Viral protein R polymorphisms in the pathogenesis of HIV-associated acute ischaemic stroke: a case-control study

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MCMKAT002

SUBMITTED TO THE UNIVERSITY OF CAPE TOWN

In fulfilment of the requirements for the degree

Master of Medicine (MMed) in Medicine

Faculty of Health Sciences

UNIVERSITY OF CAPE TOWN

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Date of Submission

28/02/2021

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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: 768/2018) and by Groote Schuur Hospital.

Objectivity

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

Acknowledgements and Contributions

Dr Kathleen Bateman was the principal supervisor of the MMed and assisted with proof-reading of the finished article before submission to the Journal of NeuroVirology.

Professor Marc Combrinck was a co-supervisor and assisted with proof-reading of the finished article before submission to the Journal of NeuroVirology.

Dr Alan Stanley was involved in clinical data, blood sample and CSF collection from the participants for original study on HIV infection and young stroke (HREC REF: 178/2010).

Professor Susan Engelbrecht supervised the sequencing of the Vpr in 2017 and provided input and advice on data analysis. She also submitted the sequences to GenBank and assisted with proof-reading of the finished article before submission to the Journal of NeuroVirology.

Professor Alan Bryer was a co-supervisor, and Principal Investigator of this project. He also assisted with proof-reading of the finished article before submission to the Journal of NeuroVirology.

List of abbreviations used in the text

Abbreviation	Explanation
AIDS	Acquired immunodeficiency syndrome
AP-1	Activator protein-1
ART	Antiretroviral therapy
CD	Cluster differentiation
CLAT	Crytpococcal latex antigen test
CMV	Cytomegalovirus
CNS	Central nervous system
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetraacetic acid
HAND	HIV-associated neurocognitive disorders
HIV	Human immunodeficiency virus
HIV-1	HIV type 1
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
IgG	Immunoglobulin G
IL-6	Interleukin 6
LTR	Long terminal repeat
MAFFT	Multiple alignment using Fast Fourier Transform
Nef	Negative factor
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SA	South Africa
Tat	Transactivator of transcription
TAE	Tris acetate EDTA
Tm	Primer melting temperature
TNF-α	Tumour necrosis factor alpha
TOAST	Trial of Org 10172 in Acute Stroke Treatment
USA	United States of America
Vpr	Viral protein R
VZV	Varicella Zoster virus

CHAPTER ONE

INTRODUCTION

HIV and ischaemic stroke

The incidence of ischaemic stroke in young people is increasing, particularly in developing countries. In sub-Saharan Africa, the incidence of young stroke has been estimated at 100 per 100 000 person-years, in comparison with just 7–8 per 100 000 person-years in Europe (Ekker et al., 2018). In the developed world, the increase in stroke in young individuals has been attributed mainly to an increased prevalence of hypertension, obesity, smoking, diabetes and hypercholesterolaemia (George, Tong & Bowman, 2017).

Similarly, the incidence of ischaemic stroke in HIV-infected individuals is also on the rise (Ovbiagele & Nath, 2011). Although traditional cardiovascular risk factors do influence HIV-associated stroke aetiology, HIV itself is recognized as an independent risk factor for stroke, particularly in those under the age of 45 years (Chow et al., 2012; Benjamin, Corbett, et al., 2016).

In 2019, the number of people living with HIV in South Africa (SA) constituted 19.7% of the total global HIV-infected population. 11.8% of new infections and 10.4% of all AIDS-related deaths occurred in SA (UNAIDS, 2020). With South Africa at the epicentre of the HIV epidemic, there is an urgent need to clarify the mechanisms by which HIV increases stroke risk.

Stroke Aetiology and Endothelial Dysfunction in HIV-infected individuals

Common mechanisms of ischaemic stroke in people living with HIV include HIV-associated vasculopathy, opportunistic infections, cardio-embolism (from HIV-associated cardiac dysfunction) and coagulopathy (relating to HIV-associated hyperviscosity syndromes) (Benjamin et al., 2012; Benjamin, Bryer, et al., 2016).

People with HIV demonstrate a persistent elevation of serum biomarkers of endothelial activation, dysfunction, inflammation and haemostasis (Graham et al., 2013), suggesting that most people living with HIV have a degree of endothelial dysfunction, ranging from sub-clinical changes to detectable vessel wall disease.

HIV vasculopathy has been defined as "any abnormality of intracranial or extracranial cerebral blood vessels resulting directly or indirectly from HIV but excluding vasculitis associated with HIV infection or neoplastic involvement of the vessels" (Benjamin et al., 2012). Histologically, vessel abnormalities in HIV exhibit a range of pathological changes. Macroscopically, aneurysmal dilatation, stenosis or occlusion has been found in HIV-infected individuals with stroke (Chetty, Batitang & Nair, 2000). On a microscopic level, there is neovascularization, vessel wall inflammation, concentric intimal hyperplasia with hyalinization and fragmentation of the elastic lamina. Additionally, there is leucocytoclastic vasculitis of the vasa vasorum and peri-adventitial vessels. All these changes have been described in the absence of atherosclerosis or other infection (Benjamin et al., 2012).

The exact mechanism by which HIV-infected individuals develop endothelial dysfunction and the range of HIV-associated vasculopathies seen is unknown. Occlusive thrombotic events causing stroke are mediated by the recruitment of leucocytes, adhesion and aggregation of platelets, activation of blood clotting and derangement of fibrinolysis (Maggi, Ingrassia & D'Annunzio, 2008). Exposure of the vascular endothelium to HIV-infected CD4+ cells, monocytes and macrophages, free virus, HIV-1 proteins and virus-induced pro-inflammatory cytokines is thought to result in production of reactive oxygen species, expression of cellular adhesion molecules and release of chemoattractants (Benjamin et al., 2012; Pillay, Ramdial & Naidoo, 2015). These processes are involved in the initiation of vascular dysfunction, which then progresses to occlusive vascular disease. A key step may be the transmigration and subsequent trapping of infected monocytes into the subendothelial space, where they release cytokines and sustain the inflammatory process that disrupts normal endothelium (Benjamin et al., 2012).

Viral protein toxicity as a potential contributor to HIV-associated stroke

Viral protein toxicity is one of the mechanisms by which HIV may cause endothelial dysfunction and vasculopathy. Secreted HIV-1 proteins have been associated with detectable changes in vascular endothelium (Kanmogne, Kennedy & Grammas, 2002; Mu et al., 2007; Kline & Sutliff, 2008). The regulatory and accessory proteins, in particular, such as Viral protein R (Vpr), Transactivator of transcription (Tat) protein and Negative factor (Nef) have been implicated in endothelial dysfunction (Kline & Sutliff, 2008; Wang et al., 2015). These proteins may have the capacity to impair endothelial function in the absence of ART and even without actively-replicating virus (Hansen et al., 2013).

As described above, endothelial dysfunction in HIV-infected individuals is likely a result of the combination of various pathogenic processes, including the activity of the virus itself, opportunistic infections, and traditional cardiovascular risk factors. Previous research suggests that Viral protein R is associated with endothelial dysfunction (Kline & Sutliff, 2008; Hansen et al., 2013). The mechanisms by which it could contribute to endothelial dysfunction are unclear, but may involve its effect on macrophages, induction of TNF-α production, and increase in viral replication rate. Furthermore, with disease progression, the concentrations of extra-cellular Vpr increase, and it has a pivotal role in reactivating latently infected cells, allowing the pathogenic effects of HIV to continue in the later stages of disease. Various functions of Vpr may be independent of ART, which would allow some of the pathogenic effects of HIV-1 to continue even in treated individuals (Acheampong et al., 2002; Ferrucci, Nonnemacher & Wigdahl, 2011; Power et al., 2012; González, 2017)

Recently, Dampier and colleagues demonstrated that specific amino acids in Vpr were associated with variations in neurocognitive status in people living with HIV. Four amino acid variations at three positions in the sequence were associated with reduction or increase in the Global Deficit Score, a measure of cognitive function (Dampier et al., 2017). This suggests that sequence variations within Vpr and other

viral proteins may explain the unpredictable progression of HIV-associated disease in different individuals.

Position	G2 arrest	Cell death	DCAF1 binding	LTR transactivation	Oligomerization	Virion incorporation	Viral replication	Disease progression
Q3		Q3R						Q3R
W18	W18	W18R				W18A		
E21				E21P				
L22	L22	L22			L22A	L22E		
L23					L23F			
E25		E25K				E25K		
K27	K27M	K27M						
A30						A30F		
F34		F34I						
R36					R36W		R36W	R36W
I37								I37
G41								G41N/S
A55								A55
I63	I63E	I63E						I63T
L64	L64P	L64P/A/R		L64P	L64P	L64P		
Q65	Q65R		Q65A/R					
L67	L67E	L67E						
L68					L68M		L68M	L68M
I70		I70S			I70S			
F72						F72L	F72L	F72L
R73	R73S			R73S			R73S	
I74	I74E	I74E						
C76	C76A				C76A	C76A		
R77		R77Q/H/A					R77Q	R77Q
H78	H78R							
S79	S79A							
R80	R80A	R80A		R80A				
R85								R85Y
Q86							Q86R	
R90	R90K						R90K	

Figure 1. Structure and functions of Vpr, with known polymorphisms that influence disease activity. (Gonzalez et al., 2017)

In addition to endothelial dysfunction, as depicted in Figure 1, Vpr polymorphisms have been associated with various clinical effects, including viral replication and disease progression (González, 2017), which may contribute to the development of advanced HIV disease and its consequences, such as HIV-associated cardiomyopathy or coagulopathy. In addition, Vpr has effects on macrophage phagocytosis(Dumas et al., 2015), which may impair the immune response and increase susceptibility of individuals to opportunistic infections. These may contribute to other causes of stroke in HIV that are not directly related to endothelial damage.

RATIONALE FOR RESEARCH

Although Vpr amino acid variations have been explored within the context of HIV-associated neurocognitive Disorders (HAND) and neuronal apoptosis, it is less clear whether variations in Vpr are associated with alterations in its other extracellular properties, which may impact on HIV-associated endothelial dysfunction and the development of vasculopathy, as well as more advanced HIV disease, which may contribute to other causes of HIV-associated stroke.

Therefore, further investigation into the Viral protein R in South African stroke and non-stroke participants who have been clinically well-characterised may elucidate any potential role in endothelial damage and stroke.

RESEARCH QUESTION

Are amino acid sequence variations within HIV-1 Viral protein R associated with acute ischaemic stroke in HIV-infected individuals?

HYPOTHESIS

There are variations in Viral protein R amino acid sequences that may distinguish between HIV-infected individuals with acute ischaemic stroke and HIV-infected non-stroke controls.

AIMS AND OBJECTIVES

1. Compare the baseline characteristics, cerebrovascular risk factors and HIV-related factors between an HIV- infected young stroke group and HIV-infected non-stroke controls.

2. To describe stroke aetiology in a group of HIV-1 Subtype C infected individuals with stroke

3. To describe and compare the amino acid sequences of Subtype-C Viral protein R between HIV-infected individuals with acute ischaemic stroke, and HIV-infected individuals without stroke.
    3.1 To visualise the amino acid sequence, compare the amino acid composition and variability, and identify signature sites in the amino acid alignments that are distinct to the stroke group relative to the control group

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CHAPTER TWO

Title:

Viral protein R polymorphisms in the pathogenesis of HIV-associated acute ischaemic stroke: a case-control study

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Keywords:

HIV, stroke, viral protein R

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ABSTRACT

Background: HIV-1 viral proteins have been implicated in endothelial dysfunction, which is a major determinant of ischaemic stroke risk in HIV-infected individuals. Polymorphisms in HIV-1 viral protein R (Vpr) may alter its potential to promote endothelial dysfunction, by modifying its effects on viral replication, reactivation of latent cells, upregulation of pro-inflammatory cytokines and infection of macrophages. **Methods:** We analysed Vpr polymorphisms and their association with acute ischaemic stroke by comparing Vpr signature amino acids between 54 HIV-infected individuals with acute ischaemic stroke, and 80 age-matched HIV-infected non-stroke controls. **Results:** Isoleucine at position 22 and serine at position 41 were associated with ischaemic stroke in HIV. Individuals with stroke had lower CD4 counts and CD4 nadirs than controls**.** These polymorphisms are more common in individuals with stroke compared to South African subtype C and the control group consensus sequences. **Conclusions:** Signature Vpr polymorphisms have been identified that are more common in people with acute ischaemic stroke in HIV compared to HIV-infected individuals without acute ischaemic stroke. These may be involved in increased stroke risk by promoting endothelial dysfunction and susceptibility to opportunistic infections, as well as more advanced HIV disease. Therapeutic targeting of HIV-1 viral proteins may present an additional mechanism of decreasing stroke risk in HIV-infected individuals.

BACKGROUND

The increased risk of cardiovascular disease and ischaemic stroke in people living with HIV (Order, Virales; family, Retroviridae; subfamily, Orthoretrovirinae; genus, Lentivirus; species, Human immunodeficiency virus) has been associated with HIV-induced endothelial dysfunction.(Feinstein et al., 2016) Serum biomarkers of endothelial activation, dysfunction, inflammation and haemostasis are persistently elevated in HIV-infected individuals, even those well-suppressed on antiretroviral therapy (ART).(Graham et al., 2013) This suggests that endothelial dysfunction- ranging from chronic asymptomatic changes to clinical events related to overt vessel wall disease, such as ischaemic stroke- is common in HIV-infected individuals. Whilst the causes of stroke in HIV are multifactorial, HIV itself is an independent risk factor for stroke, particularly in those under the age of 45 years.(Chow et al., 2012; Benjamin, Corbett, et al., 2016) Well-recognized causes of stroke in HIV, such as traditional vascular risk factors, can be addressed with lifestyle modification and lipid- or glucose-lowering medication. However, the mechanisms by which HIV itself increases the risk of stroke are less clear and there is a need to understand the pathogenesis of HIV-associated vessel wall disease, so that more targeted therapies can be developed.

HIV vasculopathy has been defined as "any abnormality of intracranial or extracranial cerebral blood vessels resulting directly or indirectly from HIV but excluding vasculitis associated with HIV infection or neoplastic involvement of the vessels"(Benjamin et al., 2012). Vessel abnormalities in HIV exhibit a range of histopathological changes which are seen in the absence of atherosclerosis or other infections.(Chetty, Batitang & Nair, 2000; Benjamin et al., 2012) HIV-associated vasculopathy is thought to be due to exposure of the endothelium to free virus and viral proteins, infected immune cells, and upregulation of pro-inflammatory cytokines. (Benjamin et al., 2012) The regulatory and accessory proteins, Viral protein R (Vpr), Transactivator of transcription (Tat) protein and Negative factor (Nef), have been implicated in endothelial dysfunction.(Kline & Sutliff, 2008; Wang

et al., 2015) Notably, these proteins may have the capacity to impair endothelial function independent of ART or actively-replicating virus.(Hansen et al., 2013)

The Viral Protein R, although better known for its neurotoxic potential, has several other properties by which it may contribute to endothelial dysfunction. Vpr is known to be neurotoxic *in vitro.* It may, however, contribute to endothelial dysfunction through several other mechanisms. Vpr promotes viral replication and reactivation of latently infected cells, which may increase exposure of vascular endothelium to free virus and viral particles. Vpr is associated with disease progression, it upregulates certain pro-inflammatory cytokines and is essential for productive infection of macrophages.(Roux et al., 2000; Romani & Engelbrecht, 2009; Ferrucci, Nonnemacher & Wigdahl, 2011; Guenzel, Hérate & Benichou, 2014; Kamori et al., 2017)

Vpr amino acid polymorphisms may alter the clinical effects of this viral protein,(Jacquot et al., 2009; Dampier et al., 2017; González, 2017; Kamori et al., 2017) highlighting the possibility that sequence variations within Vpr and other viral proteins may partly explain the unpredictable progression of HIV-associated disease in different individuals. Variants in the primary amino acid sequence of Vpr may alter its extracellular effects, thereby impacting on endothelial dysfunction and stroke risk. In this study we examined Vpr sequences in a clinically well-characterised cohort of South African individuals infected with HIV-1 Subtype C, to determine whether there is an association between certain Vpr variants and ischaemic stroke.

METHODS

Study Design and Recruitment

This was a case-control study, analysing demographic, clinical, laboratory data and blood samples from 54 HIV-1 Subtype C-infected individuals with acute ischaemic stroke, and 80 HIV-1 Subtype C-infected non-stroke controls. These individuals were a subset of a larger cohort of young HIV-infected individuals, recruited between 1st August 2010 and 30th June 2013 for a study on HIV infection and stroke. Sixty-two HIV-infected individuals with acute ischaemic stroke were enrolled at Groote Schuur Hospital in Cape Town, South Africa. Ninety-nine HIV-infected non-stroke controls, matched for age, gender and ART status, were recruited from two community health centres in the Cape Town area during the same time period. To minimize the influence of traditional risk factors for stroke that accumulate with increasing age, all participants were between 18 and 45 years old. Approval for the sub-study was granted by the Human Research Ethics Committee of the University of Cape Town for the use of patient data from the original cohort for this study (HREC REF: 768/2018)

INCLUSION AND EXCLUSION CRITERIA

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, see Table 1.

Table 1. Modified inclusion and exclusion criteria

	Modified Inclusion Criteria	Modified Exclusion Criteria
Cases	• HIV-infected (ELISA positive) • Age >18 years and <45 years • Written consent obtained • Available Viral protein R sequences from existing data*	• HIV-uninfected • Age <18 years or >45 years • Haemorrhagic stroke, subarachnoid, sub-dural or epidural haemorrhage • Non-Subtype-C Viral protein R sequences*
Controls	• HIV-infected (ELISA positive) • Age >18 years and <45 years • Written consent obtained • Available Viral protein R sequences from existing data*	• Non-Subtype C Viral protein R sequences*

*Additional criteria for this sub-study

Clinical and laboratory assessment

The original cohort was comprehensively investigated for vascular risk factors, stroke aetiology and HIV-related parameters and treatment status. Stroke aetiology was determined using modified TOAST criteria. (Benjamin, Bryer, et al., 2016)

The demographic, clinical and laboratory data were analysed for this study using information from the 134 individuals whose Vpr sequences were determined to be HIV-1 Subtype C. All analyses were performed by the student, Dr Kate McMullen. The initial laboratory work was also done by Dr Kate McMullen as part of her Master of Science in Medicine. We used GraphPad Prism Version 8.0.0 for Mac, (GraphPad Software, La Jolla California USA, www.graphpad.com) to perform statistical analysis. Continuous measurements were reported with means and standard deviations (SD), where the data were normally distributed, and compared with two-sample t-tests with unequal variances. Continuous variables of data with a skewed distribution were reported with median and interquartile ranges (IQR) and

compared using 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. We selected an alpha value of 0.05.

Genomic DNA isolation from EDTA buffy coats

DNA was isolated from 200µl of prepared buffy coat using the Macherey-Nagel NucleoSpin® Blood Kit for extraction of genomic DNA from blood (Macherey-Nagel GmbH & Co. KG, Dueren, Germany). Proviral DNA was eluted in 100 microlitres (µl) of pre-heated buffer solution.

DNA amplification and sequencing

We used the Promega GoTaq® Flexi Kit, according to manufacturer's instructions (Promega Corporation, Madison, WI, USA), for the polymerase chain reaction (PCR). We amplified *vpr* (HXB2 5559-5850) and *tat* exon 1 (HXB2 5831-6045) as a single fragment and separated them 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 (Thermo Fisher Scientific, Waltham, MA USA). For the pre-nested reaction, we used the primers Vif-1 (5'-GGGTTTATTACAGGGACAGCAGAG-3') and CATH-4R (5'-GTACCCCATAATAGACTGTGACC-3'), under the following conditions: initial denaturation at 94°C for 2minutes, then 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 68°C for 2 minutes. The final elongation step was performed at 68°C for 10 minutes. The nested reaction proceeded as follows, using Vif-1F (5'-GGAATTTGGGTCATGGAGTCTCCATA-3')/Tat-1_OR (5'-CTCATTGCCACTGTCTTCTGC-3'), with initial denaturation at 94°C for 2minutes, 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 68°C for 1 minute. The final elongation step was performed at 68°C for 10 minutes. The gene fragments were separated by 0.8% agarose gel electrophoresis. 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, with the Promega 1kb DNA ladder 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. The PCR purifications were performed with the Nucleospin® Gel and PCR Clean-up kit, according to instructions (Macherey-Nagel GmbH & Co. KG, Dueren, Germany). The DNA was diluted to a concentration of 15-25 ng/µl and Sanger sequencing was done using the Applied Biosystems BigDye® Terminator v3.1 Cycle Sequencing Kit, according to the user guide (Thermo Fisher Scientific, Waltham, MA USA). The sequences were converted into raw data files with the Applied Biosystems 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA USA).

Determination of the final cohort for Vpr analysis

The HXB2 *vpr* reference sequence was imported and used to assemble the sequence contigs in Sequencher version 5.2.4 (Gene Codes Corporation, Ann Arbor, MI USA). A multiple alignment of all sequences was created with Multiple Alignment using Fast Fourier Transform (MAFFT) within Geneious version R11 (www.geneious.com). The sequences were then codon-aligned, translated into amino acids and exported in fasta format. The HIV-1 subtype of each sequence was determined with the REGA HIV-1 Subtyping tool(De Oliveira et al., 2014). Non-Subtype C sequences were excluded to minimise the influence of inter-subtype genetic variation on signature pattern analysis. Fifty-four sequences from the total of 62 HIV-infected individuals with acute stroke and 80 of the 99 HIV-infected controls were classified as HIV-1 Subtype C. The clinical and sequence data for these 134 individuals were used for this study on HIV-1 Subtype C Vpr.

Nucleotide sequences were submitted to GenBank with accession numbers MW321656 - MW321789

Amino Acid Sequence Analysis

We used Viral Epidemiology Signature Pattern Analysis (VESPA), available at https://www.hiv.lanl.gov/content/sequence/VESPA/vespa.html to look for signature amino acids distinguishing the stroke from the control group. (Korber & Myers, 1992) Consensus sequences for both the stroke and control groups were determined with the Advanced Consensus Maker (https://www.hiv.lanl.gov/content/sequence/CONSENSUS/consensus.html). Creation of consensus sequences was to compare Vpr polymorphisms seen in this cohort with the consensus sequence for global Subtype C, as well as a consensus of all 1942 South African Subtype C Vpr sequences, both downloaded from the HIV Sequence Database (www.hiv.lanl.gov). We used SeqPublish (https://www.hiv.lanl.gov/content/sequence/SeqPublish/seqpublish.html) to align the sequences, using the HIV Sequence Database HIV-1 Subtype C Vpr consensus as the reference sequence.

RESULTS

Patients with stroke had more advanced HIV disease than controls

Traditional risk factors for stroke in the two groups are shown in Table 1. The groups were similar in age and gender, with no difference in the proportion of individuals with hypertension, diabetes or those who were current smokers. Waist circumference, and rates of alcohol or substance use were also similar. Although the stroke group had worse fasting lipid parameters than the control group, LDL-C values were at a level that has been associated with a low risk of atherosclerotic cardiovascular disease (Grundy et al., 2018) and missing data in the control group may have compromised the validity of this comparison.

Table 1. Demographics and risk factors for stroke

	Strokes n=54	Controls n=80	P value[a]
Mean age, years (SD)	32.7 (5.8)	33.0 (6.3)	0.240
Female	36 (66.7)	51 (63.8)	0.729
Hypertension	9 (16.7)	8 (10.0)	0.255
Diabetes	3 (5.6)	0 (0.0)	0.063
Mean waist circumference, cm (SD)	87.1 (13.2)	89.9 (15.7)	0.305
Smoker	15/53 (28.3)	16/70 (22.9)	0.491
Median pack years (IQR)	6.0 (7.0)	2.5 (3.75)	**0.025**
Alcohol use	15/53 (28.3)	29/75 (38.7)	0.224
Substance use	4 (7.4)	4 (5)	0.714
Fasting lipogram	**n=46**	**n=17**	
Mean total cholesterol, mmol/L (SD)	4.04 (1.3)	3.17 (0.53)	**0.0119**
Mean LDL, mmol/L (SD)	2.54 (1.3)	1.76 (0.6)	**0.0251**
Mean Trigs, mmol/L (SD)	1.21 (0.5)	0.71 (0.3)	**0.0012**
Mean HDL, mmol/L (SD)	0.97 (0.4)	0.71 (0.3)	**0.0194**

Abbreviations: cm, centimetres; IQR, interquartile range; mmol/L, millimoles per litre; SD, standard deviation; Trigs, triglycerides; wk, week
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 derived with the t-test or two-sample Wilcoxon rank-sum (Mann-Whitney) test.

In contrast, Table 2 shows that individuals in the stroke group had more advanced HIV disease than controls. Participants with ischaemic stroke had lower CD4 counts and CD4 nadirs than their non-stroke counterparts. Whilst median viral loads were similar between groups, there was a trend towards a higher median viral load in the stroke group, with a larger range of values. Almost 27% of individuals in the stroke group had defaulted ART prior to their stroke, whilst no controls had defaulted therapy.

Table 2. HIV disease severity and treatment status

	Strokes n=54	Controls n = 80	P-value[a]
CD4+ T-lymphocyte count			
Median CD4 count, cells/µl (IQR)	206 (276)	329 (291)	**0.008**
Median CD4 nadir, cells/µl (IQR)	120 (236.8)	183 (179.5)	**0.009**
Viral load			
Median viral load, log_{10} copies/ml (range)	4.68 (0-6.4)	4.14 (0-5.8)	0.056
Antiretroviral therapy			
Prior ART	26 (48.1)	43 (53.8)	0.525
Defaulted ART[b]	7 (26.9)	0	**0.001**

Abbreviations: ART, 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 derived with the t-test or two-sample Wilcoxon rank-sum (Mann-Whitney) test.
[b]Expressed as No. (%) of those previously on ART

Stroke Aetiology

The most common cause of stroke in this cohort was HIV-associated vasculopathies (33.3%). Varicella zoster virus (VZV) vasculopathy (29.6%), determined by positive CSF VZV PCR or positive monospecific CSF VZV-IgG index, was the second most common cause. Cardio-embolism accounted for 16.7% of strokes, whilst strokes due to atherosclerotic vasculopathy (with associated vascular risk factors) were relatively rare, comprising only 3.7% of strokes. All causes of stroke are depicted in Figure 2.

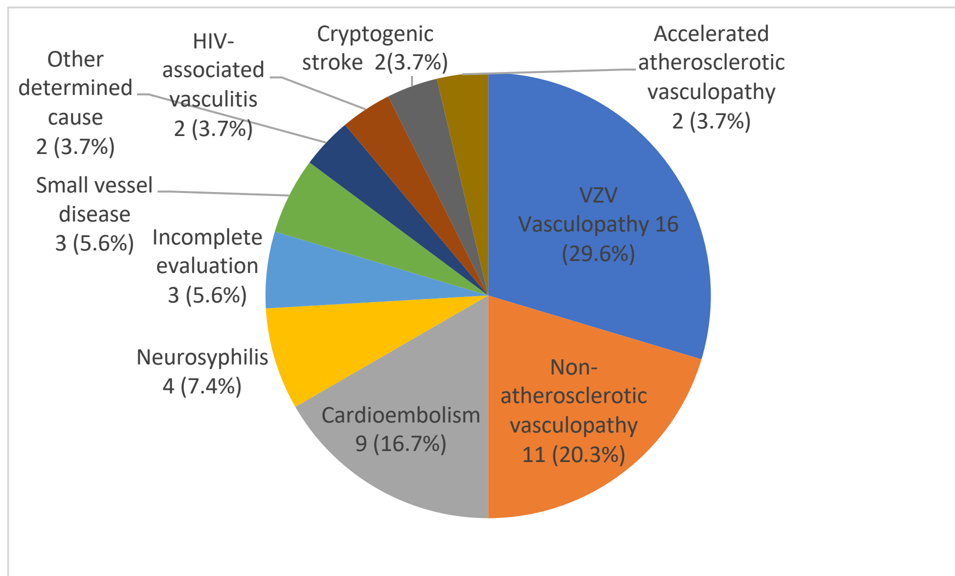

Figure 2. Stroke Aetiology. Number of patients with each cause (percentage of total)

Four amino acids at two positions in Vpr are associated with the presence or absence of acute ischaemic stroke in HIV-1 Subtype C infected individuals

The results of the VESPA analysis are depicted in Table 3. At positions 22 and 41, isoleucine and serine were the signature amino acids for the stroke group, when compared to controls. Leucine and glycine were associated with the non-stroke control group at these same positions in the Vpr amino acid sequence.

Table 3. Results of VESPA analysis

Signature Position	Most common Amino Acid	Control group frequency (%)	Stroke group frequency (%)	Variant associated with non-stroke controls	Variant associated with acute ischaemic stroke
Position 22	Leucine	57.5	42.6	22L	22I
	Isoleucine	33.8	51.9		
Position 41	Glycine	36.3	33.3	41G	41S
	Serine	33.8	46.3		

Subtype C Vpr in the stroke group differs from the control group and South African Subtype-C Vpr at positions 22 and 41

The consensus sequence alignment (Figure 1.) compares the consensus sequences of this cohort of cases and controls with the consensus sequences of global and South African Subtype C Vpr. The sequence alignment demonstrates that the stroke and control group Vpr consensus sequences were more similar to the South African Subtype C Vpr consensus sequence than the global Subtype C Vpr reference sequence. Notably, the stroke group Vpr consensus sequence had polymorphisms that were different from both the control consensus sequence and the South African subtype C Vpr consensus sequence at positions 22 and 41. These are highlighted with red boxes.

Figure 1.

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A) CONSENSUS_C           MEQAPEDQGP QREPYNEWTL ELLEELKQEA VRHFPRPWLH SLGQYIYETY GDTWTGVEAI IRILQQLLFI HFRIGCQHSR IGILRQRRAR NGASRS
B) 1942_ZA_C_Vpr         ---P------ ---------- ---------- ---------- G---H----- ---------- ---------- ---------- ---------- ------
C) 54_Strokes_Consensus  ---P------ ---------- -I-------- ---------- ---------- ---------- ---------- ---------- ---------- ------
D) 80_Controls_Consensus ---P------ ---------- ---------- ---------- G--------- ---------- ---------- ---------- ---------- ------
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Figure 3. Sequence alignment comparing: A) the Vpr consensus sequence of global Subtype C, B) the Vpr consensus sequence of South African Subtype C and the Vpr consensus sequences of the C) stroke and D) control groups

Sub-analysis of individuals with both polymorphisms

Sixteen individuals (29.6%) in the stroke group had both polymorphisms, compared to 9 (11.3%) of the controls. The most common cause of stroke in those with both polymorphisms was HIV-associated vasculopathies (37.5%). VZV vasculopathy was in fact more common in those participants who did not harbour both polymorphisms (28.9%), with HIV-associated vasculopathies accounting for only 18.4% of strokes without both 22I and 41S. There was no significant difference in CD4 count, CD4 nadir or viral load between individuals with both polymorphisms and individuals without both polymorphisms. There was also no significant difference in CD4 count, CD4 nadir or viral load between those with Glycine at position 41 and those with Serine at position 41.

DISCUSSION

This study has demonstrated that there are two signature amino acids at positions 22 and 41 in Vpr distinguishing a group of HIV-1 Subtype C infected individuals with acute ischaemic stroke, from non-stroke controls. Furthermore, we have shown that these polymorphisms in Vpr also distinguish this group of individuals with acute ischaemic stroke from the consensus of known Subtype C Vpr sequences in the South African population.

An important limitation of this study is the cross-sectional study design. Nevertheless, baseline clinical and laboratory characteristics between groups are suitably similar, justifying an analysis of the influence of HIV-specific factors in ischaemic stroke in this cohort. However, this was a retrospective study, with additional data analysis done on an existing control group which had been recruited some years before. These polymorphisms may be an indicator of immunosuppression and this deserves further scrutiny. More advanced immunosuppression in itself may contribute to the pathogenesis of ischaemic stroke in HIV-infected individuals. The stroke group had more advanced HIV disease, with higher rates of treatment interruption, suggesting that HIV itself had a prominent role to play in the development of ischaemic stroke in these individuals. Furthermore, HIV-associated vasculopathies were the most common cause of stroke seen in this cohort, followed by Varicella Zoster Virus (VZV) vasculopathy. The intriguing possibility exists that Vpr polymorphisms may have a role not only in promoting HIV-induced endothelial dysfunction but may also increase susceptibility to opportunistic infections. An additional important limitation was that Sanger sequencing, rather than next-generation sequencing, was used. 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 may be 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).

Although it has been postulated that Vpr has a potential role in endothelial dysfunction,(Kline & Sutliff, 2008; Wang et al., 2015) this is the first study, to our knowledge, to investigate whether there is an association between Vpr variants and acute ischaemic stroke. Given that other studies have demonstrated a correlation between various Vpr polymorphisms and clinical disease,(Jacquot et al., 2009; Dampier et al., 2017; González, 2017; Kamori et al., 2017) we postulate from our results that 22I and 41S may directly or indirectly contribute to the pathogenesis of HIV-associated endothelial dysfunction, as well as other infective vasculopathies, such as VZV, in the cerebral vasculature.

Disease progression

The severity of endothelial dysfunction in HIV-infected individuals has been correlated with more advanced HIV disease.(de Larranaga et al., 2003) Studies have shown that Vpr is intimately involved in viral replication by influencing reverse transcription and participating in transactivation of the long terminal repeat (LTR).(Romani & Engelbrecht, 2009),(Guenzel et al., 2012) Vpr provides a distinct replication advantage for the virus by inducing cell-cycle arrest in G2 phase, in which proviral transcription rate is highest.(Goh et al., 1998) Polymorphisms in Vpr have also been correlated with viral load and CD4 count, both of which are measures of disease progression.(Kamori et al., 2017) In this cohort, the stroke group had evidence of more severe immunocompromise, with lower CD4 counts and CD4 nadirs, as well as a higher rate of treatment interruption. This may have contributed to a greater degree of HIV-associated endothelial dysfunction, increasing stroke risk. However, the two polymorphisms associated with acute ischaemic stroke in this study were not associated with significant differences in immune status, indicating that the role of Vpr variants in stroke is likely multifactorial, and more complex than simply a direct association with more advanced disease.

Macrophage infection, transmigration and cytokine production

Macrophages have a critical role in the development of atherosclerotic vascular disease in HIV-uninfected and HIV-infected individuals.(Crowe et al., 2010; Libby et al., 2010) Similarly, transmigration of infected macrophages, with subsequent subendothelial trapping and release of pro-inflammatory cytokines, seems to be a key step in the pathogenesis of HIV-associated vasculopathy.(Benjamin et al., 2012) Activated macrophages have been implicated in the expression of cellular adhesion molecules, which aid in immune cell translocation.(Grome et al., 2017) In HIV-infected individuals, Vpr is essential for successful viral replication in the macrophage/monocyte line, enabling productive infection in these cells and enhancing the ability of HIV-1 to replicate in terminally-differentiated macrophages.(Connor et al., 1995; Vodicka et al., 1998; Guenzel, Hérate & Benichou, 2014) Furthermore, tumour necrosis factor-alpha (TNF-α) is produced mainly by activated macrophages, and is a potent stimulator of production of other cytokines.(Turner et al., 2014) Extracellular Vpr has been associated with excessive production of TNF-α, as well as IL-6 and IL-8 in numerous cell types.(Roux et al., 2000; Acheampong et al., 2002; Ferrucci, Nonnemacher & Wigdahl, 2011) Persistent and excessive cytokine release may contribute to the inflammation that disrupts normal endothelium, particularly via cytokine production from trapped subendothelial macrophages.

Opportunistic infections

Certain vasculotropic opportunistic infections, such as varicella zoster virus and neurosyphilis, are recognised causes of stroke in HIV.(Benjamin, Bryer, et al., 2016) These infections may independently or synergistically contribute to vascular injury in an HIV-infected individual, culminating in vessel occlusion and ischaemic stroke.(Benjamin et al., 2012) Vpr impairs phagosome maturation in macrophages.(Dumas et al., 2015) This effect of Vpr on macrophage phagocytosis may impair the immune response and increase susceptibility of individuals to opportunistic infections. Failure to clear pathogens may lead to the establishment of opportunistic infections that cause vasculitis. In this cohort, opportunistic infections were the second most common cause of stroke, which may have been

due to a combination of more advanced immunosuppression and the Vpr-mediated effect on macrophage phagocytosis.

Amino acid variations at positions 22 and 41

There is still much that is unknown about the clinical effect of specific polymorphisms in Vpr. Certain amino acids at position 22 have been associated with cell cycle arrest, cell death, and virion incorporation.(González, 2017) Position 41 has also been correlated with changes in viral load and CD4 count, with research suggesting an association of G41S with disease progression.(González, 2017; Kamori et al., 2017) However, participants in this study with serine at position 41 did not have significantly different CD4 counts, CD4 nadirs or viral load compared to those with glycine at position 41. This suggests that the role of Vpr variants in stroke is likely multifactorial, and more complex than simply a direct association with more advanced disease. A finding of interest was that HIV-associated vasculopathies comprised a larger proportion of stroke cause in the subset of individuals with stroke who had both 22I and 41S polymorphisms compared to those who did not. This raises the possibility of a more important role of Vpr variants in transmigration of infected macrophages and the development of HIV-associated endothelial dysfunction.

In summary, further studies are needed to elucidate the potential effects of 22I and 41S on the specific functions of Vpr that may affect vascular endothelium, such as disease progression, macrophage infection and cytokine release, as well as susceptibility to opportunistic infections.

CONCLUSION

This study has demonstrated identified signature Vpr polymorphisms that are more common in people with acute ischaemic stroke in HIV compared to HIV-infected individuals without acute ischaemic stroke. These may be involved in increased stroke risk by promoting endothelial dysfunction and susceptibility to opportunistic infections, as well as more advanced HIV disease. This finding has both mechanistic

and clinical implications, which deserve further scrutiny. Research into the effects of HIV proteins on HIV-specific disease pathogenesis is beginning to demonstrate the complexity of HIV protein sequence variation and its potential impact on disease course in individual patients. It is becoming clearer that HIV-1 viral proteins may have significant involvement in HIV disease pathogenesis, a process which is not fully controlled by suppressing viral replication with current ART. A fuller understanding of the extent and mechanisms of viral protein pathogenesis may lead to the development of more target-specific therapies to address the problem of ongoing HIV-specific morbidity in the antiretroviral era.

ACKNOWLEDGEMENTS AND FUNDING

KM was funded by the Discovery Foundation Academic Fellowship Award and the South African Medical Association Research Master's Scholarship. MIC was funded by the South African National Research Foundation "Incentive Funding for Rated Researchers" scheme: Grant number 85526.

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APPENDICES

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II. OFFICAL ETHICS APPROVAL LETTER FROM FACULTY RESEARCH ETHICS COMMITTEE

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APPENDIX I. PROTOCOL

The HIV-1 Viral protein R in HIV-associated acute ischaemic stroke: a case-control study

Proposal for Masters in Medicine (MMed) as part of the requirements of registrar training at the University of Cape Town

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EXPECTED DURATION OF PROJECT: 01/10/2018-31/01/2022

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APPENDIX A: CONSENT FORM FOR ORGINAL STUDY I

SUMMARY

BACKGROUND

The incidence of ischaemic stroke in young people is increasing worldwide. There has been a similar rise in the incidence of ischaemic stroke in HIV-infected individuals.

19% of the global HIV-infected population lives in South Africa, which is at the epicentre of the HIV epidemic. As such, this country is disproportionately burdened with HIV-associated disease, including HIV-associated ischaemic stroke. Especially when untreated, HIV infection increases the risk of stroke, and has been estimated to account for a significant proportion of strokes, with a population attributable fraction of 42% in young people in some parts of Africa (Benjamin et al., 2016).

The aetiology of stroke in HIV-infected individuals is complex and multifactorial, and whilst some causes are easily identifiable, the mechanisms by which HIV itself, an independent risk factor for stroke, contributes to stroke pathogenesis, is still unclear. There is an urgent need to elucidate the mechanisms by which HIV causes endothelial dysfunction, in order to develop more effective therapies and ameliorate HIV-associated morbidity and mortality.

Viral protein toxicity is one of the putative mechanisms by which HIV may contribute to endothelial dysfunction and HIV-associated vasculopathy. Research has shown that amino acid variations in various HIV proteins may impact on HIV-associated disease pathogenesis. The accessory and regulatory proteins, including Vpr, Tat and Nef, have all been implicated in endothelial dysfunction.

Viral protein R may exert pathogenic effects on endothelium through its role in viral replication, upregulation of certain pro-inflammatory cytokines, and its effect on macrophages, which are pivotal role players in endothelial damage.

Although Vpr amino acid variations have been explored within the context of HIV-associated neurocognitive Disorders (HAND) and neuronal apoptosis, it is less clear whether variations in Vpr are associated with alterations in its other extracellular properties, which may impact on HIV-associated endothelial dysfunction and the development of vasculopathy.

Thus, sequencing and describing the Viral protein R in our cohort of South African patients may further clarify any role it may have in endothelial dysfunction and stroke.

RESEARCH QUESTIONS, HYPOTHESIS, OBJECTIVES

The main question of this proposed research is whether there are variations in the amino acid sequence of HIV-1 Viral protein R that are associated with acute ischaemic stroke in HIV-infected individuals. The hypothesis is that there will be signature amino acids in Vpr that are unique to the stroke group, compared with non-stroke controls.

Our primary objective is to perform signature pattern analysis on the Viral protein R from the stroke and control groups, to determine whether there are amino acid variations that are associated with acute ischaemic stroke. Our secondary objectives are to compare the baseline characteristics of all the participants, to establish whether the groups are similar with regards to other risk factors for stroke, as well as to describe stroke aetiology in the stroke group.

METHODS

This is a case-control study, using two groups from a larger study on stroke and HIV infection and acute ischaemic stroke. Recruitment of participants has already been completed. Secondary data analysis will commence with 62 HIV-infected acute stroke patients, and 99 HIV-infected non-stroke controls, recruited from 2010 to 2013 in the Cape Town area.

Secondary Data Analysis

Demographic, clinical and laboratory data was collected and entered into an electronic database when the participants were enrolled into the original study. The subsequent sub-study, done for the purposes of an MSc in 2017, used stored buffy coats to sequence the Tat protein from the cohort. The *vpr* gene was sequenced at the time of that study, because it overlaps the Tat protein in the HIV genome, and primer reliability and availability meant that the Vpr protein sequence was obtained as a by-product of *tat* sequencing. This proposed sub-study, for the purposes of an MMed, will undertake secondary data analysis of the Vpr protein amino acid sequences, as well as the associated demographic, clinical and laboratory data for all participants in whom the Vpr protein can be analysed.

ETHICAL CONSIDERATIONS

Both the main study and the subsequent sub-study that took place in 2017 were approved by the Human Research Ethics Committee of the University of Cape Town (HREC REF: 178/2010, and HREC REF: 086/2017). This is a low risk study, with no further involvement needed from the original participants. Consent for secondary or further analysis of the clinical data and laboratory work on the stored blood samples was obtained on enrolment into the main study. The results of the current study will have no immediate implications for clinical care. There will be no direct benefit to the participants, but we hope our findings will contribute towards the understanding of the pathogenesis of stroke in HIV-infected individuals.

BACKGROUND

Ischaemic stroke and HIV infection

The incidence of ischaemic stroke in young people is increasing, particularly in developing countries. In sub-Saharan Africa, the incidence of young stroke has been estimated at 100 per 100 000 person-years, in comparison with just 7–8 per 100 000 person-years in Europe (Ekker et al., 2018). In the developed world, the increase in stroke in young individuals has been attributed mainly to the higher prevalence of traditional risk factors such as hypertension, obesity, smoking, diabetes and hypercholesterolaemia (George, Tong & Bowman, 2017).

Similarly, the incidence of ischaemic stroke in HIV-infected individuals is also on the rise (Ovbiagele & Nath, 2011). Whilst traditional cardiovascular risk factors do play a role in HIV-associated stroke aetiology, HIV itself is recognized an independent risk factor for stroke, particularly in those under the age of 45 years (Chow et al., 2012; Benjamin et al., 2016).

In 2016, the number of people living with HIV in South Africa (SA) constituted 19% of the total global HIV-infected population. 15% of new infections and 11% of all AIDS-related deaths occurred in SA (UNAIDS, 2016). With South Africa at the epicentre of the HIV epidemic, there is an urgent need to clarify the mechanisms by which HIV increases stroke risk.

Endothelial Dysfunction in HIV-infected individuals

People with HIV demonstrate a persistent elevation of serum biomarkers of endothelial activation, dysfunction, inflammation and haemostasis (Graham et al., 2013). This would suggest

that all HIV-infected individuals are subject to a spectrum of endothelial dysfunction, which may range from sub-clinical changes to overt vessel wall disease.

HIV vasculopathy has been defined as “any abnormality of intracranial or extracranial cerebral blood vessels resulting directly or indirectly from HIV but excluding vasculitis associated with HIV infection or neoplastic involvement of the vessels” (Benjamin et al., 2012). Histologically, vessel abnormalities in HIV exhibit a range of pathological changes. Macroscopically, aneurysmal dilatation, stenosis or occlusion has been found in HIV-infected individuals with stroke (Chetty, Batitang & Nair, 2000). On a microscopic level, there is neovascularization, vessel wall inflammation, concentric intimal hyperplasia with hyalinization and fragmentation of the elastic lamina. Additionally, there is leucocytoclastic vasculitis of the vasa vasorum and peri-adventitial vessels. All these changes have been described in the absence of atherosclerosis or other infection (Benjamin et al., 2012).

The exact mechanism by which HIV-infected individuals develop endothelial dysfunction and the range of HIV-associated vasculopathies seen is unknown. Occlusive thrombotic events causing stroke are mediated by the recruitment of leucocytes, adhesion and aggregation of platelets, activation of blood clotting and derangement of fibrinolysis (Maggi, Ingrassia & D’Annunzio, 2008). Exposure of the vascular endothelium to HIV-infected CD4+ cells, monocytes and macrophages, free virus, HIV-1 proteins and virus-induced pro-inflammatory cytokines is thought to result in production of reactive oxygen species, expression of cellular adhesion molecules and release of chemoattractants (Benjamin et al., 2012; Pillay, Ramdial & Naidoo, 2015). These processes are involved in the initiation of vascular dysfunction, which then progresses to occlusive vascular disease. A key step may be the transmigration and subsequent trapping of infected monocytes into the subendothelial space, where they release cytokines and sustain the inflammatory process that disrupts normal endothelium (Benjamin et al., 2012).

Viral protein toxicity is one of the mechanisms by which HIV may cause endothelial dysfunction and vasculopathy. Secreted HIV-1 proteins have been associated with endothelial damage,

dysfunction and remodelling (Kanmogne, Kennedy & Grammas, 2002; Mu et al., 2007; Kline & Sutliff, 2008). The regulatory and accessory proteins, in particular, such as Viral protein R (Vpr), Transactivator of transcription (Tat) protein and Negative factor (Nef) have been implicated in endothelial dysfunction (Kline & Sutliff, 2008; Wang et al., 2015). These proteins may have the capacity to impair endothelial function in the absence of ART or actively replicating virus (Hansen et al., 2013).

Vpr, although better known for its propensity for neurotoxicity, has several functional properties by which it may contribute to endothelial dysfunction in HIV-infected individuals.

Viral protein R Structure

Vpr is one of the HIV-1 accessory proteins, a 14kDa protein comprised of 96 amino acids. It is expressed in the later stages of viral replication (Morellet et al., 2003).

Its various regions or domains have been correlated with different functions (Power et al., 2012). The structure of HIV-1 Vpr protein is characterized by three well-defined α-helices at amino acid positions, 17–33, 38–50, and 54–77. These α-helices are surrounded by flexible N- and C-terminal domains. The three helices contain residues that define a hydrophobic core, around which the protein folds (González, 2017). The N-terminal may be responsible for its cytopathic effects, whilst the C-terminal could be involved in alterations in the cell cycle, including modulation of apoptosis, cell cycle arrest, and defects in mitosis (Romani & Engelbrecht, 2009).

Vpr in HIV-infected individuals

Vpr assists with stimulation of transactivation of viral transcription (Romani & Engelbrecht, 2009). Its cytopathogenicity may vary depending on the type of cell affected (Huang et al., 2000). Vpr is able to gain access to the extra-cellular compartment and function as an extra-cellular soluble protein, and is present in the peripheral blood of infected individuals. Soluble Vpr, free

of cell and virion, causes detrimental effects to bystander cells, and is also able to enter a number of different cell types. The concentration of extracellular Vpr is positively associated with disease progression (Ferrucci, Nonnemacher & Wigdahl, 2011).

Clinical effects of Vpr in HIV-infected individuals

Neurotoxicity

Vpr causes neuronal and astrocyte injury and death through apoptosis, accomplished by the activation of caspases (Power et al., 2012). Neurotoxicity is mediated via the alteration of ion concentrations, and disruption of the delicate electrochemical balance within the CNS. This results in cellular dysfunction and death, with excessive build-up of extracellular glutamate (Ferrucci, Nonnemacher & Wigdahl, 2011). Clinically, this is likely the mechanism by which Vpr is involved in neurocognitive impairment in HIV-infected individuals (Dampier et al., 2017).

Viral replication

Vpr has a pivotal role in the fidelity of reverse transcription and reduces the rate of error accumulation and mutation in HIV-1 (Power et al., 2012). The protein modulates transcription of the viral genome and facilitates reverse transcription, participates in the production of early viral transcripts and increases viral replication rate (González, 2017). Vpr stimulates the HIV-1 long terminal repeat (LTR) by activating Activator protein 1 (AP-1) and Nuclear factor kappa beta (NFκβ) (Romani & Engelbrecht, 2009).

Cell cycle arrest

In-vitro studies have demonstrated the ability of Vpr to induce cell-cycle arrest. Cell-cycle arrest in G2 phase provides a distinct replication advantage to the virus. Transcription of HIV-1 proviral DNA is highest in the G2 phase, when the HIV-1 LTR promotor is most active (Romani &

Engelbrecht, 2009; González, 2017). This capacity for cell-cycle arrest is retained in the presence of ART (Poon, 1998).

Cell death and latency

A well-known property of Vpr is the promotion of apoptosis in a variety of cells. However, cell death induced by Vpr may also be independent of caspases and have hallmarks of necrosis (Bolton et al., 2002). Huang et al, using concentrations of Vpr similar to those seen *in vivo*, also found evidence of both apoptotic and necrotic processes in astrocytes. These authors hypothesised that Vpr had different effects on the mechanism of cell death depending on the cell type (Huang et al., 2000). Vpr can also halt the induction of apoptosis, which spares infected cells to function as latently-infected viral reservoirs, to be activated at a later stage of infection. It either promotes or inhibits apoptosis on the basis of its concentration and location (Ferrucci, Nonnemacher & Wigdahl, 2011). Vpr induces anti-apoptotic pathways in infected macrophages, promoting cell-latency and facilitating long-term survival, which provides an advantage to the virus (González, 2017). In the later stages of HIV-infection, latently-infected cells exposed to Vpr reactivate viral transcription and release newly synthesized viral particles (Ferrucci, Nonnemacher & Wigdahl, 2011).

The role of Vpr in macrophages

Although Vpr is not essential for viral replication in T-cell lymphocytes and activated peripheral blood lymphocytes, it is required for viral infection and replication in the macrophage line. In the presence of Vpr, HIV-1 replication can continue even in non-replicating macrophages (Poon, 1998; Power et al., 2012). In addition, extracellular Vpr augments viral particle release from infected macrophages and impairs macrophage phagocytosis, which may contribute to the establishment of opportunistic infections in HIV-infected persons (González, 2017).

Cytokine release and promotion of inflammation

Tumour necrosis factor-alpha (TNF-α) is produced chiefly by activated macrophages, and is a potent stimulator of production of other cytokines (Turner et al., 2014). Extracellular Vpr promotes excessive production of TNF-α (Acheampong et al., 2002; Ferrucci, Nonnemacher & Wigdahl, 2011), which may contribute to the inflammation disrupting normal endothelium, particularly via cytokine production from trapped sub-endothelial macrophages.

Vpr variations and polymorphisms

Certain regions in the amino acid sequence of Vpr have been correlated with its intra- and extra-cellular functions. Specific domains and residues in the protein sequence are associated with virus cytopathogenicity and with disease progression. Figure 1 provides an overview of the various functional regions and some of the polymorphisms that have been associated with alterations in Vpr activity. The consensus sequence is represented by the internationally-accepted HXB2 Vpr. * symbols indicate positions which have fully conserved residues in HIV-1 Vpr isolates from different subtypes. H1, H2, and H3 refer to the three α-helices. Relevant residues are outlined in rectangles with the following colour code: red = leucine-rich domain, blue=mitochondrial membrane permeabilization (MMP)-inducing sequence. The two HF/SRIG motifs, which are correlated with HIV pathogenicity, are shadowed in blue (González, 2017).

Position	G2 arrest	Cell death	DCAF1 binding	LTR transactivation	Oligomerization	Virion incorporation	Viral replication	Disease progression
Q3		Q3R						Q3R
W18	W18	W18R				W18A		
E21				E21P				
L22	L22	L22			L22A	L22E		
L23					L23F			
E25		E25K				E25K		
K27	K27M	K27M						
A30						A30F		
F34		F34I						
R36					R36W		R36W	R36W
I37								I37
G41								G41N/S
A55								A55
I63	I63E	I63E						I63T
L64	L64P	L64P/A/R		L64P	L64P	L64P		
Q65	Q65R		Q65A/R					
L67	L67E	L67E						
L68					L68M		L68M	L68M
I70		I70S			I70S			
F72						F72L	F72L	F72L
R73	R73S			R73S			R73S	
I74	I74E	I74E						
C76	C76A				C76A	C76A		
R77		R77Q/H/A					R77Q	R77Q
H78	H78R							
S79	S79A							
R80	R80A	R80A		R80A				
R85								R85Y
Q86							Q86R	
R90	R90K						R90K	

Figure 1. Structure and functions of Vpr, with known polymorphisms that influence disease activity. (Gonzalez et al., 2017)

The potential contribution of Vpr to endothelial dysfunction in HIV-infected individuals

As described above, endothelial dysfunction in HIV-infected individuals is likely a result of the combination of various pathogenic processes, including the activity of the virus itself, opportunistic infections, and traditional cardiovascular risk factors. Previous research suggests that Viral protein R is associated with endothelial dysfunction (Kline & Sutliff, 2008; Hansen et al., 2013). The mechanisms by which it could contribute to endothelial dysfunction are unclear, but may involve its effect on macrophages, induction of TNF-α production, and increase in viral replication rate. Furthermore, with disease progression, the concentrations of extra-cellular Vpr

increase, and it has a pivotal role in reactivating latently-infected cells, allowing the pathogenic effects of HIV to continue in the later stages of disease. Various functions of Vpr may be independent of ART, which would allow some of the pathogenic effects of HIV-1 to continue even in treated individuals.

Recently, Dampier and colleagues demonstrated that specific amino acids in Vpr were associated with variations in neurocognitive status in people living with HIV. Four amino acid variations at three positions in the sequence were associated with reduction or increase in the Global Deficit Score, a measure of cognitive function (Dampier et al., 2017). This suggests that sequence variations within Vpr and other viral proteins may explain the unpredictable progression of HIV-associated disease in different individuals.

Rationale for research

Although Vpr amino acid variations have been explored within the context of HIV-associated neurocognitive Disorders (HAND) and neuronal apoptosis, it is less clear whether variations in Vpr are associated with alterations in its other extracellular properties, which may impact on HIV-associated endothelial dysfunction and the development of vasculopathy.

Thus, sequencing and describing the Viral protein R in our cohort of South African patients may further clarify any role it may have in endothelial dysfunction and stroke.

DEFINING THE RESEARCH

RESEARCH QUESTION

Are variations in the amino acid sequence of HIV-1 Viral protein R associated with acute ischaemic stroke in HIV-infected individuals?

HYPOTHESIS

There are differences in Viral protein R amino acid sequences between HIV-infected individuals with acute ischaemic stroke and HIV-infected non-stroke controls.

AIMS AND OBJECTIVES

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

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.

    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. To describe and compare the amino acid sequences of Subtype-C Viral protein R between HIV-infected individuals with acute ischaemic stroke, and HIV-infected individuals without stroke.

3.1 To visualise the HIV-1 Viral protein R amino acid sequences in HIV-infected individuals with and without stroke
3.2 To compare amino acid composition and variability of HIV-1 Viral protein R sequences between the stroke and control groups
3.3 To identify signature sites in the amino acid alignments that are distinctly representative of the stroke group relative to the control group

METHODOLOGY

STUDY DESIGN

This will be a sub-study of a larger study entitled 'Stroke and HIV-infection: a study of markers of endothelial dysfunction and ultrasonographic vascular phenotypes', which was designed to investigate the association between HIV and stroke in young adults. This study prospectively enrolled a cohort of young stroke patients who were characterized clinically, biochemically and radiologically in order to improve the likelihood of determining an accurate cause of stroke. It included HIV-infected individuals presenting with acute stroke together with two control groups: HIV-infected non-stroke controls and HIV-uninfected acute ischaemic stroke patients.

A sub-study of this larger study was undertaken in 2017 in fulfilment of the requirements for the degree of Master of Science in Medicine (Medicine), entitled 'The role of the HIV-1 Tat protein in acute stroke: more than just a transactivator of transcription?'.

This protocol outlines the plan for an additional sub-study, using data collected in the larger study entitled 'Stroke and HIV-infection: a study of markers of endothelial dysfunction and ultrasonographic vascular phenotypes' and the previous sub-study, 'The role of the HIV-1 Tat protein in acute stroke: more than just a transactivator of transcription?'

The design of this proposed sub-study will be a case-control study, with analysis of existing data from the two above-mentioned studies.

STUDY SETTING AND CHARACTERISTICS OF STUDY POPULATION

The stroke group was recruited from Groote Schuur Hospital, 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 community health centres were situated in Gugulethu and Crossroads.

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

RECRUITMENT OF THE STUDY PARTICPANTS

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. 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, where appropriate. The consent form for the original study, in English and isiXhosa, can be found in Appendix A.

To minimize the influence of traditional risk factors for stroke, which accumulate with increasing age, the participants were all aged between 18 and 45 years.

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 1. Modified inclusion and exclusion criteria

	Modified Inclusion Criteria	Modified Exclusion Criteria
Cases	• HIV-infected (ELISA positive) • Age >18 years and <45 years • Written consent obtained • Available Viral protein R sequences from existing data*	• HIV-uninfected • Age <18 years or >45 years • Haemorrhagic stroke, subarachnoid, sub-dural or epidural haemorrhage • Non-Subtype-C Viral protein R sequences*
Controls	• HIV-infected (ELISA positive) • Age >18 years and <45 years • Written consent obtained • Available Viral protein R sequences from existing data*	• Non-Subtype C Viral protein R sequences*

*Additional criteria for this sub-study

Efforts to minimize bias include the blinding of the technicians performing the analyses on the blood samples to the clinical information (stroke vs non-stroke).

Vulnerable populations, such as those individuals who were unable to give consent, were not included. The same standard of medical care was given to all individuals approached for the study, whether they were included in the study or not.

Haemorrhagic strokes (n=3) were excluded from this sub-study because of small numbers and the possibility that haemorrhagic stroke might have a different pathogenesis compared with ischaemic stroke.

Viral protein R amino acid sequences that, after subtyping, are determined to be non-Subtype C, will also be excluded. The other subtypes will be excluded from signature pattern analysis, as the significant genetic variation between subtypes could introduce confounding and reduce baseline similarity between the case and control groups.

SAMPLE SIZE

The main study enrolled 63 HIV-infected young strokes and 99 HIV-infected controls. The estimated sample size for the main study was calculated using existing studies on endothelial dysfunction in HIV. Using a two-tailed t-test, with an α of 0.05, power of 0.8, a sample size of 128 would have been sufficient to produce an effect size of 0.5. A total number of 162 were recruited and enrolled, which would have given the main study sufficient power.

We will exclude one HIV-infected individual with stroke who did not have blood samples taken on enrolment into the study, and therefore will commence with a total of 62 HIV-infected strokes and 99 HIV-infected controls.

Sequence analysis, which will take place once the study has commenced, will further determine the sample size. Once the sequences have been sub-typed, all non-Subtype C sequences will be excluded.

The final sample size will consist of all HIV-infected individuals with available Viral protein R sequence data, that are determined to be Subtype C only.

DATA COLLECTION AND CAPTURING

The main study, 'Stroke and HIV-infection: a study of markers of endothelial dysfunction and ultrasonographic vascular phenotypes' collected demographic, clinical, ultrasonographic, brain and vessel imaging data, captured it onto a case report form and entered into a Microsoft Excel Spreadsheet. The results of the laboratory tests including serum chemistry, full blood count, CD4 count, viral load, lipogram, coagulation studies and endothelial biomarkers have also been entered.

Sequence data was collected via clinical laboratory work for the previous sub-study, 'The role of the HIV-1 Tat protein in acute stroke: more than just a transactivator of transcription?'. This has already been captured and added to the existing database.

COMPLETED DATA ANALYSIS

Blood tests and imaging performed between 2010 and 2013

All participants had 30 millilitres of whole blood taken on enrolment into the original study. Blood samples were taken within the first five days of stroke onset in the stroke group, and on enrolment in the control group. 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.

Most of the stroke group participants had cerebrospinal fluid (CSF) taken by lumbar puncture for analysis. A very small number of controls (4/99) had lumbar punctures performed. 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. Both groups had duplex Doppler imaging of the carotid arteries to determine the intima-media thickness. All of the stroke participants 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.

Sanger sequencing performed in 2017

For the Tat protein sub-study, I had used the stored EDTA buffy coats for the DNA procurement, PCR and Sanger sequencing of part of the HIV-1 proviral genome.

For the PCR, the Promega GoTaq® Flexi Kit was used, according to manufacturer's instructions (Promega Corporation, 2013). We amplified *vpr* (HXB2 5559-5850) and *tat* exon 1 (HXB2 5831-6045) as a single fragment. The *vpr* gene lies in close proximity to, and overlaps, *tat* exon 1. For the purposes of cost-effectiveness, primer reliability and simplification of the PCR process, we amplified 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).

During the Tat protein sub-study, the *vpr* sequences were separated from the *tat* sequences, captured and stored into the database without any further analysis.

PROPOSED DATA ANALYSIS FOR THE PURPOSE OF THIS MMED SUB-STUDY

1. The archived amino acid sequences of Viral protein R will be read, separated into case and control sequences, and analysed for this MMed project.
2. Demographic, clinical and laboratory data will then be analysed for the individuals in whom the Vpr sequences are readable, and are determined to be Subtype C.

DETAILED METHODOLOGY

Clinical laboratory work performed in the previous sub-study HREC REF: 086/2017

Proviral DNA extraction

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.

Polymerase chain reaction

For the PCR, the Promega GoTaq® Flexi Kit was used, according to manufacturer's instructions (Promega Corporation, 2013). The *vpr* (HXB2 5559-5850) and *tat* exon 1 (HXB2 5831-6045) were amplified as a single fragment and then 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 to isolate *tat* and *vpr* are summarized below.

Table 2. 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 3. 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

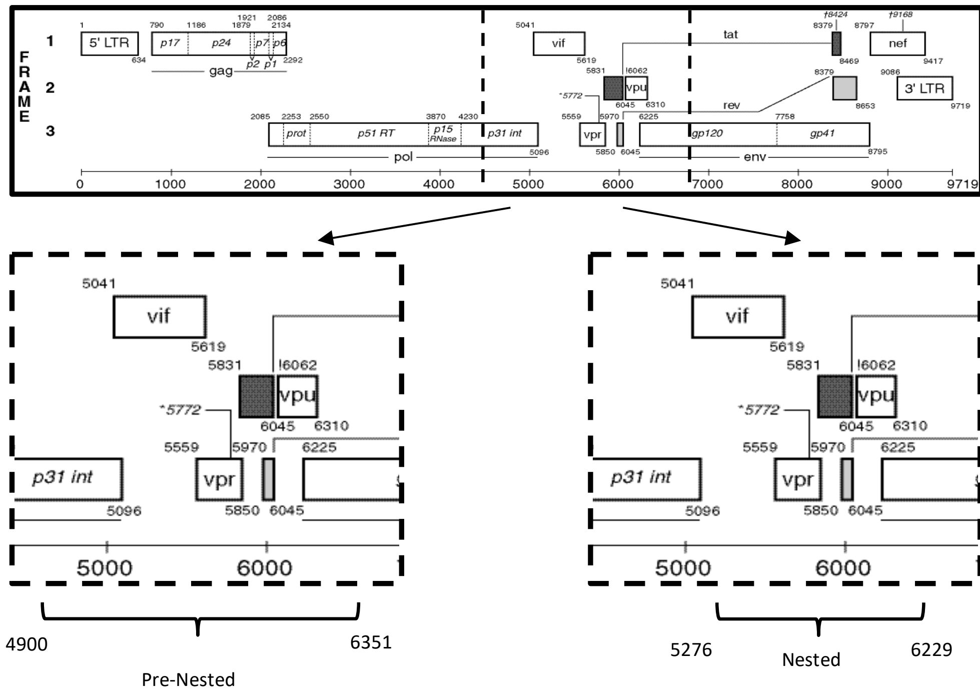

Figure 2. Approximate locations of the pre-nested & nested PCR fragments for *tat* exon 1 & *vpr*. Adapted from HIV Genome Map (www.hiv.lanl.gov)

Table 4. 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 5. 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

Gel electrophoresis and visualisation

The gene fragments were separated by 0.8% agarose gel electrophoresis. 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. The 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.

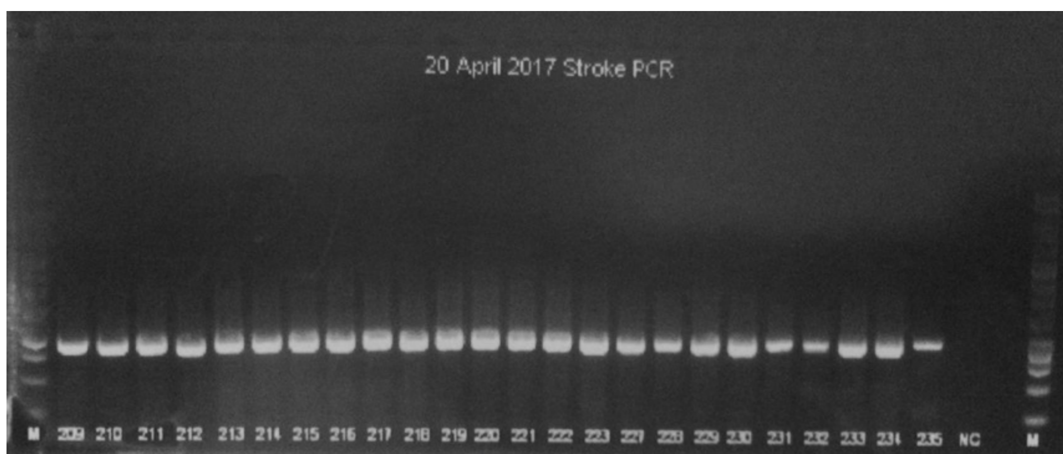

Figure 3. An example of an image acquired after gel electrophoresis and fluorescence

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.

DNA Sanger sequencing

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 6. 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 7. 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 8. 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	

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

Table 9. 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).

Data analysis to be performed in this sub-study

Acquisition and preparation of final sequences for analysis

The HXB2 *vpr* reference sequence will be 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 will be manually checked, then exported in fasta format into Geneious version R11 (Biomatters Limited, 2017). Only sequences that are readable will be used. Any sequences where the nucleotide at a certain position is unclear will be excluded, and not used for further analysis.

A multiple alignment of the entire cohort will be created using Multiple Alignment using Fast Fourier Transform (MAFFT) within Geneious. The sequences will then codon-aligned and manually checked. The sequences will be separated into stroke and control groups, and realigned. All nucleotide sequences will thereafter be translated into amino acids, realigned and manually re-checked to confirm the alignments.

Quality Control

Quality analysis will be performed on all sequences using the Quality Control Tool, accessed via www.hiv.lanl.gov.

Subtyping of the study cohort

The study cohort will be subtyped using the jumping profile Hidden Markov Model (Zhang et al., 2006). This can be cross-checked with REGA HIV-1 Subtyping tool (De Oliveira et al., 2014). Non-Subtype C sequences will be excluded from the subsequent analyses, as the significant genetic variation between subtypes could influence the amino acid signature patterns in this study.

Consensus sequences

Consensus sequences for the stroke and control groups will be created using the Advanced Consensus Maker, SeqPublish, AnalyzeAlign and Entropy tools (all accessed via www.hiv.lanl.gov) to cross-check accuracy. The consensus sequences will be used to visualize dataset similarity.

Visualization of dataset similarity

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

Visualization and analysis of site-specific variability

The CLC Sequence Viewer (Qiagen Bioinformatics, 2017) will be 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.

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 used to identify unique signature amino acids in the Tat protein exon 1 in the previous sub-study (HREC REF: 086/2017).

Signature pattern analysis will be 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).

The VESPA amino acid frequency calculations for all analyses will be cross-checked with AnalyzeAlign.

Significance of signature pattern analysis

Fisher's exact test will be used to determine the significance of the amino acid signature patterns identified by VESPA. P-values will be determined for signature amino acids in the stroke group relative to the control group. A p-value of ≤ 0.05 will be used to represent statistical significance of an amino acid mutation at that position.

Sequence logos

Sequence logos will also be created 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 by graphically representing 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. Sequence logos will be created using Weblogo 3.0. (Crooks et al., 2004) and cross-checked with AnalyzeAlign.

Signature amino acid mutation search

Any amino acid variations between the groups at variations at positions identified with signature pattern analysis will then be explored with the HIV Mutation Browser. This database contains mutation data collated from all HIV-related scientific literature. The HIV Mutation Browser can be used to find literature describing the mutation phenotype, as well as any functional effect of the substitution. (Davey et al., 2014).

PRELIMINARY DATA

Findings from the sub-study on the Tat protein have been detailed in my Msc (Medicine) thesis, which was submitted in February 2018 for graduation in December 2018. We found that there were two signature amino acids in the Tat protein exon 1 that distinguished the HIV-infected stroke group from the control group. Furthermore, we found that the HIV-infected stroke group had more traditional cerebrovascular risk factors, was more immunocompromised, and more likely to have interrupted treatment than controls. These findings support the idea of further examining the contribution of additional viral proteins to HIV-associated endothelial dysfunction and stroke risk.

ANALYSIS OF CLINICAL DATA TO BE PERFORMED IN THIS SUB-STUDY

Statistical analysis was performed on the clinical data for the main study cohort using Stata (StataCorp LLC, Texas, USA). The shape of data distributions was evaluated with Kernel Density Plots to determine whether data is normally distributed or skewed.

Statistical analysis for this sub-study will be done with Microsoft Excel and Graphpad Prism 7.0c. Continuous measurements will be reported with mean and standard deviation, where the data were normally distributed, and comparisons between the groups will be done with a two-sample t-test with unequal variances. Where the data were obviously skewed on the Kernel Density Plots, continuous variables will be reported with median and interquartile range (IQR), and comparisons will be made with the two-sample Wilcoxon rank-sum (Mann-Whitney) test. Counts (No.), and percentages (%), will be 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 will be used as the cut-off for significance in the analyses. Where there are a large number of missing values, the actual number of values analysed will be reported in the results tables. Graphs will be constructed using Microsoft Excel and Graphpad Prism 7.0c.

ETHICAL CONSIDERATIONS

This study conforms to the guidelines of the 2013 version of the World Medical Association Declaration of Helsinki-Ethical Principles for Medical Research Involving Human Subjects.

The initial research protocol for the Stroke and HIV Infection study was approved by the Human Research Ethics Committee of the Faculty of Health Sciences and University of Cape Town (HREC REF: 178/2010). The subsequent sub-study was also approved by the Human Research Ethics Committee of the Faculty of Health Sciences and University of Cape Town (HREC REF 086/2017) and has institutional approval from Groote Schuur Hospital. Subsequent annual progress reports have also been submitted and are up to date.

All participants recruited to the study were treated as per stroke protocols currently used at Groote Schuur and affiliated hospitals. HIV infection, opportunistic infections, and risk factors for stroke were all treated as per the hospital and national guidelines that were current at the time of the main study, with the consent of the participants. All those with newly-diagnosed stroke were referred to in-hospital or external rehabilitation services. Other benefits to the participants from the original study was that they underwent comprehensive assessment and optimal medical management of their condition.

PRIVACY AND CONFIDENTIALITY

Precautions to protect the confidentiality of the patients were maintained throughout the main study and subsequent sub-study. All data and records are identified by an assigned research number, rather than name of the patient. Paper records of the data are kept in the Division of

Neurology at Groote Schuur Hospital. Only the researchers involved in the study have access to the patients' names or personal information. At no point in this sub-study, or in the publication of any research pertaining to this sub-study, will the name or any other identifying data of the participants be used. The electronic data are accessible only to the researchers currently working on this study. Only the assigned research number, and not the names of the patients, are in the electronic data records.

INFORMED CONSENT PROCESS

All potential participants received a consent form, in which the purposes, aims and objectives of the study were described. The forms were available in English and isiXhosa. Participants whose home language was Afrikaans had a translator to go through the form with them and ensure complete understanding. The consent form described the potential benefits and harms of the study, as well as the option to have their blood samples stored for future use. The patients were also encouraged to ask the recruiting doctor or nurse if they had any further questions about the study once they had read the form. This was to ensure that participants and caregivers had full comprehension of the process of being involved in the original study.

This process was voluntary, and any individuals who decided not to participate in the study were given the same standard of medical care that was offered to all study participants. At no point did the decision of non-participation compromise medical care of any individuals that were approached by the investigators.

Once they had decided to participate in the study, all participants, or their primary caregiver (if cognition was impaired) signed the consent form, a copy of which they were allowed to keep.

CONSENT FORMS

Recruitment for the main study was completed in 2013. Please see Appendix A for the isiXhosa and English version of the consent form. There has been no alteration to the original consent forms.

REIMBURSEMENT FOR PARTICIPATION

During the data collection period, patients were not paid to be involved in the study, but were reimbursed for travel expenses if further follow-up visits required transport to the hospital.

POTENTIAL BENEFITS

The results of this study will not directly benefit the participants, but will further our understanding of the mechanisms by which HIV infection may be associated with stroke. We wish to further understand the association between HIV-1 proteins, which are still secreted in virally-suppressed individuals, and ischaemic stroke, so that effective therapies can be developed to reduce cerebrovascular disease risk in HIV-infected individuals.

LIMITATIONS OF THE PROPOSED STUDY

Study participants

The clinical information and data from blood samples that will be used in this sub-study were obtained from participants originally recruited for a larger study from 2010-2013. The larger study was not powered for the specific analysis of viral protein toxicity, 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. This may influence the results seen in the clinical analysis. For transparency, variables with a large number of missing values will be indicated in the results tables.

Study design

This study evaluates the prevalence of risk factors, clinical outcomes and the dominant quasispecies of the virus at a single time point. Longitudinal follow-up will not be possible in this study due to resource and time constraints. Follow-up could also have assessed whether any control participants developed ischaemic stroke. However, the HIV virus is constantly evolving due to host selection pressures. Any control participants who developed stroke or stroke participants who developed recurrent stroke would have to undergo re-sampling at the time of new stroke onset to determine whether they had acquired new mutations in the proviral DNA since enrolment into the main study.

Sources of bias

Potential for 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. 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 geographical discrepancy may result in differences in ethnic profile between the groups.

Sequencing methods

Sanger sequencing, rather than next-generation sequencing, was used to obtain the *vpr* 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). 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 or paracrine effect on neighbouring cells and endothelium (Dampier et al., 2016).

Analysis of Subtype-C

Analysis of the Viral protein R signature patterns will be confined 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 for 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 Viral protein R in acute ischaemic 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.

Lack of functional *in vitro* studies

This project will not include functional studies of any signature amino acids identified. Functional analysis would have enabled us to determine whether any amino acid variation translates into altered effect of the Viral protein R on endothelial cells *in vitro*.

COMMUNICATION AND DISSEMINATION

Principle 36 of the current Declaration of Helsinki, which guides research on humans, states that: "Researchers, authors, sponsors, editors and publishers all have ethical obligations with regard to the publication and dissemination of the results of research. Researchers have a duty to make publicly available the results of their research on human subjects and are accountable for the completeness and accuracy of their reports. All parties should adhere to accepted guidelines for ethical reporting. Negative and inconclusive as well as positive results must be published or otherwise made publicly available. Sources of funding, institutional affiliations and conflicts of interest must be declared in the publication. Reports of research not in accordance with the principles of this Declaration should not be accepted for publication."

We intend to publish the results of this work in peer-reviewed journals, and look for opportunities to present the findings at local and international conferences. Results from the sub-study on the Tat protein (HREC REF: 086/2017) have already been exhibited in poster format at the 22nd International AIDS Conference in Amsterdam in July 2018.

STUDY MANAGEMENT

ROLES AND RESPONSIBILITIES

I have undertaken to perform a sub-study on the original participants of the study on HIV-associated stroke for degree purposes. My role will include analysis of the Vpr protein sequences which were obtained in conjunction with the Tat protein sequences from the blood samples of the young strokes and the controls during the previous sub-study. I will also be doing the analysis of the clinical data, and writing up of the findings. I shall submit my findings under my MMed degree to the University of Cape Town.

My principal supervisor is Dr Kathleen Bateman, a consultant neurologist at Groote Schuur Hospital. My co-supervisors/secondary supervisors are Professors Marc Combrinck and Alan Bryer.

PROJECT TIMELINES

September to December 2018	Write-up and presentation of proposal to the Departmental Research Committee at the Department of Neurology, University of Cape Town Submission for expedited review to the Human Research Ethics Committee at the University of Cape Town
October 2018 to March 2019	Submission of proposal to Professional Masters Committee (PMC) Chair and the Board of the Faculty of Health Sciences for approval Submission to Groote Schuur Hospital for institutional approval Analysis of sequence and clinical data once approval granted
March 2019 to December 2019	Writing up of findings
January 2020 to December 2021	Writing up of findings and dissertation in publication-ready or standard monograph format
January 2022	Final deadline for formal submission to the University of Cape Town

BUDGET

There is no formal budget for this MMed project. Laboratory work and acquisition of statistical software such as Prism 7.0c was completed in the previous sub-study, which was funded by the Discovery Foundation Academic Fellowship Award and the South African Medical Association Research Masters Supplementary Scholarship. This MMed will consist of secondary data analysis, for which no additional funding is required.

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APPENDIX A: CONSENT FORM FOR ORGINAL 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 nguqqirha 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 II. OFFICIAL ETHICS APPROVAL LETTER FROM FACULTY RESEARCH ETHICS COMMITTEE

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

Room E53-46 Old Main Buildin
Groote Schuur Hospita
Observatory 792
Telephone [021] 406 649
Email: sumayah.ariefdien@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

08 February 2019

HREC REF: 768/2018

Prof A Bryer
Department of Neurology
E 8
NGSH

Dear Prof Bryer

PROJECT TITLE: THE VIRAL PROTEIN R IN HIV-ASSOCIATED ACUTE ISCHAEMIC STROKE: A CASE-CONTROL STUDY (SUB-STUDY LINKED TO 086/2017) MMED Candidate - Dr K McMullen

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

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 28 February 2020.

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: Dr Kate McMullen will also be involved in this study.

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.

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

Yours sincerely

TBurgess

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI), and Declaration of Helsinki (2013) guidelines.
The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

APPENDIX III. GROOTE SCHUUR HOSPITAL INSTITUTIONAL APPROVAL

GROOTE SCHUUR HOSPITAL
Enquiries: Dr Bernadette Eick
E-mail : *Bernadette.Eick@westerncape.gov.za*

Professor Alan Bryer
MEDICINE - NEUROLOGY

E-mail: mcmullenke@gmail.com / alan.bryer@uct.ac.za

Dear Professor Bryer,

RESEARCH PROJECT: The Viral Protein R in HIV-Associated Acute Ischaemic Stroke: A Case-Control Study (Sub-Study Linked to 086/2017 (MMed Dr Kate McMullen)

Your recent letter to the hospital refers.

You are granted permission to proceed with your research, which is valid until **28 February 2020, subject to the approval of Professor Lawrence Tucker.**

Please note the following:

a) Your research may not interfere with normal patient care.
b) Hospital staff may not be asked to assist with the research.
c) No additional costs to the hospital should be incurred i.e. Lab, consumables or stationary. **If access to TRACK Care/NHLS is required, kindly attach our letter of approval to the application form.**
d) **No patient folders may be removed from the premises or be inaccessible.**
e) Please provide the research assistant/field worker with a copy of this letter as verification of approval.
f) Confidentiality must always be maintained .
g) Should you at any time require photographs of your subjects, please obtain the necessary indemnity forms from our Public Relations Office (E45 OMB or ext. 2187/2188).
h) Should you require additional research time beyond the stipulated expiry date, please apply for an extension.
i) Please discuss the study with the HOD before commencing.
j) Please introduce yourself to the person in charge of an area before commencing.
k) On completion of your research, please forward any recommendations/findings that can be beneficial to use to take further action that may inform redevelopment of future policy / review guidelines.
l) Kindly submit a copy of the publication or report to this office on completion of the research.
m) At no time should any posters encouraging patients to partake in research, be displayed within a clinical area.

I would like to wish you every success with the project.

Yours sincerely

DR BERNADETTE EICK
CHIEF OPERATIONAL OFFICER
Date: 21 August 2019

C.C. Mr. L. Naidoo, Dr Laurene Booyens, Professor Ntobeko Ntusi

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Private Bag X,
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www.capegateway.go.v.za

APPENDIX IV. INSTRUCTIONS TO AUTHORS FROM JOURNAL OF NEUROVIROLOGY

Instructions for Authors

Scope

The *Journal of NeuroVirology* is dedicated to the molecular biology, pathogenesis and sequelae of viruses that have an impact upon the nervous system. JNV provides a forum to address basic science aspects of neurovirology and virus-induced neurological disorders and aims to bridge the gap between the basic and clinical sciences. The journal features full-length papers, short communications, mini-reviews on selected areas, and letters to the editors. A calendar of events and listing of positions is provided as a service to the neurovirology community. JNV will publish articles in molecular virology, genetics, neurochemistry, neuropharmacology, neurology, neurosurgery, medicine, neuro-oncology, gene therapy, developmental neurobiology and pediatrics, and aging and gerontology as they relate to neurotropic viruses.

Submission of Manuscripts

Three complete copies of each manuscript, including figures and tables, should be submitted directly to the editorial office:

Kamel Khalili, Ph.D., Editor-in-Chief
Journal of NeuroVirology
Center for Neurovirology and Cancer Biology
College of Science and Technology - Temple University
Biology-Life Sciences Building - 1900 North 12th Street
Philadelphia, PA 19122, USA
Tel: (215) 204-0680 Fax: (215) 204-0681
email: jnv@neurovirol.com

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The original manuscript and 1 set of high quality figures should be marked as such. Revised manuscripts should be marked as such and should include the manuscript number. Please enclose a cover letter including the corresponding author's complete address, telephone and fax numbers. To expedite the review process, authors may recommend 3-5 reviewers. Provide the name, address, telephone and fax numbers and area of expertise for each. Note that recommended reviewers will be used at the editor's discretion. Acknowledgment of receipt of the manuscript by the editorial office will be sent to the corresponding author, including a manuscript number that should be used for all subsequent correspondence.

Editorial Policy

All contributions and general correspondence regarding editorial matters should be addressed to the editorial office. Manuscripts submitted to the journal must represent reports of original research. Manuscripts will be sent for anonymous review by at least 2 referees, either Editorial Board members or others of similar standing in the field. Authors will be notified of acceptance, rejection, or need for revision within 2 months. When a manuscript is returned for revision, it should be returned to the editors within 2 months, otherwise it may be considered withdrawn. Accepted papers appear in the journal as soon as possible, normally 3-5 months after acceptance. By publishing an article in the *Journal of NeuroVirology*, authors agree to make freely available to colleagues any plasmids, viruses, antibodies, nucleic acids, and living materials such as microbial strains and cell lines, eg, used in the research reported and that are not available from commercial suppliers.

Preparation of Manuscripts

All manuscripts should be in English, double-spaced, on one side of the paper. Margins should be 1 inch. Please use these sections: Title page, Abstract, Introduction, Results, Discussion, Materials and Methods, Acknowledgments, References, Tables, Figure legends. Number each page (Title page is 1). Please indicate placement of each figure and table. Papers of over 45 pages (including tables and figures) are discouraged. Papers not following this format may be returned.

The title should be brief and contain no abbreviations. Include each author's full name and address. A corresponding author should be indicated with telephone, fax, and e-mail addressprovided. Please provide a running title of not more than 50 characters, along with 3 to 6 keywords, using standard MeSH-Medline subject headings, for indexing. Terms not contained within the title are preferred.

Abstracts should be 1 paragraph not exceeding 250 words. Please avoid abbreviations/reference citations.The Introduction should assume the reader is knowledgeable in the field and should therefore be as brief, not exceeding 1500 words.

References

Authors will be responsible for the accuracy of references. In the text, cite references by name and date. No more than 2 authors may be cited per reference; if there are more than 2 authors, use 'et al.' Please use the following style:

Journal

Lipton, HL, Dal Canto, MC (1976). Theiler's virus-induced demyelination: Prevention by immunosuppression. *Science,* 192, 62-64.

Book

Griffin, DE (1998). HIV infection of the brain: Viruses, cytokines, and immune regulatory factors associated with dementia. In *The Neurology of AIDS.* Gendelman, HE, Lipton, SA, Epstein, L, and Swindells, S (eds). Chapman & Hall: New York, pp. 73-85.

Roos, R. (1992) *Molecular Neurovirology.* Humana Press: New Jersey.

More than one article from the same author(s) in the same year must be identified by the letters a, b, c, etc., placed after the year of the publication. Journal abbreviations should follow Index Medicus. Personal communications should be avoided. Articles in press (state the journal in which they have been accepted) may be included. Manuscripts in preparation or submitted, but not yet accepted, may be cited in the text and should NOT be included in the list of references.

Illustrations

Illustrations submitted (line drawings, halftones, photos, photomicrographs, etc.) should be clean originals or digital files. Originals will be needed if these guidelinhes are not followed for digital files:

· 300 dpi or higher, sized to fit on journal page
· EPS, TIFF, or PSD format only
· submitted as separate files, not embedded in text files

Tables and Figures

Tables and figures should not be embedded in the text, but included as separate sheets or files. A short descriptive title should appear above each table with a clear legend and any footnotes below. All units must be included. Figures should be completely labeled, taking into account necessary size reduction. Captions should be double-spaced on a separate sheet. All original figures should be clearly marked in pencil on the reverse side with the number, author's name, and top edge indicated.

Nucleotide Sequences

It is expected that GenBank/EMBL accession numbers for primary nucleotide and/or amino acid sequence data will be included in the original manuscript or be inserted upon revision. The accession number should be included as a separate paragraph at the end of the Materials and Methods sections.

Abbreviations

These should be defined in parentheses after their first mention in the text, except for the use of accepted abbreviation, such as SI symbols, which need not be defined. Use generic names when referring to drugs; trade names may be given in parentheses at first mention.

Proofs

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An offprint order form and a bill for page charges will accompany the proofs. It is anticipated that page charges will be paid by authors whose research was supported by special funds, grants, or contracts (departmental, governmental, institutional, etc.), or whose research was done as part of their official duties. If the research was not supported by any of these, a request to waive the charges may be addressed to the Editors and sent to the editorial office.

Short Communications are submitted and reviewed in the same way as full-length articles. The short communication format is intended for the presentation of observations that do not warrant full-length articles. Abstracts should be under 100 words, and text should be brief. Do not use section headings in the body of the Short Communication; instead, report methods, results, and discussion in a single section. Materials and methods should be described in the text, not in legends or table footnotes. The References section is identical to full-length articles.

Case reports should highlight novel or interesting features of virological diseases affecting the nervous system, should be less than 1000 words in length, have 15 references or fewer, and contain no more than 3 figures or tables.

Mini-reviews are brief summaries of not more than 4 single-sided pages of developments in fast-moving areas within the scope of JNV that are based on published articles. Mini-reviews may be either solicited or proffered by authors responding to a recognized need and are subject to editorial review.

Letters to the Editor should be not more than 500 words and must include data to support the writer's argument and are intended for comments on articles published previously in the journal. If the editor believes that publication of the letter is warranted, he will solicit a reply from the corresponding author of the article.

Journal of NeuroVirology
https://doi.org/10.1007/s13365-020-00936-y

Viral protein R polymorphisms in the pathogenesis of HIV-associated acute ischaemic stroke: a case–control study

Kate McMullen[1] · Kathleen Bateman[1] · Alan Stanley[1] · Marc Combrinck[2] · Susan Engelbrecht[3] · Alan Bryer[1]

Received: 12 May 2020 / Revised: 3 December 2020 / Accepted: 21 December 2020
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Abstract
HIV-1 viral proteins have been implicated in endothelial dysfunction, which is a major determinant of ischaemic stroke risk in HIV-infected individuals. Polymorphisms in HIV-1 viral protein R (Vpr) may alter its potential to promote endothelial dysfunction, by modifying its effects on viral replication, reactivation of latent cells, upregulation of pro-inflammatory cytokines and infection of macrophages. We analysed Vpr polymorphisms and their association with acute ischaemic stroke by comparing Vpr signature amino acids between 54 HIV-infected individuals with acute ischaemic stroke, and 80 age-matched HIV-infected non-stroke controls. Isoleucine at position 22 and serine at position 41 were associated with ischaemic stroke in HIV. Individuals with stroke had lower CD4 counts and CD4 nadirs than controls**.** These polymorphisms are unique to individuals with stroke compared to South African subtype C and the control group consensus sequences. Signature Vpr polymorphisms are associated with acute ischaemic stroke in HIV. These may increase stroke risk by promoting endothelial dysfunction and susceptibility to opportunistic infections. Therapeutic targeting of HIV-1 viral proteins may present an additional mechanism of decreasing stroke risk in HIV-infected individuals.

Keywords HIV · Stroke · Viral protein R

Background

The increased risk of cardiovascular disease and ischaemic stroke in people living with HIV (Order, Virales; family, Retroviridae; subfamily, Orthoretrovirinae; genus, Lentivirus; species, human immunodeficiency virus) has been associated with HIV-induced endothelial dysfunction (Feinstein et al. 2016). Serum biomarkers of endothelial activation, dysfunction, inflammation and haemostasis are persistently elevated in HIV-infected individuals, even those well-suppressed on antiretroviral therapy (ART) (Graham et al. 2013). This suggests that endothelial dysfunction—ranging from chronic asymptomatic changes to clinical events related to overt vessel wall disease, such as ischaemic stroke—is common in HIV-infected individuals. Whilst the causes of stroke in HIV are multifactorial, HIV itself is an independent risk factor for stroke, particularly in those under the age of 45 years (Chow et al. 2012, Benjamin et al. 2016). Well-recognised causes of stroke in HIV, such as traditional vascular risk factors, can be addressed with lifestyle modification and lipid- or glucose-lowering medication. However, the mechanisms by which HIV itself increases the risk of stroke are less clear and there is a need to understand the pathogenesis of HIV-associated vessel wall disease, so that more targeted therapies can be developed.

HIV vasculopathy has been defined as “any abnormality of intracranial or extracranial cerebral blood vessels resulting directly or indirectly from HIV but excluding vasculitis associated with HIV infection or neoplastic involvement of the vessels” (Benjamin et al. 2012). Vessel abnormalities in HIV exhibit a range of histopathological changes which are seen in the absence of atherosclerosis or other infections (Benjamin et al. 2012, Chetty et al. 2000). HIV-associated vasculopathy is thought to be due to exposure of the endothelium to free virus and viral proteins,

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Published online: 18 January 2021

infected immune cells and upregulation of pro-inflammatory cytokines (Benjamin et al. 2012). The regulatory and accessory proteins, viral protein R (Vpr), transactivator of transcription (Tat) protein and negative factor (Nef), have been implicated in endothelial dysfunction (Wang et al. 2015, Kline and Sutliff 2008). Notably, these proteins may have the capacity to impair endothelial function independent of ART or actively replicating virus (Hansen et al. 2013).

The viral protein R, although better known for its neurotoxic potential, has several other properties by which it may contribute to endothelial dysfunction. Vpr is known to be neurotoxic in vitro. It may, however, contribute to endothelial dysfunction through several other mechanisms. Vpr promotes viral replication and reactivation of latently infected cells, which may increase exposure of vascular endothelium to free virus and viral particles. Vpr is associated with disease progression, it upregulates certain pro-inflammatory cytokines and is essential for productive infection of macrophages (Romani and Engelbrecht 2009, Kamori et al 2017; Guenzel et al 2014; Roux et al 2000; Ferrucci et al 2011).

Vpr amino acid polymorphisms may alter the clinical effects of this viral protein (Kamori et al 2017, Dampier et al. 2017, Jacquot et al. 2009, González 2017), highlighting the possibility that sequence variations within Vpr and other viral proteins may partly explain the unpredictable progression of HIV-associated disease in different individuals. Variants in the primary amino acid sequence of Vpr may alter its extracellular effects, thereby impacting on endothelial dysfunction and stroke risk. In this study we examined Vpr sequences in a clinically well-characterised cohort of South African individuals infected with HIV-1 Subtype C, to determine whether there is an association between certain Vpr variants and ischaemic stroke.

Methods

Study design and recruitment

This was a case–control study, analysing demographic, clinical, laboratory data and blood samples from 54 HIV-1 subtype C-infected individuals with acute ischaemic stroke, and 80 HIV-1 subtype C-infected non-stroke controls. These individuals were a subset of a larger cohort of young HIV-infected individuals, recruited between 1 August 2010 and 30 June 2013 for a study on HIV infection and stroke. Sixty-two HIV-infected individuals with acute ischaemic stroke were enrolled at Groote Schuur Hospital in Cape Town, South Africa. 99 HIV-infected non-stroke controls, matched for age, gender and ART status, were recruited from two community health centres in the Cape Town area during the same time period. To minimize the influence of traditional risk factors for stroke that accumulate with increasing age, all participants were between 18 and 45 years old. Approval for the study was granted by the Human Research Ethics Committee of the University of Cape Town for the use of patient data from the original cohort for this study (HREC REF: 768/2018).

Clinical and laboratory assessment

The original cohort was comprehensively investigated for vascular risk factors, stroke aetiology and HIV-related parameters and treatment status. Stroke aetiology was determined using modified TOAST criteria (Benjamin et al. 2016).

The demographic, clinical and laboratory data were analysed for this study using information from the 134 individuals whose Vpr sequences were determined to be HIV-1 subtype C. We used GraphPad Prism Version 8.0.0 for Mac (GraphPad Software, La Jolla, CA, USA, www.graphpad.com) to perform statistical analysis. Continuous measurements were reported with means and standard deviations (SDs), where the data were normally distributed and compared with two-sample *t* tests with unequal variances. Continuous variables of data with a skewed distribution were reported with median and interquartile ranges (IQRs) and compared using 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.

Genomic DNA isolation from EDTA buffy coats

DNA was isolated from 200 µl of prepared buffy coat using the Macherey–Nagel NucleoSpin® Blood Kit for extraction of genomic DNA from blood (Macherey–Nagel GmbH & Co. KG, Dueren, Germany). Proviral DNA was eluted in 100 µl of pre-heated buffer solution.

DNA amplification and sequencing

We used the Promega GoTaq® Flexi Kit, according to manufacturer's instructions (Promega Corporation, Madison, WI, USA), for the polymerase chain reaction (PCR). We amplified *vpr* (HXB2 5559-5850) and *tat* exon 1 (HXB2 5831-6045) as a single fragment and separated them 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 (Thermo Fisher Scientific, Waltham, MA USA). For the pre-nested reaction, we used the primers Vif-1 (5′-GGGTTTATTACAGGGACAGCAGAG-3′) and CATH-4R (5′-GTACCCCATAATAGACTGTGACC-3′), under the following conditions: initial denaturation at 94 °C

for 2 min, then 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 68 °C for 2 min. The final elongation step was performed at 68 °C for 10 min. The nested reaction proceeded as follows, using Vif-1F (5′-GGAATTTGGGTCATGGAGTCTCCATA-3′)/Tat-1_OR (5′-CTCATTGCCACTGTCTTCTGC-3′), with initial denaturation at 94 °C for 2minutes, 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 68 °C for 1 min. The final elongation step was performed at 68 °C for 10 min. The gene fragments were separated by 0.8% agarose gel electrophoresis. Gels were prepared with agarose powder and 1× Tris–acetate-EDTA (TAE) buffer. GR Green Nucleic Acid Stain was added to the agarose gel solution at a ratio of 1 µl:10 ml. The samples were loaded with Promega 6× Blue/Orange Loading Dye, with the Promega 1 kb DNA ladder as a marker. Each gel contained one negative control. The electrophoresis was run at 80 V and 400 mA for 35 min. The UVIprochemi II D-77 LS-26M gel documentation system was used for fluorescence and image acquisition of the proviral DNA fragments. The PCR purifications were performed with the Nucleospin Gel and PCR Clean-up kit, according to instructions (Macherey–Nagel GmbH & Co. KG, Dueren, Germany). The DNA was diluted to a concentration of 15–25 ng/µl, and Sanger sequencing was done using the Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit, according to the user guide (Thermo Fisher Scientific, Waltham, MA USA). The sequences were converted into raw data files with the Applied Biosystems 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA USA).

Determination of the final cohort for Vpr analysis

The HXB2 *vpr* reference sequence was imported and used to assemble the sequence contigs in Sequencher version 5.2.4 (Gene Codes Corporation, Ann Arbor, MI USA). A multiple alignment of all sequences was created with Multiple Alignment using Fast Fourier Transform (MAFFT) within Geneious version R11 (www.geneious.com). The sequences were then codon-aligned, translated into amino acids and exported in fasta format. The HIV-1 subtype of each sequence was determined with the REGA HIV-1 Subtyping tool (De Oliveira et al 2014). Non-Subtype C sequences were excluded to minimise the influence of inter-subtype genetic variation on signature pattern analysis. 54 sequences from the total of 62 HIV-infected individuals with acute stroke and 80 of the 99 HIV-infected controls were classified as HIV-1 Subtype C. The clinical and sequence data for these 134 individuals were used for this study on HIV-1 Subtype C Vpr.

Nucleotide sequences were submitted to GenBank with accession numbers MW321656—MW321789.

Amino acid sequence analysis

We used Viral Epidemiology Signature Pattern Analysis (VESPA), available at https://www.hiv.lanl.gov/content/sequence/VESPA/vespa.html to look for signature amino acids distinguishing the stroke from the control group (Korber and Myers 1992). Consensus sequences for both the stroke and control groups were determined with the Advanced Consensus Maker (https://www.hiv.lanl.gov/content/sequence/CONSENSUS/consensus.html). Creation of consensus sequences was to compare Vpr polymorphisms seen in this cohort with the consensus sequence for global Subtype C, as well as a consensus of all 1942 South African Subtype C Vpr sequences, both downloaded from the HIV Sequence Database (www.hiv.lanl.gov). We used SeqPublish (https://www.hiv.lanl.gov/content/sequence/SeqPublish/seqpublish.html) to align the sequences, using the HIV Sequence Database HIV-1 Subtype C Vpr consensus as the reference sequence.

Results

Patients with stroke had more advanced HIV disease than controls

Traditional risk factors for stroke in the two groups are shown in Table 1. The groups were similar in age and gender, with no difference in the proportion of individuals with hypertension, diabetes or those who were current smokers. Waist circumference and rates of alcohol or substance use were also similar. Although the stroke group had worse fasting lipid parameters than the control group, LDL-C values were at a level that has been associated with a low risk of atherosclerotic cardiovascular disease (Grundy et al. 2018) and missing data in the control group may have compromised the validity of this comparison.

In contrast, Table 2 shows that individuals in the stroke group had more advanced HIV disease than controls. Participants with ischaemic stroke had lower CD4 counts and CD4 nadirs than their non-stroke counterparts. Whilst median viral loads were similar between groups, there was a trend towards a higher median viral load in the stroke group, with a larger range of values. Almost 27% of individuals in the stroke group had defaulted ART prior to their stroke, whilst no controls had defaulted therapy.

Table 1 Demographics and risk factors for stroke

	Strokes, $n = 54$	Controls, $n = 80$	P value[a]
Mean age (years) (SD)	32.7 (5.8)	33.0 (6.3)	0.240
Female	36 (66.7)	51 (63.8)	0.729
Hypertension	9 (16.7)	8 (10.0)	0.255
Diabetes	3 (5.6)	0 (0.0)	0.063
Mean waist circumference (cm) (SD)	87.1 (13.2)	89.9 (15.7)	0.305
Smoker	15/53 (28.3)	16/70 (22.9)	0.491
Median pack years (IQR)	6.0 (7.0)	2.5 (3.75)	0.025
Alcohol use	15/53 (28.3)	29/75 (38.7)	0.224
Substance use	4 (7.4)	4 (5)	0.714
Fasting lipogram	$n = 46$	$n = 17$	
Mean total cholesterol (mmol/L) (SD)	4.04 (1.3)	3.17 (0.53)	0.0119
Mean LDL (mmol/L) (SD)	2.54 (1.3)	1.76 (0.6)	0.0251
Mean Trigs (mmol/L) (SD)	1.21 (0.5)	0.71 (0.3)	0.0012
Mean HDL (mmol/L) (SD)	0.97 (0.4)	0.71 (0.3)	0.0194

IQR interquartile range, *SD* standard deviation

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 derived with the *t* test or two-sample Wilcoxon rank-sum (Mann-Whitney) test

Stroke aetiology

The most common cause of stroke in this cohort was HIV-associated vasculopathies (33.3%). Varicella zoster virus (VZV) vasculopathy (29.6%), determined by positive CSF VZV PCR or positive monospecific CSF VZV-IgG index, was the second most common cause. Cardio-embolism accounted for 16.7% of strokes, whilst strokes due to atherosclerotic vasculopathy (with associated vascular risk factors) were relatively rare, comprising only 3.7% of strokes.

Four amino acids at two positions in Vpr are associated with the presence or absence of acute ischaemic stroke in HIV-1 subtype C infected individuals

The results of the VESPA analysis are depicted in Table 3. At positions 22 and 41, isoleucine and serine were the signature amino acids for the stroke group, when compared to controls. Leucine and glycine were associated with the non-stroke control group at these same positions in the Vpr amino acid sequence.

Table 2 HIV disease severity and treatment status

	Strokes, $n = 54$	Controls, $n = 80$	P value[a]
CD4+ T-lymphocyte count			
Median CD4 count, cells/μl (IQR)	206 (276)	329 (291)	0.008
Median CD4 nadir, cells/μl (IQR)	120 (236.8)	183 (179.5)	0.009
Viral load			
Median viral load, $\log_{10}$ copies/ml (range)	4.68 (0–6.4)	4.14 (0–5.8)	0.056
Antiretroviral therapy			
Prior cART	26 (48.1)	43 (53.8)	0.525
Defaulted ART[b]	7 (26.9)	0	0.001

ART 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 derived with the *t* test or two-sample Wilcoxon rank-sum (Mann-Whitney) test

[b]Expressed as no. (%) of those previously on ART

Table 3 Results of VESPA analysis

Signature position	Most common amino acid	Control group frequency (%)	Stroke group frequency (%)	Variant associated with non-stroke controls	Variant associated with acute ischaemic stroke
Position 22	Leucine	57.5	42.6	22L	22I
	Isoleucine	33.8	51.9		
Position 41	Glycine	36.3	33.3	41G	41S
	Serine	33.8	46.3		

Subtype C Vpr in the stroke group differs from the control group and South African subtype C Vpr at positions 22 and 41

The consensus sequence alignment (Fig. 1) compares the consensus sequences of this cohort of cases and controls with the consensus sequences of global and South African subtype C Vpr. The sequence alignment demonstrates that the stroke and control group Vpr consensus sequences were more similar to the South African subtype C Vpr consensus sequence than the global subtype C Vpr reference sequence. Notably, the stroke group Vpr consensus sequence had polymorphisms that were different from both the control consensus sequence and the South African subtype C Vpr consensus sequence at positions 22 and 41. These are highlighted with red boxes.

Sub-analysis of individuals with both polymorphisms

16 individuals (29.6%) in the stroke group had both polymorphisms, compared to 9 (11.3%) of the controls. The most common cause of stroke in those with both polymorphisms was HIV-associated vasculopathies (37.5%). VZV vasculopathy was in fact more common in those participants who did not harbour both polymorphisms (28.9%), with HIV-associated vasculopathies accounting for only 18.4% of strokes without both 22I and 41S. There was no significant difference in CD4 count, CD4 nadir or viral load between individuals with both polymorphisms and individuals without both polymorphisms. There was also no significant difference in CD4 count, CD4 nadir or viral load between those with glycine at position 41 and those with Serine at position 41.

Discussion

This study has demonstrated that there are two signature amino acids at positions 22 and 41 in Vpr distinguishing a group of HIV-1 Subtype C infected individuals with acute ischaemic stroke, from non-stroke controls. Furthermore, we have shown that these polymorphisms in Vpr also distinguish this group of individuals with acute ischaemic stroke from the consensus of known Subtype C Vpr sequences in the South African population.

An important limitation of this study is the cross-sectional study design. Nevertheless, baseline clinical and laboratory characteristics between groups are suitably similar, justifying an analysis of the influence of HIV-specific factors in ischaemic stroke in this cohort. The stroke group had more advanced HIV disease, with higher rates of treatment interruption, suggesting that HIV itself had a prominent role to play in the development of ischaemic stroke in these individuals. Furthermore, HIV-associated vasculopathies were the most common cause of stroke seen in this cohort, followed by VZV vasculopathy. The intriguing possibility exists that Vpr polymorphisms may have a role not only in promoting HIV-induced endothelial dysfunction but may also increase susceptibility to opportunistic infections. An additional important limitation was that Sanger sequencing, rather than next-generation sequencing, was used. 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 may be sufficient to estimate the response of the virus to selection pressures, such as antiretroviral therapy, host immune response and environmental factors, and also most

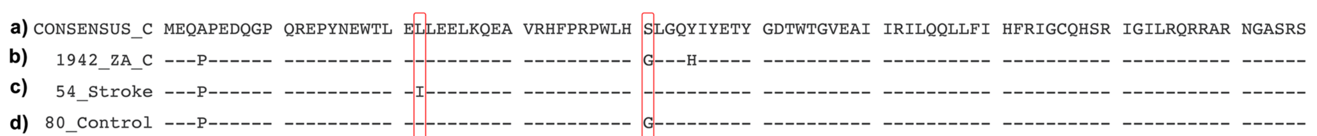

Fig. 1 Sequence alignment comparing **a**) the Vpr consensus sequence of global subtype C, **b**) the Vpr consensus sequence of South African subtype C and the Vpr consensus sequences of the **c**) stroke and **d**) control groups

likely to have the largest bystander effect (Dampier et al. 2016).

Although it has been postulated that Vpr has a potential role in endothelial dysfunction (Wang et al. 2015, Kline and Sutliff 2008), this is the first study, to our knowledge, to investigate whether there is an association between Vpr variants and acute ischaemic stroke. Given that other studies have demonstrated a correlation between various Vpr polymorphisms and clinical disease (Kamori et al 2017, Dampier et al. 2017, Jacquot et al. 2009, González 2017), we postulate from our results that 22I and 41S may directly or indirectly contribute to the pathogenesis of HIV-associated endothelial dysfunction, as well as other infective vasculopathies, such as VZV, in the cerebral vasculature.

Disease progression

The severity of endothelial dysfunction in HIV-infected individuals has been correlated with more advanced HIV disease (de Larranaga et al. 2003). Studies have shown that Vpr is intimately involved in viral replication by influencing reverse transcription and participating in transactivation of the long terminal repeat (LTR) (Romani and Engelbretch 2009, Guenzel et al. 2012). Vpr provides a distinct replication advantage for the virus by inducing cell-cycle arrest in G2 phase, in which proviral transcription rate is highest (Goh et al. 1998) Polymorphisms in Vpr have also been correlated with viral load and CD4 count, both of which are measures of disease progression (Kamori et al. 2017). In this cohort, the stroke group had evidence of more severe immunocompromise, with lower CD4 counts and CD4 nadirs, as well as a higher rate of treatment interruption. This may have contributed to a greater degree of HIV-associated endothelial dysfunction, increasing stroke risk. However, the two polymorphisms associated with acute ischaemic stroke in this study were not associated with significant differences in immune status, indicating that the role of Vpr variants in stroke is likely multifactorial, and more complex than simply a direct association with more advanced disease.

Macrophage infection, transmigration and cytokine production

Macrophages have a critical role in the development of atherosclerotic vascular disease in HIV-uninfected and HIV-infected individuals (Crowe et al. 2010; Libby et al, 2010). Similarly, transmigration of infected macrophages, with subsequent subendothelial trapping and release of pro-inflammatory cytokines, seems to be a key step in the pathogenesis of HIV-associated vasculopathy (Benjamin et al. 2012). Activated macrophages have been implicated in the expression of cellular adhesion molecules, which aid in immune cell translocation (Grome et al. 2017). In HIV-infected individuals, Vpr is essential for successful viral replication in the macrophage/monocyte line, enabling productive infection in these cells and enhancing the ability of HIV-1 to replicate in terminally differentiated macrophages (Guenzel et al. 2014; Vodicka et al. 1998; Connor et al. 1995). Furthermore, tumour necrosis factor-alpha (TNF-α) is produced mainly by activated macrophages and is a potent stimulator of production of other cytokines (Turner et al. 2014). Extracellular Vpr has been associated with excessive production of TNF-α, as well as IL-6 and IL-8 in numerous cell types (Roux et al. 2000; Ferrucci et al. 2011; Acheampong et al. 2002). Persistent and excessive cytokine release may contribute to the inflammation that disrupts normal endothelium, particularly via cytokine production from trapped subendothelial macrophages.

Opportunistic infections

Certain vasculotropic opportunistic infections, such as VZV and neurosyphilis, are recognised causes of stroke in HIV (Benjamin et al. 2016). These infections may independently or synergistically contribute to vascular injury in an HIV-infected individual, culminating in vessel occlusion and ischaemic stroke (Benjamin et al. 2012). Vpr impairs phagosome maturation in macrophages (Dumas et al. 2015). This effect of Vpr on macrophage phagocytosis may impair the immune response and increase susceptibility of individuals to opportunistic infections. Failure to clear pathogens may lead to the establishment of opportunistic infections that cause vasculitis. In this cohort, opportunistic infections were the second most common cause of stroke, which may have been due to a combination of more advanced immunosuppression and the Vpr-mediated effect on macrophage phagocytosis.

Amino acid variations at positions 22 and 41

There is still much that is unknown about the clinical effect of specific polymorphisms in Vpr. Certain amino acids at position 22 have been associated with cell cycle arrest, cell death and virion incorporation (González 2017). Position 41 has also been correlated with changes in viral load and CD4 count, with research suggesting an association of G41S with disease progression (Kamori et al. 2017; González 2017). However, participants in this study with serine at position 41 did not have significantly different CD4 counts, CD4 nadirs or viral load compared to those with glycine at position 41. This suggests that the role of Vpr variants in stroke is likely multifactorial, and more complex than simply a direct association with more advanced disease. A finding of interest was that HIV-associated vasculopathies comprised a larger proportion of stroke cause in the subset of individuals with stroke who had both 22I and 41S polymorphisms compared

to those who did not. This raises the possibility of a more important role of Vpr variants in transmigration of infected macrophages and the development of HIV-associated endothelial dysfunction.

In summary, further studies are needed to elucidate the potential effects of 22I and 41S on the specific functions of Vpr that may affect vascular endothelium, such as disease progression, macrophage infection and cytokine release, as well as susceptibility to opportunistic infections.

Conclusion

This study has demonstrated an association between Vpr polymorphisms and acute ischaemic stroke in HIV-infected individuals, particularly with relation to HIV-associated vasculopathies. This finding has both mechanistic and clinical implications, which deserve further scrutiny. Research into the effects of HIV proteins on HIV-specific disease pathogenesis is beginning to demonstrate the complexity of HIV protein sequence variation and its potential impact on disease course in individual patients. It is becoming clearer that HIV-1 viral proteins may have significant involvement in HIV disease pathogenesis, a process which is not fully controlled by suppressing viral replication with current ART. A fuller understanding of the extent and mechanisms of viral protein pathogenesis may lead to the development of more target-specific therapies to address the problem of ongoing HIV-specific morbidity in the antiretroviral era.

Funding KM was funded by the Discovery Foundation Academic Fellowship Award and the South African Medical Association Research Master's Scholarship. MIC was funded by the South African National Research Foundation "Incentive Funding for Rated Researchers" scheme: Grant number 85526.

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