarkers.

Proline, the signature residue at position 21 in the stroke group, was associated with a relative increase in IL-6 compared with other amino acids, including alanine, which was the most common amino acid at position 21 in the control group. It suggests that the predominance of proline at position 21 in the stroke group may have contributed to the upregulation of IL-6, which is a pro-inflammatory cytokine, and may increase stroke risk (see section 2.4.2). In most studies, functions of the Tat protein, such as upregulation of certain cytokines, have been localised to a single domain. However, studies have also shown that other domains can perform the same function, albeit less strongly, or have a supporting role (see Table 2.2). Whilst the upregulation of IL-6 has been localised to the basic domain (Ambrosino et al., 1997), the association of IL-6 with the N-terminal in this study provides evidence to support the theory that more than one region contributes to the transcription of various genes.

Lysine, the most common amino acid in the control group at position 29, was significantly associated with a relative decrease in MCP-1, compared with histidine, and other amino acids. Histidine is the signature residue at this position in the stroke group. The Tat protein has been shown to upregulate MCP-1, which is primarily responsible for macrophage chemotaxis (Weiss et al., 1999). Macrophage/monocyte invasion and sub-endothelial trapping are postulated to be integral to the development of HIV-associated vasculopathy (see section 2.3.5.2). This finding is physiologically plausible, as position 29 lies within the chemotactic domain described by Albini *et al.* (Albini, Benelli, et al., 1998). The predominance of lysine at this position in the control group would suggest that it may have a relatively reduced effect on chemoattraction of inflammatory cells to vascular endothelium, and therefore on Tat-mediated endothelial dysfunction, than histidine or other amino acids at this position.

Further work on the Tat protein's influence on endothelial and pro-inflammatory biomarkers is needed. Tat could emerge as having an integral role in endothelial disruption. It may be possible to develop therapies that counter the Tat protein's contribution to the chronic persistent inflammation seen in HIV. It is yet unclear as to how effectively statin therapy reduces subclinical vascular disease in HIV (d'Ettorre et al., 2016), and we await the

conclusion of the current REPRIEVE trial for further clarification (National Institute of Allergy and Infectious Diseases, 2017). However, statin therapy has directly and indirectly been shown to downregulate the expression or activation of certain endothelial biomarkers induced by the Tat protein (Chauhan et al., 2007; Sui et al., 2007; Andras et al., 2008), and may attenuate the paracrine effects of the Tat protein on vascular endothelium. Additionally, there is currently a Tat vaccine that shows promise in the development of Tat-neutralising antibodies in human subjects (Loret et al., 2016).

Specific amino acids in the HIV-1 Tat protein are associated with modulation of IL-6 and MCP-1

This study demonstrated how the Tat protein may propagate endothelial activation and dysfunction in young HIV-infected individuals. Specific amino acids were associated with relative increases or decreases in biomarkers of endothelial activation. Future work could include improving statistical models, with the aim of creating a model that would examine all 72 positions, and their effect on the endothelial markers measured in this cohort. Further evidence supporting the role of the Tat protein in upregulation of endothelial biomarkers would emphasise the importance of the current trials into statin therapy and Tat vaccines. Neutralisation of Tat could assist in reducing HIV-associated endothelial dysfunction and cardiovascular disease.

6.10 Potential limitations of this study

6.10.1 Study participants

The clinical information and blood samples utilised in this sub-study were obtained from participants originally recruited for a larger study four to seven years prior to this research. The advantage of using participants from a larger study was that the contributions made by the original participants could be honoured by allowing additional research to examine multiple aspects of the role of HIV infection in stroke. However, the larger study was not powered for the specific analysis of the Tat protein, and the sample size was therefore fixed for this particular sub-study. There were also some missing values for certain variables, most notably fasting lipogram, and carotid intima-media thickness. This was handled with pair-wise deletion, and for variables where there was a large number of missing values, the actual number of individuals analysed was indicated in the results table.

6.10.2 Study design

This study evaluates the prevalence of associative factors and clinical outcomes at a single time point in the sample. Longitudinal follow-up was not possible in this study due to resource and time constraints. Follow-up analyses could have examined whether the biomarkers measured in the stroke group decreased after the acute ischaemic event. Longitudinal follow-up could also have assessed whether any control participants developed ischaemic stroke. However, the Tat protein is constantly evolving due to host selection pressures. The predominant quasispecies in an individual can change over time, and any control participants who developed stroke would have to undergo re-sampling at the time of new stroke onset to determine whether they had acquired new mutations in the Tat protein since enrolment into the present study. It would have been interesting to follow-up on those control participants who possessed the mutation that was predominant in the stroke group, to see if they will develop ischaemic stroke.

6.10.3 Sources of bias

A potential sampling and selection bias exists in this study. Cases and controls were recruited by convenience sampling. As all participants were recruited through the stroke services of a tertiary hospital and its affiliated secondary hospitals, there may have been bias in favour of more severe strokes. Patients presenting to primary facilities with minor strokes may not have been referred for further assessment. This study took place in Cape Town, South Africa. The Groote Schuur Stroke Service receives patients from a wider area of Cape Town, increasing the chance of more accurate representation of the city's population. The controls were recruited from two community health centres, each of which serves a specific geographical area of the Cape Town metropole. The difference in ethnic profile seen between the groups may have been a result of this geographical discrepancy. Nevertheless, almost 90% of the stroke group was of similar ethnic background to the control group, meaning that the groups were actually quite similar at baseline.

6.10.4 VZV testing

VZV-specific IgG testing, the recommended method for VZV vasculopathy diagnosis, was not available in our setting. To diagnose VZV vasculopathy, we used VZV-PCR, which is less sensitive than VZV-specific antibody testing (Nagel et al., 2007). This may have led to in under-diagnosis of VZV as a cause of stroke. However, the prevalence of VZV-vasculopathy in our cohort was similar to that of a recent study of HIV-infected individuals with stroke that had access to VZV-specific antibody testing in the CSF (Benjamin et al., 2017). The distinction between VZV vasculopathy and non-atherosclerotic vasculopathy is currently difficult, due to the similarities between VZV vasculopathy and other vasculopathies on clinical, radiographic and CSF findings (Nagel et al., 2007, 2008).

6.10.5 Sequencing methods

Sanger sequencing, rather than next-generation sequencing, was used to obtain the *tat* exon 1 sequence in the peripheral blood of each participant. This differs from next-generation sequencing, which can identify all the quasispecies present within an individual (Barzon et al., 2011). However, although there is always intra-individual variability, a number of dominant sequence clusters exist (Yin et al., 2012). Sanger sequencing identifies the most prevalent sequence (Dampier et al., 2017), which is sufficient to estimate the response of the virus to selection pressures, such as antiretroviral therapy, host immune response and environmental factors, and also most likely to have the largest bystander effect (Dampier et al., 2016).

6.10.6 Analysis of Subtype-C

Analysis of Tat protein signature patterns was limited to Subtype-C sequences. An advantage of this is that Subtype-C represents 52% of all HIV-1 infections worldwide, which means that our findings may be relevant to the majority of HIV-infected individuals globally. However, the evidence for geographical variation in Subtype-C isolates means that our findings may not translate to Subtype-C outside of Sub-Saharan Africa. As this study is the first to look at the Tat protein in stroke in South Africa, we hope that it may prompt research in other geographical regions, to see whether similar amino acid variations exist in stroke patients elsewhere.

6.10.7 *In vitro* studies

Functional studies of the identified signature amino acids were not in the scope of this research project. Functional analysis would have enabled us to determine whether these amino acid substitutions altered the action of the Tat protein on endothelial cells *in vitro*. However, *in vitro* studies are not always transferable to the biological activity of the protein *in vivo*. The difficulty of ascertaining which amino acid substitutions are clinically relevant is demonstrated by the debate around the C31S mutation in the Tat protein. The C31S motif, thought to be the cause of reduced neurovirulence and pro-

inflammatory properties *in vitro*, has more recently been demonstrated to have less clinical or neuroimaging relevance than was originally postulated (Campbell et al., 2007; Paul et al., 2014, 2017; Dara et al., 2015).

6.11. Summary of discussion

Chronic persistent inflammation in HIV is a prominent driver of endothelial dysfunction, and contributes to the elevated risk of cardiovascular disease and ischaemic stroke in these individuals (Feinstein et al., 2016). HIV-associated endothelial dysfunction and HIV-associated vasculopathy are thought to be due to the inflammatory state generated by the effects of the virus.

This study demonstrated that there were multiple factors that may cause inflammation and endothelial dysfunction to reach a critical threshold at which thrombosis and occlusion occur, and that the Transactivator of Transcription protein may have a role in HIV-associated inflammation and endothelial dysfunction.

- Young HIV-infected individuals with acute ischaemic stroke have a higher prevalence of traditional cardiovascular risk factors compared with those without stroke.
- Immunocompromise is a significant risk factor for ischaemic stroke: a low CD4 nadir, low CD4 count, treatment interruption and high viral load are associated with ischaemic stroke.
- The majority of strokes in young, immunocompromised HIV-infected individuals are caused by certain forms of HIV-associated vasculopathy and opportunistic infections, and in this study, were associated with a lower recent CD4 count, and not with IRIS.
- The various forms of HIV-associated vasculopathy occur at a range of CD4 counts, with a tendency for non-atherosclerotic vasculopathy and HIV-associated vasculitis to occur at CD4 counts < 200 cells/μl. The forms of HIV-associated vasculopathy that occur at higher CD4 counts

may need additional risk factors to reach a critical threshold at which stroke occurs.

- Specific amino acids at positions 21 and 29 in exon 1 of the HIV-1 Tat protein are associated with ischaemic stroke and could contribute to HIV-associated endothelial dysfunction and HIV-associated vasculopathy by increasing viral replication and chemoattraction of inflammatory cells to the vascular endothelium. These signature residues were also shown to modulate IL-6 and MCP-1, which are key pro-inflammatory biomarkers.
- A signature amino acid at position 58 in exon 1 of the HIV-1 Tat protein distinguishes strokes due to alternative mechanisms from strokes due to HIV-associated vasculopathy. The differences in stroke aetiology may be related to the modulation of chronic persistent inflammation by Tat protein variants.

The study does have limitations, which include a fixed sample size, potential sampling bias, and lack of *in vitro* studies. However, it was always intended to be a preliminary exploration into the association of Tat protein variants with ischaemic stroke, and to assess the potential for further research. The study, with its depth of clinical information accompanying the viral protein analysis, has provided information about the Tat protein, as well as synergistic risk factors that may influence ischaemic stroke in young South African HIV-infected individuals.

CHAPTER SEVEN: CONCLUSION

This study is, to our knowledge, the first to look at the Tat protein in the clinical context of HIV-associated acute ischaemic stroke. It has added to the growing body of knowledge around the impact of HIV-1 proteins on disease risk and pathogenesis in HIV-infected individuals.

The exact pathogenesis of HIV-associated endothelial dysfunction and the extent of its contribution to the development of ischaemic stroke is still unclear. It is acknowledged that HIV-associated inflammation and endothelial dysfunction have a prominent, and, in some individuals, independent role in the pathogenesis of ischaemic stroke.

A state of chronic persistent inflammation has become synonymous with HIV infection, and likely underpins HIV-associated endothelial dysfunction. Most HIV-infected individuals have a degree of HIV-induced endothelial dysfunction, the extent and progression of which may be determined by several factors. HIV-associated endothelial dysfunction, represented by HIV-associated vasculopathy, may be sufficient to produce clinical ischaemia, or may need additional factors to reach a critical threshold at which thrombosis and occlusion occur.

I conducted a cross-sectional analysis of two groups: cases which had reached this critical threshold and developed ischaemic stroke, and a control group, which had not. A large proportion of the entire cohort was virally unsuppressed, making it highly likely that individuals in both groups had a degree of HIV-associated endothelial dysfunction. However, there were several factors that distinguished the stroke from the control group which could have enhanced the progression of endothelial dysfunction to thrombosis, occlusion, and clinical ischaemia.

Firstly, the severity of HIV-associated endothelial dysfunction in the stroke group could have been influenced by their lower CD4 count, lower CD4 nadir,

higher viral load and higher rate of treatment interruption. Secondly, the stroke group had a higher prevalence of certain cardiovascular risk factors, and specific opportunistic infections, which could have acted synergistically with the virus to enhance inflammation and oxidative stress on the vascular endothelium. Finally, the focus of this study was the HIV-1 Tat protein, and its potential to contribute to HIV-associated endothelial dysfunction. Sequence analysis in this cohort demonstrated that there were indeed genetic attributes of the Tat protein that distinguished the stroke group from controls. The two signature amino acids identified were in regions important for viral replication and chemoattraction of inflammatory cells to the vascular endothelium. Furthermore, statistical analysis demonstrated that different residues at these signature positions were associated with biomarkers of inflammation, supporting the idea that the Tat protein may indeed be integral to the development of HIV-associated endothelial dysfunction and stroke.

This study, conducted at the epicentre of the HIV-epidemic, demonstrated that amino acid variations in the HIV-1 Subtype-C Tat protein may have a role in HIV-associated endothelial dysfunction. Overall, the clinical and virological findings also support the idea that the severity of HIV-associated endothelial dysfunction, and an individual's progression to clinical ischaemia, is likely to be a multi-step, and multi-factorial phenomenon.

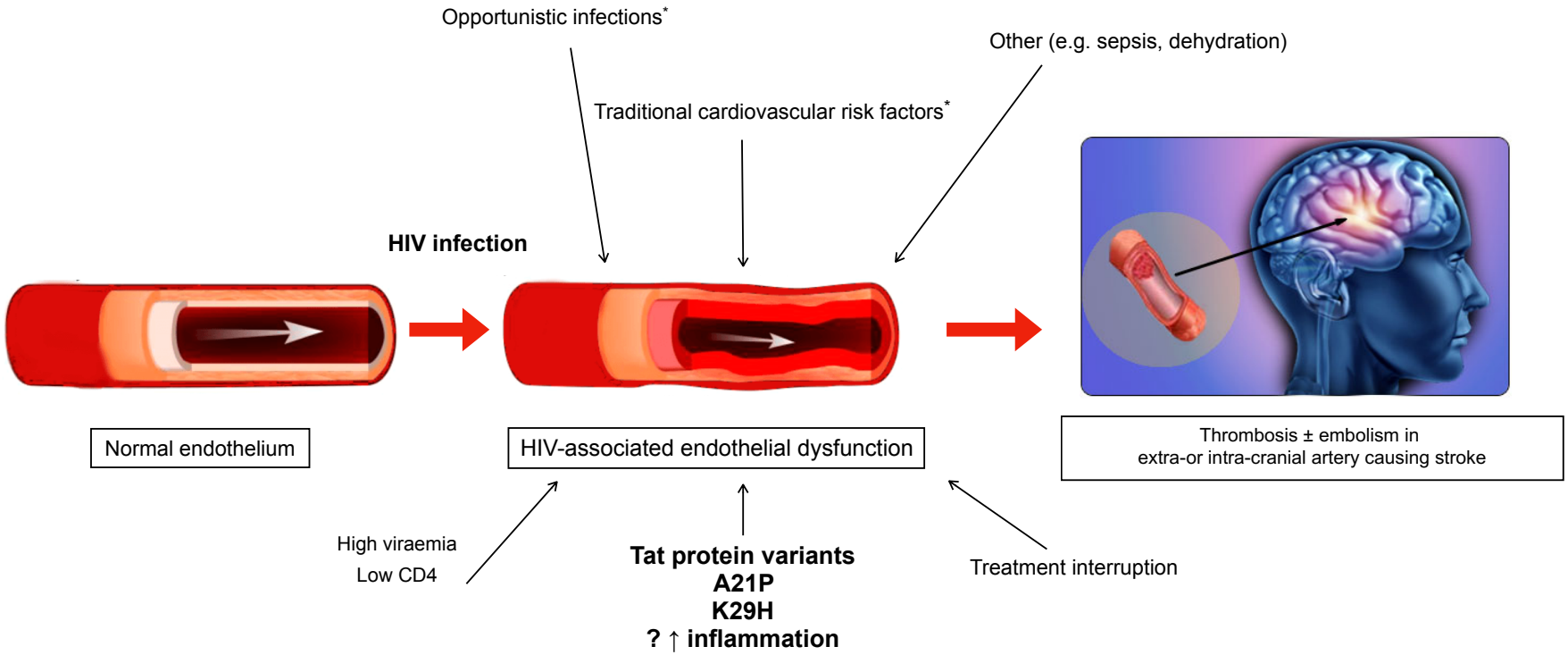


Figure 7.1. Model for the progression of HIV-associated endothelial dysfunction to stroke. Variants in the Tat protein may increase inflammation. Multiple factors can independently or cumulatively contribute to a threshold at which thrombosis and occlusion occurs.

*These factors may independently cause stroke in HIV
Images adapted from https://thumbs.dreamstime.com/z/normal-artery-inflamed-narrowed-artery-artery-aneurysm-cross-section-57964874.jpg and
http://78.media.tumblr.com/2d15aba34db2a245a96175f709a0cfed/tumblr_inline_n2mydp7hNb1r41umo.jpg

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APPENDICES

Appendix A: Ethics approval

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 404 7682 • **Facsimile** [021] 406 6411
Email: nosi.tsama@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

08 March 2017

HREC REF: 086/2017

Prof A Bryer
E8 Neurology
NGSH

Dear Prof Bryer

PROJECT TITLE: THE ROLE OF THE HIV-1 TAT PROTEIN IN ACUTE STROKE: MORE THAN JUST A TRANSACTIVATOR OF TRANSCRIPTION? (Masters candidate- Kate McMullen)

Thank you for submitting your response letter to the Faculty of Health Sciences Human Research Ethics Committee dated 28th February 2017.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th March 2018.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the student Kate McMullen will be involved in this study.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval before the research may occur.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

pp T J Burgess
PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

HREC 086/2017

Appendix B: English and IsiXhosa participant consent form for original study

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

Icwecwe-ngcaciso novumo lomthathi nxaxheba

TITLE OF THE RESEARCH PROJECT: Stroke and HIV Infection: A study of endothelial dysfunction and ultrasonographic vascular phenotypes.

Intloko ndaba yophando: Ukufa kwemizwa nosuleleko lwesandulela ngculaza:

PRINCIPAL INVESTIGATOR: Prof. Alan Bryer

Umphandi oyintloko: Prof Alan Bryer

ADDRESS: Department of Neurology, E8 Groote Schuur Hospital, Anzio Road, Observatory, 7925

CONTACT NUMBER: 021-404-3198

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

Uyamenywa ukuba uthabathe inxaxheba koluphando. Nceda uthathe ixesha ufunda ingcombolo oyinikwe apha, ethi icacise ngenkcukacha zoluphando. Nceda ubuze abaphandi okanye ugqirha nangayiphi na imibuzo emalunga nalo neliphi na ibakala loluphando ongayivisisisiyo. Ibaluleke kakhulu into yokuba waneliseke kwaye uluqonde ngokupheleleyo ukuba olu phando lungantoni kwaye ungabandakanyeka njani kulo. Kwakhona, ukuthatha kwakho inxaxheba lungokuzigqatsa kwaphela kwaye ukhululekile ukuba ungaliyeka nanini. Ukuba awuvumi, oku akunakuchaphazela kakubi nangayiphi na indlela. Ukwakhululekile ukuba ubuye umva koluphando nakwesiphi na isigaba, nokuba ubusowuvumile ukuthabatha inxaxheba.

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

Olu phando luvunyiwe liqumrhu lezophando kwicandelo lezempilo leDyunivesithi yeNtshona Koloni kwaye luqhutywa ngendlela eyamkelekileyo ngokwimikhombandlela nemiqathango yehlabathi ngokukaHelsinki, ngokwemikhombandlela yezempilo efanelekileyo yoMzantsi Afrika nangokwemikhombandlela esemgaqweni yequmrhu lophando ngezempilo.

What is this research study all about?

- *This study aims to investigate stroke in young people. In many cases there is an obvious cause but in some cases we do not find the cause.*
- *Some people with HIV develop strokes. We are interested in finding out what causes stroke in HIV and in those without HIV.*
- *In order to find out what causes these strokes we need to compare people who have HIV with those who do not and those who have strokes to those who do not.*
- *Patients who are eligible to enter to the study will be asked to sign this form. A nurse or a doctor will examine you and talk to you about your background. In addition we will take a few tubes of blood and do an ultrasound scan of your blood vessels.*
- *The blood samples will be used directly for the study.*
- *Not everyone who comes to the clinic or hospital will be asked to participate. We will choose people who are eligible, depending on whether they have other medical problems or not.*
- *Apart from the examinations and tests, the study will not offer special treatment or medication. Agreeing to participate will not change your treatment in any way; you will get exactly the same care as everyone else. If another medical problem is found, you will be referred for treatment at your nearest hospital or clinic. Any treatment related to HIV/AIDS you will also receive at your normal clinic.*
- *The study will be conducted at your hospital as well as at local clinics.*

Lungantoni kanye olu phando?

- Olu phando lujonge ukuphanda ngokufa kwamalungu omzimba kubantu abatsha. Kumaxesha amaninzi iba sisizathu esazekayo kodwa ngamanye amaxesha asiyi sazeke isizathu sokufa kwamalungu.
- Abanye abantu abanentsholongwane kagawulayo baye bachatshazelwe sisifo sokufa kwamalungu omzimba. Sinomdla wokwazi ukuba senziwa ntoni esisifo sokufa kwamalungu omzimba kubantu abanentsholongwane kagawulayo nakwabangenayo le ntsholongwane.
- Ukuze sifumane ukuba kwenziwa yintoni oku kufa kwamalungu omzimba kufuneka sithelekise abantu abanentsholongwane kagawulayo nabo bangenayo kunye nabo banesifo sokufa kwamalungu omzimba nabo bangenaso.
- Abantu abanelungelo lokungena koluphando bazakucelwa ukuba batyikitye olu xwebhu. Umongikazi okanye ugqirha bazakuxilonga bathethe nangemvelaphi yakho. Ngaphezu koku kuzakutsalwa ibhotilana ezimbalwa zegazi kwenziwe nohlolo ngomatshini wokuhlola imithambo yegazi.
- Eli gazi litsaliweyo lilo ekuzakwenziwa kulo olu phando.
- Ayinguye wonke umntu oza esibhedlele onokuthabatha inxaxheba kolu phando. Sizakukhetha abantu abakulungeleyo, kuxhomekeke ukuba abanazo na ezinye izigulo.
- Ngaphandle konyango nemvamvanyo, olu phando alizi kunika lunyango okanye amachiza akhethekileyo. Ukuvuma ukuthabatha inxaxheba akuzi kutshintsha lunyango nangayiphi indlela, uzakufumana ukhathalelo njengaye wonke umntu ondwendwele isibhedlele. Ukuba kuye kwafunyanwa enye ingxaki yempilo, uyakuthunyelwa kwisibhedlele okanye kwiziko lezempilo elikufuphi nawe. Naluphi na unyango olunxulumene ne sandulela ngculaza kunye nengculaza nalo uyakulufumana kwisibhedlele sakho sesiqhelo.
- Olu phando luzakuqhutywa kwisibhedlele okanye kwiKliniki yakho.

Why have you been invited to participate?

- *You have been invited to participate, because blood vessel problems in young people with stroke are not properly understood. Our study may help us to understand why young people develop stroke so that we can treat this problem better in future.*

Kutheni umenywe ukuba uthabathe inxaxheba?

- Umenywe ukuba uthabathe inxaxheba, kuba ingxaki zemithambo yegazi kubantu abatsha abagula kukufa kwamalungu omzimba aziqondisiseki ncam. Uphando lwethu lungasinceda siqonde ukuba kutheni abantu abatsha bebanokufa kwamalungu omzimba ukuze sibenokuyinceda le ngxaki ngcono kwixesha elizayo

What will your responsibilities be?

- *You will be required to attend the study visits on time and to participate as fully as possible. This means that you will answer questions as fully and honestly as possible. If there are questions you do not want to or cannot answer, you should say so.*
- *The initial examination will be part of your hospital stay or clinic visit. In addition we will see you in six months for another ultrasound test and to take some more blood.*

Izakubayintoni inxaxheba yakho?

- Kuzakufuneka ukuba uhambe utyelelo lwabaphandi ngexesha abakuxelele ngalo kwaye udlale indima kangangoko. Oku kutsho ukuba uzakuphendula imibuzo ngokugcweleyo nangokunyanisekileyo kangangoko. Ukuba kukho imibuzo ongafuni okanye ongenakwazi ukuyiphendula, ungatsho njalo.
- Uxilongo lokuqala liyakuba lelinye ibakala lokuhlala kwakho esibhedlele okanye lokundwendwela iziko lezempilo. Ukongeza koku sizakubona kwinyanga ezintandathu nezinye inyanga ezintandathu malunga nokuphonononga nokuthabatha elinye igazi.

Will you benefit from taking part in this research?

- *If we find any problems with you during the study, these will be treated or referred appropriately.*
- *Although you will not benefit directly by participating in this study, the information that we get will help us to treat strokes better in future.*

Ingaba ikhona inzuzo ngakuwe ngokuthabatha inxaxheba koluphando?

- Ukuba sifumana ezinye ingxaki kuwe ngelixa siqhuba uphando, ezi ziyakuqhutywa okanye uthunyelwe ngokukufaneleyo.
- Nangona ungazukufumana ngokuthe ngqo ngokuthi uthathe inxaxheba kolu phando, inkcukacha esiya kuthi sizifumane zizakusinceda ukuthi sinyange isifo sokufa kwamalungu omzimba ngcono kwixesha elizayo.

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

- *There are no risks to taking part in this study.*
- *The amount of blood needed is very small.*
- *The ultrasound scan of the blood vessel is not harmful or painful. The scanner will be pressed gently on your neck and will take about 30 to 45 minutes to complete.*
- *Your treatment will not be changed by being in the study.*
- *If there is any part of the study which you feel uncomfortable about, you should feel free to mention your feelings or concerns to any member of the study team or to your own doctor.*

Ingaba ikho na imingcipheko ebandakanyekayo ekuthatheni inxaxheba kolu phando?

- Akukho mingcipheko ekuthatheni inxaxheba kolu phando.
- Umyinge wegazi ofunekayo mncinci kakhulu.
- Ukuxilongwa ngomatshini wokuxilonga imithambo yegazi akukho buhlungu. Umatshini uzakuxinaniswa ngobunono entanyeni kwaye uthatha imizuzu engamashumi amathathu ukuya kwengamashumi amane anesihlanu khonukuze ugqibe.
- Ukunyangwa kwakho akunakutshintshwa kukuba ukoluphando.
- Ukuba kukho ndawana koluphando ongayiva kamnandi, ukhululekile ukuxela imvakalelo okanye inkxalabo yakho kulo naliphi ilungu loluphando okanye ugqirha wakho.

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

- *You are free not to participate or to withdraw at any time during the study. Your treatment will not be affected in any way. You may continue to attend your clinic. It would be helpful for the study team to let us know why you have decided not to take part, but you are free to not give a reason.*

Ukuba awufuni kuthatha nxaxheba kolu phando, zeziphi ezinye izinto onazo?

- Ukhululekile ukuba ungangathathi nxaxheba okanye ubuye umva nanini kolu phando. Unyango lwakho aluchaphazeleki nangayiphi na indlela. Ungaqhubekeka ukuhambela iziko lezempilo. Inganceda into yokuba ulazise eli qumrhu lophando ukuba kutheni ugqibe ukungathathi nxaxheba kolu phando, kodwa ke ukhululekile nokuba awunikanga sizathu.

Who will have access to your medical records?

- *The information collected about you will be treated as confidential and protected. If it is used in any publication or thesis, your identity will remain anonymous. Only the direct study team will have full access to the information. If we need to refer you to a clinic for treatment, we will provide them with the relevant information needed to treat your condition.*

Ngubani onokufikelela kwiziphumo zempilo zakho?

- Ezi ngcombolo ziqokelelweyo ngawe zizakuphathwa njengeziyimfihlo kwaye zikhuselekile. Ukuba zithe zasetyenziswa nakoluphi na upapasho, ubunakani bakho buyagcinwa buyimfihlo. Kuphela kwabaphandi abangundoqo bayakufikelela ngokupheleleyo kwingcombolo. Ukuba sifuna ukukuthumela kwiziko lempilo ukuze unyangwe, sakubanika ingcombolo ezifanelekileyo khonukuze bakwazi ukunyanga isigulo eso sakho.

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

You will not be paid to take part in the study but your transport and meal costs will be covered for each study visit- The study nurse will give you R100 for this.

Depending on your salary level the hospital may bill you for your admission and related investigations (for example CT scans,MRI scans, ultrasounds and blood tests). These ar3e tests done by your admitting doctor to try and find the cause of your stroke so that it can be treated properly. However there will be no extra costs, related to the research project, if you do take part.

Ingaba uzakuhlawulwa na ngokuthabatha inxaxheba kolu phando kwaye ingaba kukho zindleko zikhoyo na?

Awuzi kuhlawulwa ngokuthabatha inxaxheba koluphando kodwa indleko zokukhwela kunye nezokutya zizakuhlawulwa qho undwendwele malunge

noluphando. Umongikazi wophando uzakunika ikhulu lerandi malunga noku. Akuyi kubakho zindleko ngakuwe, ukuba uthabatha inxaxheba.

Is there any thing else that you should know or do?

- You should inform your family practitioner or usual doctor that you are taking part in a research study.
- You can contact Dr Alan Stanley or Prof. Bryer 021-404-3198 if you have any further queries or encounter any problems.
- You can contact the Research Ethics Committee of the Health Sciences Faculty of the University of Cape Town 021-406-6338 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

Ingaba kukho nayiphina into ofanele kukuyazi okanye ukuyenza?

- Ufanele kukwazisa ugqirha wakho wesiqhelo ukuba uthabatha inxaxheba kolu phando.
- Unganxulumana noGqirha Alan Stanley okanye uNjingalwazi Bryer kule nombolo (021) 404 3198 ukuba uneminye imibuzo okanye ujamelene neengxaki.
- Unganxulumana ne Qumrhu lononophelo ngezophando kwicala lezeMpilo kwiDyunivesiti yaseNtshona Koloni kwezinombolo (021) 406 6338 ukuba unamaxhala okanye izikhalazo ezingakhange zicaciswe ngokupheleleyo ngugqirha wophando.
- Uzakufumana eli cwecwe lengcombolo kunye noxwebhu lesivumo ukuze uzigcine.

Analysis of cerebrospinal fluid(Addition still requires Xhosa translation)

- As part of the routine investigation of young strokes many patients need a lumbar puncture. If this is the case with you we request permission to perform additional tests on this fluid to exclude a viral infection (chicken pox) that is thought to be related to stroke. If you are HIV positive we will also measure the amount of virus in your spinal fluid. This will require an extra 5ml of spinal fluid and a blood test.

Consent for Storage and Future Use of Unused blood Samples:

- After the study is completed we wish to store your blood samples for future research provided you sign a separate consent giving us permission to do this.

Isigunyaziso sokugcina nokusebenzisa kamva inxenye yegazi elingakhange lisebenze:

- Emva kokuba uphando lugqityiwe sinqwenela ukuligcina igazi ebesilithabathe kuwe ukuze silisebenzise kuphando kwixesha elizayo kuxhomekeke ukuba ulityikityile olunye uxwebhu olusigunyazisayo ukuba senze oku.

Declaration by participant/guardian/treatment partner (circle)

Imvume ngumthathinxaxheba/impelesi/iqabane lokunyanga (rhanqela)

By signing below, I …….. agree/agree on behalf of…… to take part in a research study entitled: "Stroke and HIV Infection: A study of endothelial dysfunction and ultrasonographic vascular phenotypes".

Ngokuthi utyikitye ngezantsi, mna ..... ndiyavuma/ndivuma ukumela u ..... ukuba athabathe inxaxheba koluphando elibizwa ngokuba 'Isifo sokuf akwamalungu omzimba kunye nosuleleko yintsholongwane kagawulayo: Uphando ngokungasebenzi kwamajoni amcaba angqonge imithambo yegazi

I declare that (delete whichever is NOT applicable):

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that my taking part/my relative or friend's participation in this study is **voluntary** and I/we have not been pressurised to take part.
- I/my relative or friend may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I/my relative or friend may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Ndiyavuma ukuba (cima naphina apho kungangqinelaniyo)

- Ndifundile okanye undifundele le ngcombolo noxwebhu lokuvuma kwaye ibhalwe ngelwimi endilaziyo nelivakalayo kum.
- Ndibenethuba lokubuza imibuzo kwaye imibuzo yam iphendulwe ngokwanelisayo.
- Ndiyaqonda ukuba ukuthabatha inxaxheba/ukuthabatha inxaxheba kwesihlobo sam kungokuzigqatsa kwaye andi/sikhange ndi/sintlokothiswe ukuba ndi/sithathe inxaxheba.
- Ndi/umhlobo wam angakhetha ukulishiya olu phando nangeliphi ixesha kwaye akazukujeziswa okanye athawuziswe nangayiphi na indlela.
- Ndi/umhlobo wam angacelwa ukuba alushiye uphando lungekagqibi ukwenziwa, ukuba ugqirha wophando okanye umphandi ubona kundi/mlungele, okanye ukuba andilandeli migaqo yoluphando, njengoko bekuvunyelwene.

Signed at (*place*) ..... on (*date*) ……..…….. ….
Kutyikitywe e Ngomhla

Signature of participant/ Umtyikityo womthathi nxaxheba:

……..……

OR/ Okanye

Signature of guardian/treatment partner/ Umtyikityo wempelesi/iqabane lokunyanga

.....

Signature of witness/ Umtyikityo wengqina:

1) ……

2) Declaration by investigator

I *(name)* ……..…… declare that:

- I explained the information in this document to

  ……..

- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above

2) Isivumo ngumphandi

Mna (igama) ..... ndiyavuma ukuba:

- Ndicase zonke inkcukacha ezikolu xwebhu ku

  .....

- Ndimkhuthazile ukuba abuze imibuzo kwaye ndithathe ixesha elaneleyo ukuyiphendula.
- Ndiyoneliseka ukuba uve ngokwaneleyo zonke inkcukacha zophando, njengoko kucaciswe ngentla apha.

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

Ityikitywe e (indawo) ngo (umhla)

Signature of investigator/ Utyikityo lomphandi:

.....

Consent for Storage and Future Use of Unused blood Samples:

Uvumo lokugcinwa nokusetyenziswa kamva kwegazi elingakhange lisebenze:

If any of the blood samples I have provided for this research project is unused or leftover when the project is completed then

Ukuba naliphina kweligazi ndinikeze ngalo malunga noluphando luthe alwasebenza okanye lwashiyeka xa uphando lugqitywa ke

(Tick **one** choice from each of the following boxes)
(Korekisha enye kolu luhlu lulandelayo)

☐ I wish my blood sample to be destroyed immediately.

Ndinqwenela ukuba igazi lam luchithwe kwangoko.

☐ I want my blood sample to be destroyed after ____ years.

Ndifuna ukuba igazi lam luchithwe emva kweminyaka emi.....

☐ I give permission for my blood sample to be stored indefinitely

Ndinika imvume ukuba igazi lam lugcinwe kangangoko.

AND (if the sample is to be stored)
Kwaye(ukuba igazi lizakugcinwa)

☐ I give permission for my blood samples to be stored and used in future research but only on the same subject as the current research project : "Stroke and HIV Infection"

Ndinika imvume ukuba igazi lam ligcinwe kwaye lusetyenziswe kuphando olulandelayo kodwa kuphela kumba ofana nalo uqhuba ngoku: 'Ukufa kwamalungu omzimba kunye nosuleleko yintsholongwane kagawulayo'

☐ I give my permission for my blood samples to be stored and used in future research of any type which has been properly approved.

Ndinika imvume ukuba igazi lam ligcinwe kwaye lisetyenziswe kolunye uphando olulandelayo nokuba lololuphi na ihlobo lezigulo kodwa lube lolugunyaziswe ngokusemthethweni.

☐ I give permission for my blood samples to be stored and used in future research except for research about [NAME TYPE OF RESEARCH]

Ndinika imvume ukuba igazi lam lugcinwe ukuba lisetyenziswe kuphando oluzayo ngaphandle kophando olunge (bhala igama lesifo)

AND

Kwaye

☐ I want my identity to be removed from my blood samples.

Ndifuna ubumna bususwe kwisixa segazi lam.

☐ I want my identity to be kept with my blood samples.

Ndifuna ubumna bugcinwe kwisixa segazi lam.

I have read the information, or it has been read to me. I have had the opportunity to ask questions about it and my questions have been answered to my satisfaction. I consent voluntarily and understand that I have the right to withdraw my consent without this affecting the current research study or my medical care.

Ndizifundile zonke inkcukacha, okanye ndiye ndazifundelwa. Ndiye ndanethuba lokubuza imibuzo ngazo kwaye imibuzo yam iphenduleke ndoneliseka. Ndivuma ngokuzigqatsa kwaye ndiyayiqonda ukuba ndinelungelo lokubuya umva koluphando ngaphandle kokuba oku kuphazamise olu phando okanye ukhathalelo lwam kwezonyango.

Print Name of Participant/ Bhala igama lomgqatswa

_____

Signature of Participant/ Tyikitya Mgqatswa

_____

Date/ Umhla _____

If illiterate
A literate witness must sign (if possible, this person should be selected by the participant and should have no connection to the research team). Participants who are illiterate should include their thumb print as well.

Ukuba awukwazi kufunda
Ingqina elikwaziyo ukufunda malityikitye (ukuba kunokwenzeka, lomntu kufanele akhethwe kumgqatswa kwaye angabinabudlelane nabaphandi). Abagqatswa abangakwazi kufunda kufanele bafake kunye nomgximfizo kabhontsi.

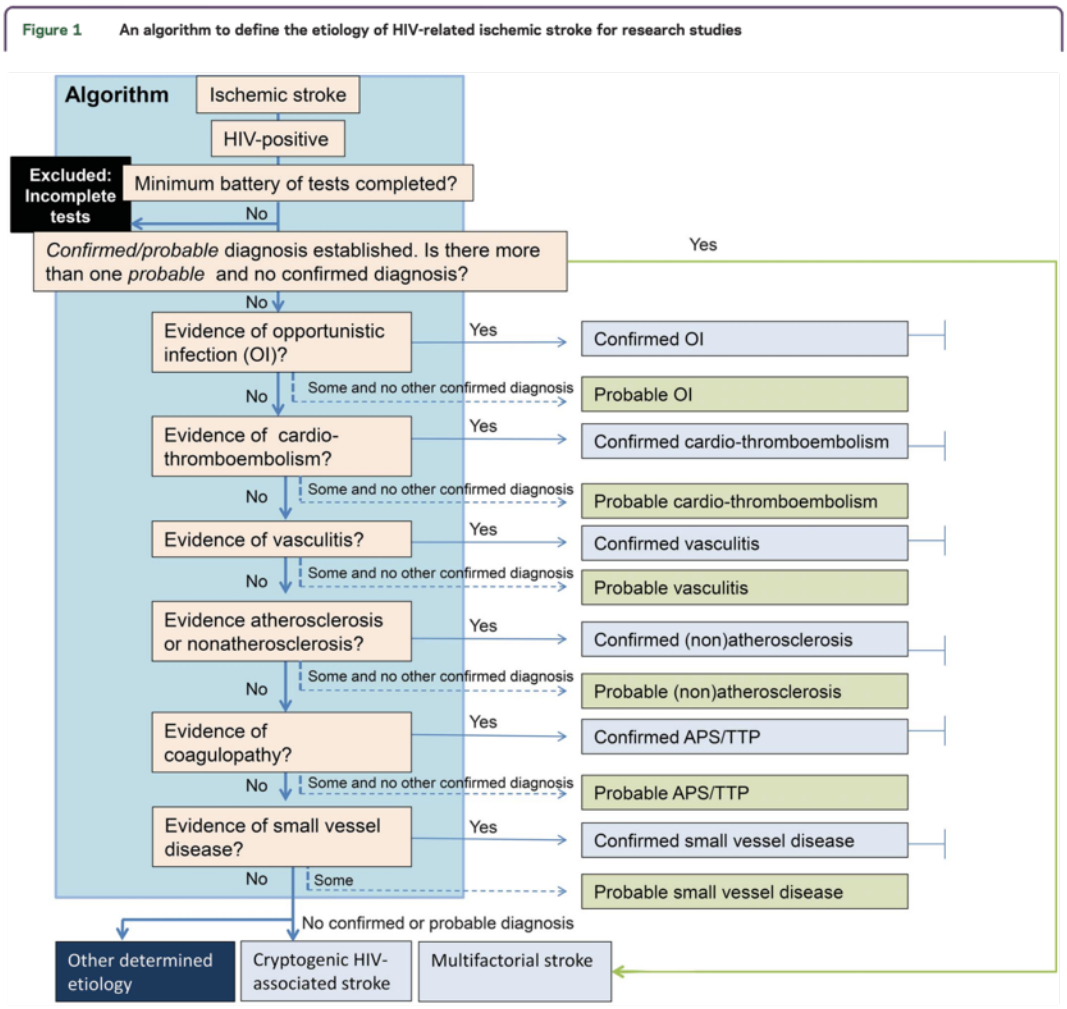
I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Ndiye ndabanobungqina ngokufunda ngokuchanekileyo uxwebhu lovumo kumgqatswa orhanelekayo, kwaye umgqatswa lowo uye wanethuba lokubuza imibuzo. Ndiyaqinisekisa ukuba umgqatswa uvume ngokuzithandela.

Print name of witness_____ AND Thumb print of participant
Signature of witness _____
Date _____
Day/month/year
I have accurately read or witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.
Print Name of Researcher_____
Signature of Researcher _____
Date _____
Day/month/year
Copy provided to participant _____ (initialed by researcher)

Appendix C: Classification of arterial ischaemic stroke in HIV

(Benjamin, Bryer, et al., 2016)



Minimum battery of tests include the following: HIV test, full blood count and blood film and urine dipstix assay, anticardiolipin antibodies, lupus anticoagulant, anti-β_2-glycoprotein, hemoglobin for sickle cell disease, serum syphilis treponemal (immunoassay + agglutination test) and nontreponemal tests, chest x-ray, CSF—microscopy, biochemistry, India ink and acid fast bacilli stains, blood culture, tuberculosis culture, unenhanced CT, ECG, and carotid/vertebral duplex ultrasound and echocardiography. APS = antiphospholipid syndrome; OI = opportunistic infection; TTP = thrombotic thrombocytopenic purpura.

Table 3 Diagnostic criteria for the etiology of HIV-related arterial ischemic stroke

Etiology	Confirmed	Probable	Tests
Tuberculosis infection	Brain histopathology/CSF evidence of MTB (AFB, culture, or PCR-positive), plus evidence of endarteritis obliterans on histology.[18]	A score ≥12, based on clinical, CSF, cerebral brain imaging criteria, or evidence of TB elsewhere.[18]	*Minimum:* TB CSF microscopy, biochemistry, AFB stain, unenhanced head CT and chest x-ray. *Optimum:* CSF/brain histopathology/sputum TB culture/TB PCR, and head MRI with contrast.
Cryptococcus	Brain histopathology/CSF evidence of *Cryptococcus* (positive India ink, culture, or antigen), plus evidence of endarteritis obliterans on histology.	Evidence of *Cryptococcus* in the blood (culture/antigen) but a negative CSF.	*Minimum:* Blood culture/CSF India ink stain. *Optimum:* CSF culture/antigen detection, brain histopathology.[20]
Syphilis[a]	Brain histopathology confirmation of Tp spirochetes by immunohistochemistry with associated endarteritis obliterans *or* active disease in the blood (positive [Tp EIA + TPPA] + [VDRL or RPR] of more than a 4-fold change in titer]) *and* positive CSF VDRL/RPR.[5,24]	Active disease in the blood (positive [Tp EIA + TPPA] + [VDRL or RPR] of more than a 4-fold change in titer]) and >20 CSF white cells and negative CSF VDRL/RPR.	*Minimum:* (Tp EIA + TPPA) + (VDRL or RPR) blood tests, CSF microscopy and biochemistry. *Optimum:* VDRL/RPR/chemokine (C-X-C motif) ligand 13.
VZV	Brain histopathology evidence of vasculitis and isolation of VZV (in situ hybridization, PCR, or antigen/antibody detection using immunohistochemistry) or positive monospecific intrathecal VZV-IgG index/CSF VZV PCR.[29,e11]	Varicella zoster in a trigeminal or cervical distribution within 12 wk, before the onset of stroke, in the absence of histology or laboratory confirmation[e11] and CSF VZV PCR-negative (or CSF testing not available) and blood VZV-IgG–positive.[29]	*Minimum:* Nil in the presence of a typical rash, blood VZV-IgG. *Optimum:* CSF VZV-IgG index (the index will be determined by serum/CSF ratio of albumin and VZV-IgG),[31] PCR, or brain histopathology examination.
Cardio-thromboembolism	Presence of *high-risk* cardio-thromboembolic lesions as detailed in the TOAST classification or evidence of infective and marantic endocarditis.[5]	Presence of *medium-risk* cardio-thromboembolic lesions as detailed in the TOAST classification.[5]	*Minimum:* ECG and transthoracic echocardiography. *Optimum:* Holter ECG and optimal echocardiography (e.g., transthoracic with bubble contrast and consideration of transesophageal echocardiography).[5]
Accelerated atherosclerotic vasculopathy	Brain histopathology consistent with atherosclerosis, irrespective of age or exposure to vascular risk factors *or* intra/extracranial carotid stenosis (≥50% or complete occlusion), supplying the affected ischemic field/a mobile thrombus in the aortic arch *and* age >45 y *or* age ≤45 y and exposed to traditional risk factors or hepatitis C.	Age >45 y or ≤45 y and exposed to traditional vascular risk factors or hepatitis C plus evidence of one of the following: (1) clinical history suggestive of extra/intracranial atherosclerosis (i.e., TIA/ischemic heart disease/peripheral vascular disease), (2) significant stenosis (≥50%) in an intra/extracranial nonaffected vascular territory, (3) nonsignificant stenosis (≥30 and <50%) in an intra/extracranial artery.	*Minimum:* Unenhanced head CT (the ideal should include magnetic resonance or CT angiography). Carotid/vertebral duplex (in the absence of noninvasive angiography). Transthoracic echocardiograph. *Optimum:* Head MRI, CT angiography or magnetic resonance angiography or digital-subtraction angiography.[5] Optimal echocardiography. Brain histopathology. Hepatitis C serology.
Nonatherosclerotic vasculopathy	Brain histopathology demonstrating intimal hyperplasia and degenerate elastica in the absence of atherosclerosis and vasculitis, irrespective of age or exposure to vascular risk factors *or* intra/extracranial carotid stenosis (≥50% or complete occlusion), supplying the affected ischemic field ± luminal thrombus with or without aneurysmal dilatation *and* age ≤45 y and absence of traditional risk factors.[34,35]	Age ≤45 y and free of exposure to traditional vascular risk factors and hepatitis C, plus evidence of one of the following: (1) significant stenosis (≥50%) supplying the nonaffected ischemic field, (2) nonsignificant stenosis (≥30 and <50%) in an intra/extracranial artery.	*Minimum:* Unenhanced head CT (the ideal should include magnetic resonance or CT angiography). Carotid/vertebral duplex (in the absence of noninvasive angiography). Transthoracic echocardiograph. *Optimum:* head MRI head with contrast, CT angiography or magnetic resonance angiography or digital-subtraction angiography.[5] Hb SS. *Optimum:* Echocardiography. Brain histopathology.
HIV-associated vasculitis	Histopathology or classic angiographic features of vasculitis within the CNS.[43,45,48,e12]	CT/MRI confirmation of an acute and/or chronic ischemic change in more than one vascular territory, involving any or all of cortical, subcortical, and deep white matter distribution.[48,e12]	*Minimum:* Unenhanced head CT (the ideal should include magnetic resonance or CT angiography). *Optimum:* Hepatitis B + C serology, CT (with contrast)/MRI (with contrast) and CT angiography or magnetic resonance angiography or digital-subtraction angiography. Histopathology examination.
Small vessel disease	Brain histopathology of small vessel disease demonstrating evidence of hyaline arteriolosclerosis and lipohyalinosis *or* at least one traditional clinical lacunar syndromes[c] and CT/MRI lesion with a diameter of ≤20 mm.[5,51]	Presence of one traditional clinical lacunar stroke syndrome[c,d] and a normal unenhanced head CT within 24 h of index stroke.[e]	*Minimum:* Unenhanced CT. *Optimum:* MRI head and histopathology examination.
Antiphospholipid syndrome[f]	Presence of anti-β_2-glycoprotein 1 antibody in combination with anticardiolipin antibody of IgG or IgM or lupus anticoagulant present in plasma serum, in medium, or high titer (i.e., >40 GPL or MPL units, or >99th percentile)[55] *and* persistence of medium to high titers of these antibodies for >12 wk.	Presence of anti-β_2-glycoprotein 1 antibody in combination with anticardiolipin antibody of IgG or IgM or lupus anticoagulant in plasma or serum, in medium or high titer (i.e., >40 GPL or MPL units, or >99th percentile).[55] Present at one time point, or on 2 occasions separated by <12 wk.	*Minimum:* Lupus anticoagulant, anticardiolipin antibody, and anti-β_2-glycoprotein 1 antibody and unenhanced CT (the ideal should include a CT, head MRI, and noninvasive angiography). *Optimum:* Histopathology, MRI; CT angiography plus CT venography or magnetic resonance angiography plus magnetic resonance venography.

Continued

Table 3 Continued

Etiology	Confirmed	Probable	Tests
Thrombotic thrombocytopenic purpura	ADAMT313 levels <10% or anti-ADAMT313 antibody.[59,60] Brain histopathology with platelet thrombi in the small vessels.[58]	1 of 2 laboratory criteria: (1) thrombocytopenia,[g] (2) microangiopathic hemolytic anemia[h,59] *and* 2 of 4 clinical or laboratory criteria: (1) fever, (2) intravascular thrombi generation, (3) renal dysfunction, (4) 1.5 times elevation of LDH.[59]	*Minimum:* FBC and blood film and urine dipstix assay and unenhanced CT. *Optimum:* Imaging to type stroke (venous stroke may be involved): head MRI and CT angiography plus CT venography or magnetic resonance angiography plus magnetic resonance venography.[48] Blood test: clotting profile, fibrinogen, U + E, LDH, ADAMTS13 levels, and anti-ADAMT313 antibody.[60] Brain histopathology.

Abbreviations: ADAMT313 = ADAM metallopeptidase with thrombospondin type 1 motif, 13; AFB = acid fast bacilli; FBC = full blood count; Hb SS = hemoglobin for sickle cell disease; IgG = immunoglobulin G; IgM = immunoglobulin M; LDH = lactate dehydrogenase; (M)TB = (*Mycobacterium*) *tuberculosis*; RPR = rapid plasma reagin; TOAST = Trial of Org 10172 in Acute Stroke Treatment; Tp EIA = treponema enzyme immunoassay; TPPA = *Treponema pallidum* particle agglutination assay; U + E = urea and electrolyte; VDRL = Venereal Disease Research Laboratory; VZV = varicella zoster virus.
Definitions assume the clinical entry criteria of ischemic arterial stroke and HIV infection.
[a] Intracranial meningovascular complications are more common than extracranial changes.
[b] RPR is less sensitive than VDRL.
[c] The following 4 syndromes were recognized: pure motor stroke, pure sensory stroke, sensorimotor stroke, and ataxic hemiparesis (including dysarthria-clumsy hand syndrome). In the absence of cortical involvement.
[d] The presence of a visual field defect, evidence of higher cerebral dysfunction (e.g., dysphasia, visuospatial disturbance, predominantly proprioceptive sensory loss) on standard clinical testing, or features that clearly localize the lesion in the vertebrobasilar distribution (e.g., gaze palsies or crossed deficits but not nystagmus or dysarthria) exclude the diagnosis of lacunar syndrome.[e13]
[e] There should be careful consideration for a psychogenic cause of symptoms in those with normal unenhanced CT within 24 hours of index stroke.[55]
[f] Histopathology confirmation is not essential for a confirmed diagnosis but, if used, thrombosis should be present without significant evidence of inflammation in the vessel wall.
[g] Thrombocytopenia = platelet count of <100,000/mm^3; microangiopathic hemolytic anemia = hemoglobin level of <12 g/dL for males and 11 g/dL for females and detection schistocytes (fragmented erythrocytes) on blood film.
[h] Fever (>37.7°C); presence of thrombi in the circulation = elevated D-dimer or fibrinogen <1.0 g/dL; renal dysfunction = anuria, oliguria, hematuria, proteinuria, or serum creatinine greater than twice the upper limit of normal, or an abnormal result for urine dipstix assay; LDH elevated at 1.5 times the upper limit of normal (due to systemic tissue ischemia and to a lesser extent hemolysis).