Analysis of HIV-1 diversity and inflammatory markers in HIV-associated neurocognitive disorders (HAND).

By

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Dissertation presented for the degree of Doctor of Philosophy in Medical Virology at Stellenbosch University

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April 2022

Declaration by the candidate

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification

This dissertation includes 4 original papers published in peer reviewed journals and 4 unpublished papers under review in peer reviewed journals. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and the co-authors. The nature and extent of the contributions of co-authors is included in the dissertation. I am the first author of the 8 manuscripts submitted for consideration. In all the 8 manuscripts I contributed to the conception, design, conducting experiments, writing the manuscripts and responding to reviewer comments with guidance from my supervisors.

a. The declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to the manuscripts presented in this dissertation.
b. No other authors contributed besides those specified in the manuscripts (as specified in the dissertation), and
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SUMMARY

HIV-associated neurocognitive disorders (HAND), which involve impairment or disruption of neurocognitive functioning have become one of the most frequent complications in adult HIV-1 infections with global estimates ranging from 42% to 45%. The screening and diagnosis for HAND relies on multiple clinical and neuro-psychometric methods. However, these methods have a low reliability because they are not precise as most of possess inadequate psychometric properties and diagnostic accuracy. Therefore, this study aimed to describe and characterise viral and immunological determinants of HAND and evaluated their relationship with specific clinical, neuromedical and neuropsychological data to identify putative easy-to-measure biological markers for diagnosis of the condition.

This study demonstrated that higher peripheral blood lymphocyte-derived HIV-1 proviral DNA is a predictor of global and domain-specific neurocognitive impairment among individuals infected with HIV-1 subtype C. The study also determined proviral load cut-off /threshold value for neurocognitive impairment and associated diagnostic accuracy. It also identified IP-10 and RANTES as a plasma chemokine bio-signature for HIV-associated neurocognitive impairment with diagnostic accuracy comparable to standard psychometric tests used to screen and describe severity of HAND. In addition, the study identified 3 viral genetic signatures for cognitive impairment, namely Lysine at codon 24, (24K) and Arginine at codon 29 (29R) on Tat protein and Tyrosine (Y) at position 45 (45Y) of Vpr. These three signature amino acids were related to classical markers for monitoring HIV infection. Finally, we identified 4 conserved fragment sequences, PEDQGPQREPYNEWTLE (5 to 21), LGQYIY (42 to 47), TYGDTW (49 to 54), PEDQGPQREPYNEW (5 to 18) on viral protein R, that were associated with higher plasma viral load and proviral load.

The study has identified novel cytokine/chemokine and viral biomarker signatures for HIV associated neurocognitive impairment with low to moderate diagnostic accuracy. The findings demonstrated a need for interdisciplinary approach to elucidate possible molecular interactions between the peripheral blood immune markers, viral signatures and the CNS that are linked to observed clinical outcomes of neurodegradation in HIV infection. The identified biomarkers can be further investigated for use as screening tools and treatment endpoints for HAND.

OPSOMMING (AFRIKAANS)

MIV-geassosieerde neurokognitiewe versteurings (HAND), wat 'n inkorting of onderbreking van die neurokognitiewe funksionering behels, het een van die mees algemene komplikasies by volwasse MIV-1-infeksies geword, met globale ramings wat wissel van 42% tot 45%. Die sifting en diagnose vir HAND is afhanklik van verskeie kliniese en neuro-psigometriese metodes. Hierdie metodes het egter 'n lae betroubaarheid omdat hulle nie presies is nie, aangesien die meeste onvoldoende psigometriese eienskappe en diagnostiese akkuraatheid het. Daarom was hierdie studie daarop gemik om virale en immunologiese determinante van HAND te beskryf en te karakteriseer en het hulle verband met spesifieke kliniese, neuromediese en neuropsigologiese data geëvalueer om vermeende maklik meetbare biologiese merkers vir die diagnose van die toestand te identifiseer.

Hierdie studie het getoon dat perifere bloed limfosiet provirale HIV-1 DNS 'n voorspeller is van neurokognitiewe inkorting onder individue wat met subtipe C-siekte het. Die studie het ook die grens / grenswaarde van die provirale las bepaal vir neurokognitiewe inkorting en gepaardgaande diagnostiese akkuraatheid. Dit word ook geïdentifiseer as 'n chemokien-bio-handtekening vir MIV-geassosieerde neurokognitiewe inkorting met diagnostiese akkuraatheid wat vergelykbaar is met standaard psigometriese toetse wat gebruik word om die erns van HAND te ondersoek en te beskryf. Daarbenewens het die studie drie virale genetiese handtekeninge vir kognitiewe inkorting geïdentifiseer, naamlik Lisien by kodon 24, (24K) en Arginien by kodon 29 (29R) op Tat -proteïen en Tyrosien (Y) op posisie 45 (45Y) van Vpr. Hierdie drie kenmerkende aminosure was verwant aan klassieke merkers vir die monitering van MIV -infeksie. Laastens het ons vier klassieke merkers op virale proteïen R geïdentifiseer, wat verband hou met 'n hoër plasma virale lading en provirale lading.

Die studie het nuwe sitokien-/chemokien- en virale biomerker handtekeninge geïdentifiseer vir HIV -geassosieerde neurokognitiewe inkorting met 'n lae tot matige diagnostiese akkuraatheid. Die bevindinge toon 'n behoefte aan 'n interdissiplinêre benadering om moontlike molekulêre interaksies tussen die periferie en die SSS wat verband hou met waargenome kliniese uitkomste van MIV -infeksie, toe te lig. Die geïdentifiseerde biomerkers kan verder ondersoek word vir gebruik as siftings instrumente en eindpunte vir behandeling.

DEDICATION

To the loving memory of my pillar my mom, gone too soon!

Esnath Razura, Mai Glory, Mbuya vaTaro. Haurovi Esnath, Esnath Haurovi!

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisors Professor Susan Engelbrecht and Professor Richard H. Glashoff and the late Dr. Graeme B Jacobs for their guidance and advice throughout the course of this work. Many thanks to all the co-authors for all the valuable insights, suggestions and contributions towards manuscripts generated during my studies.

This work would not have been possible without the generous support from Poliomyelitis Research foundation (PRF) and National Health Laboratory Service (NHLS), as well as funding from the University of Stellenbosch (Division of Medical Virology) and the government Presidential and National Scholarship. I am grateful to the principal investigators of these grants and the Division of Medical Virology for the financial support I received.

I am grateful to my family for believing in me and supporting me at all the times. To my beloved wife Patience, and my children Taropafadzwa, Tinovonga, Tehillah Jobel, Tinovimbanashe Faithful and Tariroyashe: your support has been amazing, thank you for being patient while I took time away from home studying. I am forever grateful for God's wisdom, protection and guidance!

List of abbreviations

AIDS	Acquired Immunodeficiency syndrome
cART	combination Antiretroviral treatment
BBB	Blood brain barrier
BVM-R	Brief Visual Memory Test-Revised
GDS	Global deficit score
HVLT-R	Hopkins visual learning test revised
HAND	HIV Associated Neurocognitive Disorders
MEGA	Molecular evolutionary genetic analysis
MEME	Mixed effects model of evolution
MRI	Magnetic resonance imaging
NCI	Neurocognitve impairment
CNS	Central nervous system
PCR	Polymerase chain reaction
PBMC	Peripheral blood mononuclear cells
PLWH	People living with HIV
ROI	Region of Interest
SLAC	Single likelihood ancestor counting
SAR	Signature Amino acid residue
Tat	Transactivator of transcription
VESPA	Viral epidemiology signature pattern analysis
Vpr	Viral protein R
WAIS	Wechsler adult intelligence scale
WMS	Wechsler Memory scale

THESIS STRUCTURE

This thesis comprises five chapters presented as introduction, four individual data chapters composed of eight stand-alone scientific manuscripts, four of them published, one accepted for publication and three submitted papers currently under review. The last chapter is general discussion which summarizes results detailed in the manuscripts and drawing conclusions from the findings thereof. The manuscripts are presented in the listed chapters as follows:

Chapter 2 – Peripheral blood proviral load and neurocognitive function in HIV infection

Paper 1 (Review): Ruhanya*, V.; Jacob, G.B.; Glashoff, RH.; Engelbrecht, S. Clinical relevance of total HIV DNA in peripheral blood mononuclear cell compartments as a biomarker of HIV-associated neurocognitive disorders (HAND). Viruses 2017, 9,324. doi: 10.3390/v9110324. PMID: 29088095; PMCID: PMC5707531.

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Impact factor 5.048; Cited 9 times.

Paper 2: Ruhanya, V.; Jacob, G.B.; Nyandoro, G.; Paul, R.H.; Joska, J.A.; Seedat, S.; Glashoff, RH.; Engelbrecht, S. Peripheral blood lymphocyte proviral DNA predicts neurocognitive impairment in clade C HIV. Journal of NeuroVirology 2020, 26, 920–928. PMID: 32737863; PMCID: PMC7717048.

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Impact factor 2.987; Cited 2 times

Chapter 3 – Plasma proinflammatory cytokines and neurocognitive function in HIV infection

Paper 3: Ruhanya, V.; Jacobs, G.B.; Paul, R.H, Joska, J.; Seedat, S.; Nyandoro, G.; Engelbrecht, S.; Glashoff, R. Plasma cytokine levels as predictors of global and domain-Specific Human immunodeficiency virus-associated neurocognitive impairment in treatment-naive individuals. J Interferon Cytokine Res. 2021 ;41(4):153-160. doi: 10.1089/jir.2020.0251. PMID: 33885338.

* Corresponding author / authors: Vurayai Ruhanya

Impact factor 2.032 Cited 1 time.

Paper 4: Ruhanya, V.; Jacobs, G.B.; Naidoo, S.; Paul, R.H.; Joska, J.; Seedat, S.; Nyandoro, G.; Engelbrecht, S.; Glashoff, R. Impact of plasma IP-10/CXCL10 and RANTES/CCL5 levels on neurocognitive function in HIV treatment naïve patients. AIDS Res Hum Retroviruses. 2021 Sep;37(9):657-665. doi: 10.1089/AID.2020.0203. PMID: 33472520.

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Impact factor 2.325; Cited 1 time.

Paper 5: Ruhanya, V.; Jacobs, G.B.; Paul, R.; Joska, J.; Seedat, S.; Nyandoro, G.; Engelbrecht, S.; Glashoff, R.H. Proinflammatory Cytokines and Chemokines are Related to MRI Measures of White and Gray Matter in HIV Infection. Pathogens 2021.10 (Submitted - Currently under review)

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Paper 6: Ruhanya, V.; Jacobs, G.B.; Paul, R.; Joska, J.; Seedat, S.; Nyandoro, G.; Engelbrecht, S.; Glashoff, R.H. Plasma cytokine biomarker cut-off values for HIV-associated neurocognitve impairment in adults. Viral Immunology 2021;10:1-8. http://doi.org/10.1089/vim.2021.0047. PMID.34807730.

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Impact: 2.257.

Chapter 4 – HIV diversity and HIV-associated neurocognitive impairment

Paper 7: Ruhanya, V.; Jacobs, G.B.; Paul, R.; Joska, J.; Seedat, S.; Nyandoro, G.; Glashoff, R.H.; Engelbrecht, S. HIV-1 subtype C Tat exon-1 amino acid residue 24K is a signature for neurocognitive impairment. Journal of neurovirology 2021. NJIV-D-21-00243. (Submitted – Currently under Review).

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Paper 8: Ruhanya, V.; Jacobs, G.B.; Paul, R.; Joska, J.; Seedat, S.; Nyandoro, G.; Glashoff, R.H.; Engelbrecht, S. HIV-1 subtype C Vpr amino acid residue 45Y and specific conserved fragments are associated with neurocognitive impairment and markers of viral load. Journal of neurovirology 2021. NJIV-D-21-00204 (Submitted - Currently under review)

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CHAPTER 1

1.0. INTRODUCTION

1.1.Prevalence and severity of HAND

Combination anti-retroviral therapy (cART) has increased life expectancy of HIV/AIDS patients and reduced incidence of opportunistic infections. Hence, efforts are being directed towards managing long-term HIV complications such as central nervous system (CNS) diseases which have continued to affect patients, despite adequate virological control [1]. HIV-associated neurocognitive disorders (HAND), which involve impairment or disruption of neurocognitive functioning have recently become one of the most frequent complications in adult HIV-1 infections with global estimates ranging from 42% to 45%, of which 72% are from Sub-Saharan Africa (SSA) [2, 3]. In South Africa, HAND is a significant health problem with estimated prevalence ranging from 15% to 60% depending on settings [4].

Severity of HAND can range from asymptomatic neurocognitive impairment to HIV-associated dementia [5, 6]. HIV-associated dementia (HAD) is the most severe and disabling form of HAND [7]. Although the severe manifestations of HAND have been markedly reduced by the use of cART [8], the milder forms of cognitive impairment, including asymptomatic neurocognitive impairment (ANI) and mild neurocognitive disorder (MND) are still problematic, and currently account for approximately 88% of all HAND cases [1, 9]. Asymptomatic neurocognitive impairment (ANI) is defined as acquired impairment in at least two cognitive domains without a decline in activities of daily living (ADL). There is no overall functional impairment in ANI. However, MND is characterised by changes in two or more cognitive abilities with overall functional impairment or decline in activities of daily living [9].

Despite the availability of cART, HIV-associated neurological disorders remain a substantial challenge affecting between 10 - 20% of HIV-infected people receiving cART[10]. Although the severe forms of HAND since the introduction of cART have decreased, the less severe forms (MND and ANI) remain unchanged if not increased. This could be due to the inability of antiretroviral drugs to penetrate the BBB or due to the chronic CNS and systemic immune activation that persist despite cART[11]. In some settings, the prevalence of severe forms (HAD) were reported to be very high on people receiving cART, ranging between 25 -31% [12, 13]. In SSA a meta-analysis of HAND prevalence studies using international HIV

dementia scale (IHDS) reported a pooled HAD prevalence of 30% [14] which was comparable to other settings. The screening tools for diagnosis were developed in the pre-cART era for severe forms of HAND and it is now of limited accuracy in post-cART era to detect mild forms [15].

1.2. Pathogenesis of HAND

Pathological evidence of brain invasion by HIV includes neuronal loss, synaptic and dendritic damage, astrogliosis, microgliosis and nucleated giant cell formation [15]. The presence of activated resident microglia, infiltration predominantly by monocytoid cells, including blood-derived macrophages. HIV-1 associated neuronal damage and neuronal loss have been reported in numerous regions of the central nervous system (CNS) [16]. Structural imaging of HIV patients suspected of HAND observed deep impact on grey matter and subcortical regions [17] and cerebral atrophy [18]. Pathological research has demonstrated the presence of viral particles in astrocytes, microglia, oligodendrocytes, and in a lower proportion, neurons [19]. However, the precise mechanism of HAND pathogenesis is unknown, but studies have shown that HAND is likely determined by a complex interaction between viral factors, cellular targets, and the immune response. These interactions are associated with functional alterations, damage and death of neurons culminating in cognitive impairment [20]. HIV is thought to enter the CNS early during systemic infection through different mechanisms [21, 22]. In addition to initial neuroinvasion and infection of perivascular macrophages and microglia, factors associated with progressive HIV infection in the periphery may be required to eventually trigger the development of HAND [23].

1.2.1. Neuroinvasion

There are 4 different proposed models which describe neuroinvasion by HIV-1 namely, the Trojan Horse model, direct interaction of blood brain barrier (BBB) with cell free virus, macropinocytosis and endocytosis and non-specific passage of virus from periphery into the brain. According to the “Trojan horse” hypothesis (Figure 1), HIV-infected perivascular monocytes/macrophages and lymphocytes traffic HIV from the peripheral blood to the CNS, leading to the infection of the resident microglial cells [24, 25, 26].

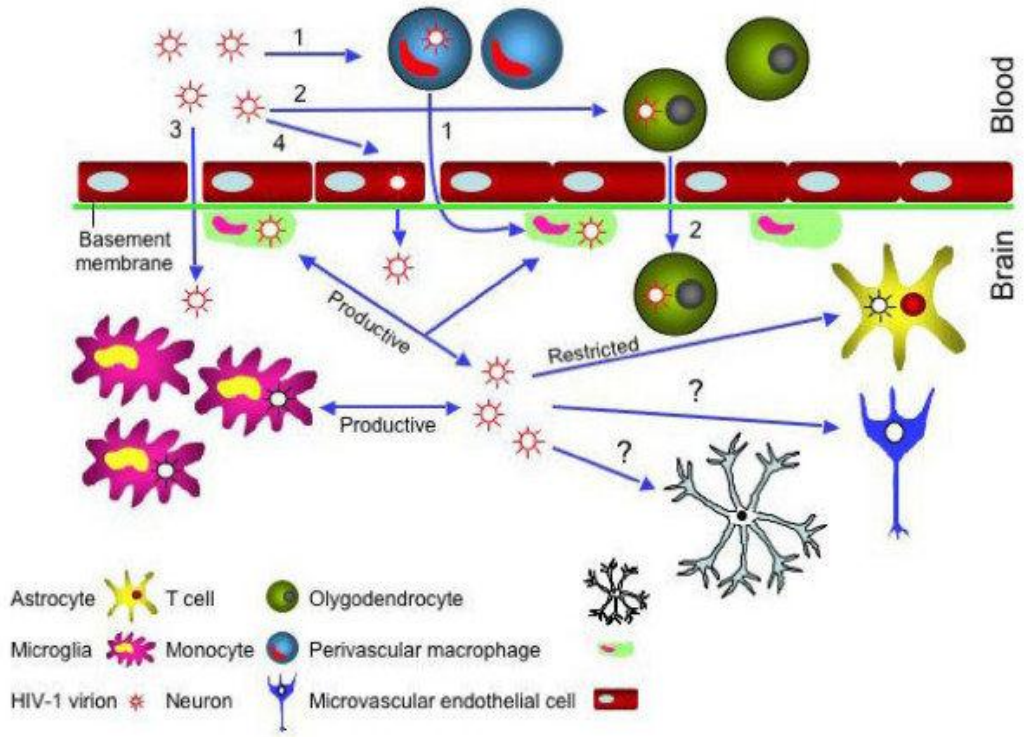

Figure 1. *Trojan horse model for HIV neuroinvasion (1) Migration of infected monocytes which differentiate into perivascular macrophage; (2). infected CD4+ T cells as a source of infection in the brain; (3) direct entrance of the virus or (4) entrance of HIV-1 by transcytosis of brain microvascular endothelial cells [27]*

According to this model, HIV-1 enters the CNS as a passenger in cells trafficking to the brain. Infected and activated monocytes and lymphocytes in the periphery cross the BBB and enter the brain as part of the routine immunologic surveillance. This model which is considered to represent the major route of HIV-1 neuroinvasion, was confirmed by in situ immunohistochemical analysis that brought evidence of virus accumulation in perivascular regions [28]. Once the virus has been replicated in macrophages and microglia within the CNS, it becomes difficult to eradicate [29], and therefore establishes chronic infection.

Transmigration of leukocytes across BBB is an active / dynamic multistep process involving both the BBB cells, the leukocytes and inflammatory mediators [30]. It involves leukocyte adhesion on endothelial cells and subsequent diapedesis across the activated vasculature. Activated T cells express adhesion molecules and metalloproteases necessary to transverse cerebral capillary endothelial cells and the basement membrane [31]. Transmigrated monocytes and T lymphocytes with specific antigens for HIV are retained in the CNS [32]. Studies on neuropathology of HAND have shown evidence of abnormal BBB structure and altered expression of Tight Junction proteins (TJPs) ZO-1 and occlude in where leukocyte infiltration were detected [33]. It was also demonstrated that the state of immune activation of

cells that constituted the BBB dictated the ability of monocytes to traverse the BBB using *in vitro* models. Similar models also showed that there was increased BBB permeability and loss of TJP when HIV-infected monocytes and this process was dependent on MCP-1/CCL2 [34].

A more contested hypothesis suggests that viral entry of HIV across the BBB is due to the transcellular uptake of the virus by certain chemokine receptors on the microvascular endothelial cells (BMECs) of the BBB such as CCR3, CXCR4 and CCR5 [35]. This model proposes that cell free HIV enters the CNS by direct-by-direct infection of the BBB. Infection of the BBB provides source HIV infectious particles closer to CNS parenchyma cells. The infected BBB cells either infect the CNS parenchyma cells or they release free virus into the CNS. Confocal microscopy has revealed free viruses transited across endothelial cells in the BBB model [36]. Studies have also revealed that human brain pericytes express the major HIV-1 receptor CD4 and co-receptors CXCR4 and CCR5 and determined that HIV-1 can replicate in these cells as determined by p24 ELISA [37]. Infection of brain pericytes results in compromised integrity of BBB allowing haematogenous spread of free viruses into the brain.

Internalization of HIV by endothelial cells or astrocyte foot processes by macropinocytosis and endocytosis is another proposed mechanism by HIV is subsequently transferred into the CNS. Ultrastructural studies have shown HIV crossing BBB by transcytosis through visicles [38]. Macropinocytosis is a unique pathway of endocytosis characterised by the nonspecific internalization of large amounts of extracellular fluid, solutes and membrane in large endocytic vesicles known as macropinosomes. Macropinocytosis in BBB EC is dependent on intact lipid rafts and MAPK signaling [39]. Lipid rifts which are plasma membrane microdomains enriched in cholesterol and sphingolipids that are involved in the lateral compartmentalization of HIV at the cell surface.

Another way by which HIV-1 enters the brain is through disruption of the BBB which may occur by altering the structure of tight junction proteins that tightly bind the microvascular endothelial cells. The BBB is a tightly regulated vascular interface that limit trafficking of macromolecules, peripheral cells, toxins and invading pathogens from the blood to the central nervous system (CNS) [40]. HIV-1 can disrupt the BBB to enhance neuroinvasion through

viral proteins which are released or secreted by HIV-1 infected cells and free viral particles [35,36]. HIV-1 viral proteins such as Tat, gp 120, Vpr and Nef induce functional changes that contribute to BBB disruption. Disruption of BBB which occurs in early infection can lead to simple entry of circulating virus into the brain (haematogenous spread). The gp120 protein can induce passage of HIV-1 by induction of adsorptive endocytosis [41] while Nef can breach BBB by increasing matrix metalloproteinase 9 (MMP-9) activity [42]. In the same manner, HIV-1 Tat stimulates MMP-9 in astrocytes and disrupt BBB integrity. Elevated Tat concentration have been shown to induce disruption of tight junctions by altering expression of tight junction proteins which may severely compromise BBB integrity.

Peripheral inflammation and BBB disruption

Peripheral HIV-1 infection causes expression of inflammatory cytokines including IL-1β, IL-6, IL-9, IL-17, IFN-γ, TNF-α, and MCP-1/CCL2, can lead to reduced tight junction (TJ) expression [43] (Figure 2). For example, claudin- 5, the most important TJ protein responsible for selective permeability of the BBB, and inflammation leads to its downregulation and BBB disruption [44]. Virus-induced systemic inflammation was also associated with degradation of occludin and some studies showed that peripheral cytokines reduced expression of ZO-1 [45, 46]. Changes in the expression of TJs are now used as indicators of BBB disruption. Studies have shown that monocyte chemoattractant protein1 (MCP-1) plays a major role in disruption of BBB by altering permeability through upregulation metalloproteinase and disruption of endothelial adherent junctions (AJ) [47]. This process is thought to be facilitated by HIV infection [48].

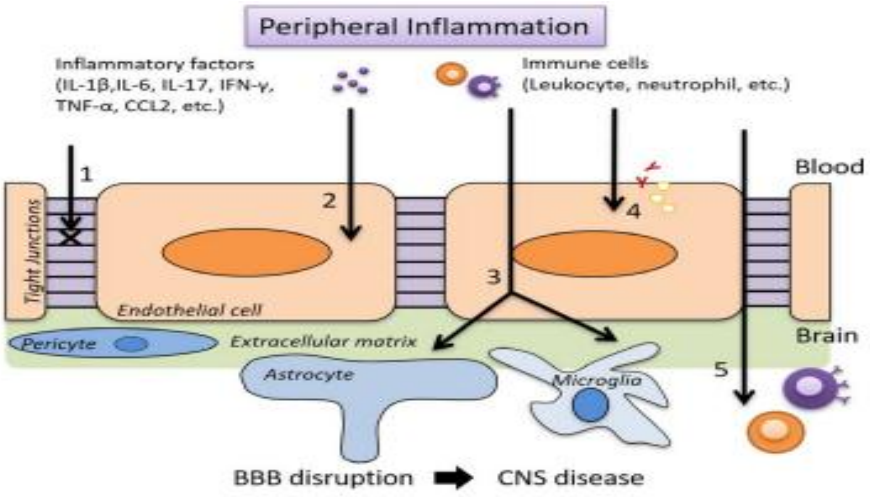

Figure 2. Mechanisms of BBB disruption induced by peripheral inflammation. (1) Changes in tight junctions; (2 damage to endothelial cells; (3) activation of astrocytes and microglia; (4) alteration of multiple transport pathways and receptors; (5) penetration of peripheral immune cells [39].

1.2.2. Neurodamage

Two models have been proposed to explain neurodegradation and the development of symptomatic HAND, namely the direct and indirect pathways. The direct pathway proposes that the viral proteins (Env gp120, Tat and Vpr) released from HIV-infected monocyte-derived cells, can cause neuronal death by direct interaction with neurons [49,50]. Among the direct effects of viral proteins on neurons is neuronal depolarization by direct excitation independent of synaptic interactions, NMDA receptor dysregulation and disruption of calcium homeostasis in the case of Tat [51].

The indirect pathway suggests that neuronal death is mediated by inflammatory cells targeting HIV proteins released by infected cells. Infected or activated macrophages release a variety of soluble cytokines and chemokines which are pro-inflammatory e.g., IL-6, GM-CSF, TNF-α and IL-1β which eventually leads to neuronal apoptosis [52]. Sensory cells like immune cells express receptors for pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) and cytokines. Therefore, infection, tissue damage by HIV-1 infection and cytokine release disturb neuronal activity [53].

In both, direct and indirect pathways of neuronal damage, macrophages/microglia infection by HIV play a significant role in various ways. Microglia are resident macrophages of the CNS and monocytes can also serve as precursors of macrophages after they have been trafficked across BBB [54]. HIV-infected macrophages produce higher levels of inflammatory ctokines and neurotoxins leading to neuroinflammation and damage. Under this inflammatory condition, macrophages derived from recently transmigrated monocytes die in a few days. Macrophage turn-over is associated with progression of disease in HIV infection. In contrast to monocyte-derived microglia, embryonically derived infected macrophages/ microglia survive for extended periods from weeks to years contributing to prolonged inflammatory state which leads to neuronal dysregulation and/or cell death. Chronic inflammatory state is a major problem in HAND. Mobility, ability to infiltrate tissues and extended survival of macrophages have been proposed as critical for generation, stability, dissemination and reactivation of HIV reservoirs in the CNS [55].

It has also been observed that monocyte-derived macrophages are more susceptible to HIV infection [56]. Therefore, these macrophages seeded in the brain are one of the major contributors to reservoirs of HIV infection. Macrophage-trophic (M-trophic) viruses are able

to efficiently enter cells with low CD4 receptors and they are compartmentalized mostly in the brain [57]. It has been suggested that infected macrophages contribute to reservoir persistence despite cART.

Although astrocytes contribute a significant population in the brain the percentage of infected cells has been determined to be very low [58]. However, when we consider the total astrocyte cell population, this type of cell is potentially an important reservoir for HIV in the brain. It has been shown that HIV-1 can achieve a state of dormancy in astrocytes and can be reactivated by a treatment with tumor necrosis factor (TNF) or interleukin (IL)-1β to produce infectious virus particles [59]. Studies using highly sensitive PCR in single astrocytes have shown that up to 20% of astrocytes could carry viral DNA [60]. The extent of virus infection in these cells was correlated with the severity of the neuropathology. A more recent study has demonstrated that astrocytes sustain long-term productive HIV-1 infection without establishment of reactivable viral latency [61]. This finding could imply that virus production by this brain cell population could contribute to the neurological disorders seen in HIV-1-infected persons.

1.3. Diagnosis of HAND

Diagnosis of HAND is important because of the clinical and functional impact on affected indviduals. The condition is associated with heightened risk of mortality, poor adherence to treatment and disruption of everyday functioning [62, 63, 64, 65, 66, 67]. However, HAND like several other neuropsychiatric conditions is difficult to diagnose because there are no standard laboratory markers or standard protocols to clinically assess the condition optimally. The screening and diagnosis for HAND relies on multiple clinical and neuro-psychometric methods. These psychometric methods are based on assessment of key cognitive domains episodic memory, motor skills, speed of information processing, language, working memory and sensory perception as described by the Frascarti criteria [68. These methods have been extensively used to define the scope and severity of HAND [69, 70. However, these methods have a low reliability because they are not precise [71]. A scoping review of screening tools for HAND in (SSA) indicated that most tools possess inadequate psychometric properties and diagnostic accuracy [72]. In this review, the reported diagnostic accuracy of tools ranged from a specificity of 37% to 81% and sensitivity of 45% to 100%.

The poor clinical validity of behavioural tools for screening and defining the spectrum of HAND has resulted in varying prevalences of the diseases emanating from misdiagnosis. Depending on the criteria for diagnosis and setting, wide margins in prevalence of 21% to 86% have been reported [73, 74]. HAND diagnosis and hence, prevalence range is affected by many factors including demographic factors (eg. sex distribution, education and country development level), psychosocial variables and clinical variables such as Hepatitis C coinfection and the type of anti-retroviral therapy (ART) used [75]. It is generally agreed that a standardized neuropsychometric assessment with culture-adapted tools are still needed in clinical settings, researchers disagree on the accuracy of these tools to detect MND and ANI. Methods for assessing HAND have a false positivity that exceeds 20% [76]. In SSA, the situation is worsened by lack of specialised training to recognize and screen using psychometric approaches, resulting in few clinics assessing this condition in routine HIV clinics.

Therefore, there is a need for easy-to-use biological phenotypes in addition to behavioural screening. The availability of reliable biomarkers would aid in diagnosis and monitoring of patients and could flag patients for more detailed psychometric assessment. In addition, having such markers would inform understanding of neuropathogenesis and therapeutic strategies to HAND [77]. Although many proposed potential biomarkers for HAND have been described, there is a lack of sensitive and highly specific candidates. The biomarkers can be divided into five categories as follows: structural imaging changes observed in neuroimaging, biomarkers of metabolic stress, soluble markers of immune activation, markers of neuronal injury and virus associated signatures. However, to date most of the markers are associated with HAD rather than the milder and subtle forms of the condition like MND and ANI.

Research has demonstrated that individuals with HAD had higher levels of circulating activated CD14 monocytes and monocytes that co-express CD69, as compared to those without HAD. The finding supports the hypothesis that activated monocytes enter the brain and subsequently trigger production of neurotoxic mediators [78]. Evidence has also accumulated that there is an association between plasma soluble CD14 and cognitive dysfunction [79]. This evidence supports that accumulation of activated macrophages/ microglia resulting from systemic immune activation triggers neurodegeneration in HAND [80]. Immune activation, particularly inflammation continue to be the hallmarks of HIV

infection which has been shown to be a predictor of clinical events including HAND [81, 82]. There is a growing interest in exploring possibility of using plasma inflammatory cytokine and chemokine biomarkers for the diagnosis of HAND. Plasma cytokines may serve as easily accessible biomarkers that complement current standard methods for diagnosis of HAND by enhancing sensitivity of diagnosis [83]. Blood based biomarkers have a great potential for clinical utilization since blood samples are a routine part of HIV clinics. A number of inflammatory markers of NCI have been identified, including, MCP-1/CCL2, IP-10/CXCL10, RANTES/CCL5 and IL-2, IL-6, Eotaxin [84, 85, 86, 87, 88]. Available data on cytokines and neurocognitive impairment in HIV infection is mainly based on correlative studies. There is need to go further and determine cytokine biomarker cut-off values HAND for sensitive screening and therapeutic interventions.

Neurological involvement of HIV infection remains a huge challenge since cART has not accomplished full protection of the nervous system. The use of standard clinical markers of HIV infection like current CD4+ count and plasma viral load for diagnosis and monitoring of HAND were shown to be inadequate in the antiretroviral therapy era. In some studies plasma viral load and CD4+ count did not appear to be important markers as they were not associated with HAND [89, 90, 91]. The markers did not adequately reflect the environment that continues to exist in the CNS. The quantity of cerebrospinal fluid (CSF) HIV RNA associated with CNS disease in several studies [92, 93, 94]. CSF HIV RNA may have a role in the management of HIV infected subjects with overt CNS diseases. Although there has been discordance in some studies, CSF has shown to be correlated with plasma viral load indicating that it reflects the progression of HIV disease [95, 96]. However, the sample is clinically expensive and not easily accessible compared to plasma. Therefore, there is need for blood based biomarkers to monitor HAND. Besides, some researchers believed that RNA replicating in the CNS is driven by independent HIV strains evolving in the CNS compartment [97]

In cases of chronic HIV infection, HIV-1 proviral DNA has been identified as a correlate of HAD. In a cross-sectional study in Hawaii, it was observed that HIV-DNA in PBMCs in HAD was 20 times higher than in normal individuals [98]. The same study also observed higher levels of HIV-1 DNA in activated inflammatory monocytes (CD 14+/CD16+) than in non-activated monocytes [99]. Individuals with HAD have a higher percentage of circulating activated monocytes/macrophages (M/MΦ), suggesting that HIV-1 proviral DNA harboured

in these reservoir cells is transported into the CNS by the same cells. Therefore, the detection of plasma viral load (VL) in patients is not sufficient as a marker of HAD but needs to be supplemented by measurement of proviral DNA load. HIV-1 proviral DNA load can also be used to measure the progression of HAND in cases where plasma viral load is undetectable [35]. However, more data is required on the diagnostic thresholds and associated accuracies.

1.4. HIV diversity and HAND

Diversity, which is the hallmark of HIV-1 impacts on every aspect of the pandemic, including disease progression, diagnosis and immune response [100]. Inter-clade and intra-clade genetic variation in HIV-1 neurotoxic genes such as env, tat and vpr have a different impact on the severity of HAND [101,102]. Genetic diversity can be estimated from nucleotide sequence data by measuring pairwise differences between sequences in an alignment or the number of polymorphic sites [103]. For HIV-1, Sanger sequencing of cDNA from total viral RNA gives the consensus sequence which is the genetic average of all the variants in virus population [104].

HIV stably persists as integrated provirus in lineages of lymphocytes and monocytes, and it is protected from biochemical decay [105]. Persistence and transactivation of proviral DNA in cellular reservoirs such as Memory T- cells have been linked to viral rebound in HIV patients. This process creates a complex archive of wild-type and mutant viruses generated throughout the life of infected patients. As a result, HIV in the host consists of evolving and interacting sub-populations. It is, therefore, imperative to analyze the genetic characteristics of viruses in peripheral blood cell components of patients with HAND and to determine how they impact on the severity of disease.

1.5. HAND sequence data and repository

It has been noted that research in HAND has been limited by the availability of HIV-1 sequence samples from both the brain and HAND assessed patients. Small sample size and variation in study methodologies have led to conflicting results in HAND research. As a result, a repository, namely the HAND database (http://www.handdatabase.org) has been created as HIV sequence resources for all HAND studies, as well as tools for studying HIV evolution in the brain and other tissue reservoirs [106, 107]. Forty-five (45%) of the sequences in the HBSD are from the variable region V3 of the envelope *env* gene and 31% of the sequences come from the near or full length of the *gp120* [108]. The database is a

valuable tool for studying HIV evolution in the brain and other tissues reservoirs, but it is limited to envelope gene sequences. The HAND database, which is an updated HIV-1 sequence resource for HAND research, has the majority of sequences obtained from the *env* gene as well. More than 90% of the samples in the HAND database are from America, Europe and Asia combined. Of all the archived sequences in the HAND database, 79% are purely subtype B.

1.6. Problem Statement

There is almost no HIV-1 sequence data in the HAND database from SSA where 72% of people suffer from different forms of HAND. There is also limited data available from HIV-1 subtype C and how the sequence diversity in proposed neurotoxic genes affect the severity of neurocognitive impairment and the immune response. In addition to the lack of sequence data, little information on differences in virus sequences derived in different cell compartments (monocyte vs. lymphocyte) and their impact on neurocognitive impairment is available.

HIV-1 may enter the CNS early in infection and a greater viral genetic diversity in the CNS, might increase the risk of viral adaptation and fitness in the CNS. Although profiling peripheral HIV provirus may not be a true reflection of CNS diversity, it has been identified as a correlate of HAND [109] and the processes that eventually lead to neurocognitive impairment emanate from peripheral blood mononuclear cells trafficking the CNS [44]. Besides, peripheral blood samples are more accessible for research, diagnostic and monitoring purposes than brain biopsy and CSF. There is limited proviral data of HAND in subtype C in SSA. In addition to virological markers, there is a growing interest in elucidating the role of plasma inflammatory markers in the pathogenesis and potential use as complimentary tests for diagnosis and monitoring of neurocognitive impairment in HIV infection. There is paucity of data on the impact plasma cytokine biomarkers on measures of cognitive function and brain volumes. We used a combination of high-through put immunoassays, sequencing proviral DNA, quantitative PCR, bioinformatic analyses and statistical models quantitative a well-described HAND cohort of 200 participants to search for novel cytokine and virus based peripheral signatures for HAND.

Research Question

What are the cytokine biomarkers and genetic signatures for neurocognitive impairment on peripheral lymphocyte-derived HIV-1 subtype C *tat* and *vpr* sequences? Is lymphocyte-derived proviral load in HIV-1 subtype C predictive of neurocognitive impairment.

Research hypothesis

There are plasma cytokine biomarkers and unique genetic signatures on HIV subtype C neurotoxic genes, *tat* and *vpr* that are associated with neurocognitive impairment.

Aim

To define novel viral and plasma immune biomarker signatures in HAND

Specific objectives

Objective 1: To assess lymphocyte derived proviral load as a predictor of HIV associated neurocognitive impairment.

Objective 2: To evaluate the relationship between plasma cytokine levels and cognitive impairment

Objective 3: To determine impact of genetic variation/diversity on HIV-1 subtype C on neurocognitive impairment.

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CHAPTER 2

PERIPHERAL BLOOD PROVIRAL LOAD AND NEUROCOGNITIVE FUNCTION IN HIV INFECTION

Two papers in this chapter were published, a research and a review article.

The work focuses on peripheral blood proviral HIV-1 DNA as a predictor of HAND. Firstly, examined different PCR assays and their potential clinical utility in HAND diagnosis. We then used an ultrasensitive HIV-1 DNA qPCR to assess this marker as a predictor of HIV-associated neurocognitive impairment.

***Paper 1*: Ruhanya**, **V**.; Jacob, G.B.; Glashoff, RH.; Engelbrecht, S. Clinical relevance of total HIV DNA in peripheral blood mononuclear cell compartments as a biomarker of HIV-associated neurocognitive disorders (HAND). Viruses 2017,9,324

The author contributions to the published paper were:

Conception and design including discussion with subject matter experts, conducting the review using systematic methodology, writing of manuscript

Clinical Relevance of Total HIV DNA in peripheral blood mononuclear cell compartments as a biomarker of HIV-associated neurocognitive disorders (HAND)

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***Abstrac**t: The pathogenesis of HIV-associated neurocognitive disorders is complex and multifactorial. It is hypothesized that the critical events initiating this condition occur outside the brain, particularly in the peripheral blood. Diagnoses of HIV-induced neurocognitive disorders largely rely on neuropsychometric assessments, which are not precise. Total HIV DNA in the peripheral blood mononuclear cells (PBMCs), quantified by PCR, correlate with disease progression, which is a promising biomarker to predict HAND. Numerous PCR assays for HIV DNA in cell compartments are prone to variation due to the lack of standardization and, therefore, their utility in predicting HAND produced different outcomes. This review evaluates the clinical relevance of total HIV DNA in circulating mononuclear cells using different published quantitative PCR (qPCR) protocols.*

The rationale is to shed light on the most appropriate assays and sample types used to accurately quantify HIV DNA load, which predicts severity of neurocognitive impairment. The role of monocytes as a vehicle for trafficking HIV into the CNS makes it the most suitable sample for determining a HAND associated reservoir. Studies have also shown significant associations between monocyte HIV DNA levels with markers of neurodamage. However, qPCR assays using PBMCs are cheaper and available commercially, thus could be beneficial in clinical settings. There is need, however, to standardized DNA extraction, normalization and limit of detection.

***Keywords**: HAND; HIV DNA; qPCR; biomarker*

1. Introduction and Brief Pathology of HAND

Highly active antiretroviral therapy (HAART) has reduced the prevalence of AIDS related opportunistic infections. Consequently, efforts are being directed at managing long-term HIV complications such as central nervous system (CNS) diseases, which have continued to affect patients, despite adequate virological control [1]. Some of the most frequent complications in patients with HIV-1 are HIV-associated neurocognitive disorders (HAND), which involve impairment or disruption of neurocognitive functioning [2]. It is estimated that 20–69% of all HIV-1 cases have neurocognitive disorders [3,4]. It is estimated that 20–69% of all HIV-1 cases have neurocognitive disorders [3,4]. There is a wide range of disorders covered in the description of HAND according to the 2007 Frascati criteria of categorising severity of neurocognitive impairment [5]. Dementia associated with HIV, HAD, is the most severe form of HAND. Mild neurocognitive disorder (MND) is characterised by minor neurocognitive impairment and asymptomatic neurocognitive impairment (ANI) is characterised by two or more cognitive abilities with no functional impairment.

The precise mechanism of the neuropathogenesis of HAND is unknown, but studies have shown that HAND is likely determined by a complex interaction between viral factors, cellular targets and the immune response. Two models have been proposed to explain neurodegradation and the development of symptomatic HAND: namely, the direct and indirect pathways (Figure 1). Both initially require HIV-1 infection of the perivascular macrophages and microglia in the brain. According to the “Trojan horse” hypothesis, HIV-infected perivascular monocytes/macrophages traffic HIV from the peripheral blood to the CNS, leading to the infection of the resident microglial cells [6].

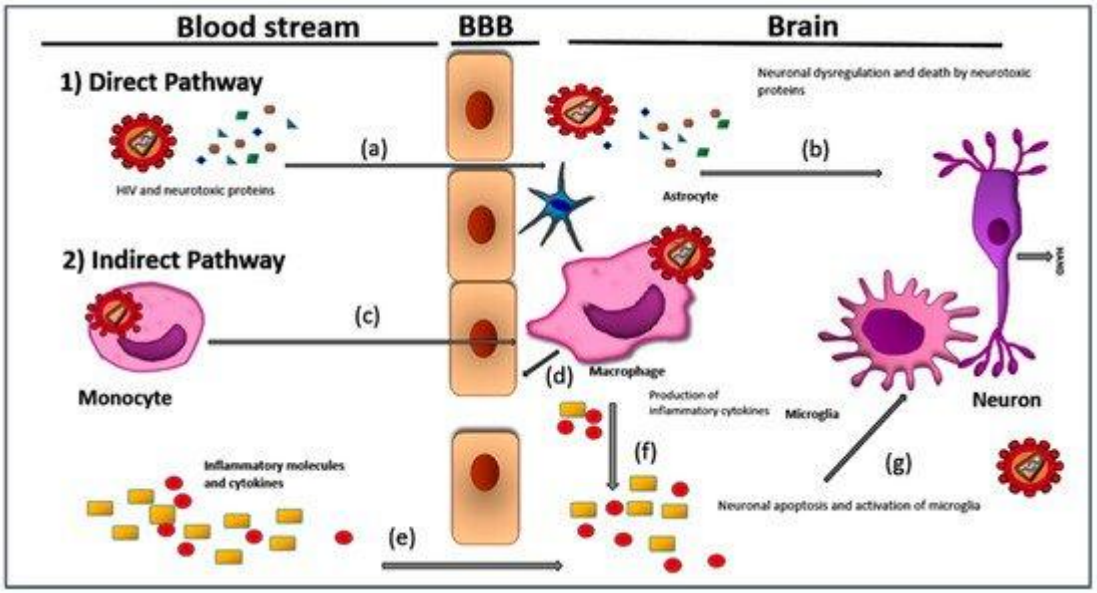

Figure 1. Mechanism of neuropathogenesis. Two pathways involved shown by arrows: (1) Direct pathway caused by HIV and released HIV proteins. (2) Indirect pathway involving secretion cytokines. (**a**) Virus particles and viral proteins shed and cross Blood brain barrier (BBB). (**b**) Neural injury caused by direct viral infection and dysregulation by viral proteins. (**c**) Infected monocyte infiltrating BBB. (**d**) Release of cytokines from infected monocytes contributing to disruption of BBB. (**e**) BBB become more permeable to cytokines present in the periphery. (**f**) More cytokines released into the brain and (**g**) cytokines disrupt normal functioning ultimately leading to neuronal apoptosis resulting in different forms of HIV associated neurocognitive disorders (HAND).

The direct pathway proposes that the viral proteins (Env gp120, Tat and Vpr), released from HIV-infected monocyte-derived cells, can cause neuronal death by direct interaction with neurons. Among the direct effects of viral proteins on neurons is neuronal depolarization by direct excitation independent of synaptic interactions, N-methyl-D-aspartate (NMDA) receptor dysregulation and disruption of calcium homeostasis in the case of Tat [7,8]. The indirect pathway suggests that neuronal death is mediated by inflammatory cells targeting HIV proteins released by infected cells. Infected or activated macrophages release a variety of soluble cytokines and chemokines, which are pro-inflammatory, including IL-6, GM-CSF TNF-α and IL-1β, which eventually leads to neuronal apoptosis [9,10].

Since there is no standard protocol to assess the condition optimally, HAND is difficult to diagnose. The screening and diagnosis for HAND relies on multiple clinical and neuro-psychometric methods, such as the World Health Organization (WHO) Auditory Verbal Learning Task (WHO-AVLT) and the Revised Brief Visual Memory Task (BVMT-R) for learning efficiency [11,12]. These methods have been used to define the scope and severity of HAND. However, there is need to compliment clinical and neuropsychometric assessments

with HIV-specific biological markers underlying symptomatic HAND. The availability of reliable biomarkers would help to diagnose patients and bring a better understanding of neuropathogenesis and therapeutic strategies to HAND [13]. Although many proposed potential biomarkers for HAND have been described, there is a lack of sensitivity and some of them are not HIV specific [14]. They include markers of monocyte activation such as soluble CD163 and CD14 (sCD163 and sCD14) [15], cytokine and chemokine receptors as well as markers of neural injury and glial dysfunction [1,16–18]. They do not distinguish between the severity and HAND subtype. No longer closely associated with neuropsychometric performance are HIV specific markers such as plasma CD4+ T-cell count and viral load [19] in this era of HAART, where prevalence of HAND in aviremic patients is common. Peripheral blood mononuclear cells (PBMCs) containing HIV DNA has been linked to HAND progression in virally suppressed patients and therapy naïve patients [11,20]. This review summarizes recent developments on the potential use of total peripheral blood mononuclear cell-associated HIV DNA as a marker of HAND, focusing on PCR quantification assays. The rationale is to highlight the potential clinical utility of this biomarker for HAND, considering the roles played by different mononuclear cell compartments (lymphocytes compared to monocytes) or unfractionated PBMCs in the proposed HAND pathogenesis models, bringing into context the suitability of different samples used in different PCR assays. A careful analysis of published PCR methods regarding sample type, sample loading quantity and quality, normalization, robustness, accuracy, sensitivity and specificity is required before total peripheral HIV DNA is used as a biomarker for HAND in clinical settings.

2. Latency and Different Forms of HIV DNA in the Periphery

Immune cells are infected by HIV through CD4 receptors, and the co-receptors CCR5 and/or CXCR4. The primary target cell is the CD4+ T lymphocyte. Following cellular infection, viral genetic material (RNA) is then reverse transcribed into HIV DNA that becomes integrated into host DNA or might exist as non-integrated linear and circular forms (1-LTR and 2-LTR circles) [21]. A subset of these infected immune cells may become memory cells that harbor HIV DNA without the production of virus (i.e., latently infected) [22,23]. This latency state may be disrupted if the cells are re-stimulated/activated or when treatment is interrupted [21,23]. The HIV DNA is persistently stored in a stable form in lineages of memory T lymphocytes and is protected from biochemical decay [23]. Monocytes may be directly infected or may be infected following phagocytic uptake of infected lymphocytes

and/or free virions. Infection may occur in the blood or at the bone marrow level [24]. Antiretroviral therapy (ART) reduces HIV RNA particles in circulating blood to undetectable levels, but it cannot eliminate HIV DNA infecting white blood cells [22]. Latently infected cells are disseminated throughout the body but are differentially concentrated in the lymphoid tissues [25]. Blood is the most accessible tissue, but not the true representative medium for measuring the latent reservoir [26]. Furthermore, the frequency of HIV infected cells is higher in the gut associated lymphoid tissue than in the blood [27–29]. Outlined earlier, the systemic HIV DNA reservoir trafficking to the CNS with activated cells (monocyte/macrophage lineages) causes activation-induced neurodamage [30,31]. The easy accessibility of peripheral blood as a clinical specimen makes it more feasible to investigate the contribution of peripheral HIV DNA reservoirs to pathogenesis of neurocognitive impairment [24,28]. Peripheral HIV DNA is an independent predictor of HIV disease irrespective of combined antiretroviral therapy and was widely used in both adult and paediatric cohorts [32–35]. The HIV DNA in peripheral blood has also been used as a prognostic marker of disease progression, especially in suppressed patients where it is the only biomarker of viral activity [35]. Therefore, it is possible to investigate the role of peripheral HIV DNA in neuropathogenesis by evaluating the association between the clinical categories of HAND (ANI, MND and HAD) and the quantities of HIV DNA. Available assays can quantify both total and unintegrated HIV DNA forms (linear, 1-LTR and 2-LTR) [36,37].

3. What Samples Can Be Used to Measure HIV DNA as a Biomarker for HAND?

Assays to quantify HIV DNA by qPCR have utilized both PBMCs and sorted cell subsets as clinical samples, but choices must be made on the most appropriate samples to collect and process [38]. The appropriate clinical specimen in HIV DNA qPCR should give a true size on the peripheral HIV-1 DNA pool and its composition [36], which enables clinical diagnosis and monitoring disease progression [39,40]. It is important to decide on the type of sample and form of DNA to use as a marker to define and monitor the progression of HAND, considering the size of the HIV reservoir in the sample and its role in the pathology of the disease. An appropriate clinical specimen gives information which reflects on the condition of the disease by correlating with findings from clinical assessments [41]. The choice of the sample should consider the stage of HIV disease as it is known that there are differences between early HIV disease and advanced stages of infection with regard to shift in tropism [42]. Advanced stages of HIV diseases shows depletion of CD4+ T lymphocytes, while there

is both expansion and increased turnover of peripheral monocytes, including CD14+/CD16+ subsets known to be associated with increased susceptibility to HIV infection [43]. Therefore, investigations should consider the clinical stage of neurocognitive impairment/severity of HAND since there is likely to be differences in quantities of PBMC subsets and the composition of different forms of HIV DNA in the cells. Sample type is very critical because it has implications, first on the quantity and forms of DNA in the cell type and, second, the role played by the cell type in HAND pathogenesis. All three forms of HIV DNA have been detected in brain tissues of patients having AIDS dementia [44]. Some studies have measured HIV DNA quantities using the whole blood, while others have used PBMC and found good correlation between the two samples [34,36]. To consider peripheral HIV DNA load as a reliable virological marker of HAND in clinical settings, a careful assessment of PBMC and its cell subsets as appropriate samples is required.

3.1. Unfractionated PBMC

Peripheral blood mononuclear cells constitute the cellular part of the blood containing cells with a round nucleus, namely monocytes, T cells, B cells, Natural killer cells (NK) and dendritic cells [45]. Peripheral blood mononuclear cells (PBMCs) are involved in many immune-related diseases and there is growing interest to use them as surrogate markers of several diseases [46,47]. These PBMCs can be obtained relatively easily from routinely collected blood samples without analytical difficulties [48]. Thus, there is a growing interest to use PBMCs diagnostically as surrogates for direct sampling of sites of infection and to investigate other disease processes such as HIV-associated neurocognitive impairment [32,49]. It has been demonstrated that HIV DNA levels in PBMCs correlate with antiretroviral therapy efficacy, suggesting that DNA quantitation is a useful tool to monitor the decay of the HIV reservoir, especially when plasma viremia is undetectable [50].

Significant positive correlations between levels of HIV DNA measured in PBMC and CSF cell pellets has been reported in HAND patients, showing that there was a link between peripheral blood HIV reservoirs which influence CNS HIV DNA quantities [51]. Measurement of HIV DNA in unfractionated PBMCs using PCR is the simplest way of quantifying the reservoir in the peripheral blood [52]. The PBMC HIV DNA levels have been linked to cortical and subcortical grey matter atrophy in patients on HAART with undetectable viral load, and it was also observed that HIV DNA in PBMCs in HAD was 20 times higher than in uninfected individuals [53,54]. Since brain biopsy and CSF are not a clinically feasible option in HAND studies, utility of PBMCs is imperative. The quantity of

HIV DNA in unfractionated PBMCs represents the true size of the peripheral HIV reservoir, in fact, because HIV DNA differentially distributed in all cell subsets are considered in PCR assays [31]. However, some studies on the association between HIV DNA in unfractionated PBMC and HAND did not show significant correlation [53]. Therefore, there is need to analyze the role of separate peripheral mononuclear cell subsets (Lymphocytes/monocytes) in neuropathogenesis.

3.2. Lymphocytes

The primary target for HIV infection in the peripheral blood are CD4 T lymphocytes, which makes them the predominant cell type harbouring HIV-1 in the peripheral blood of infected individuals [55–57]. The majority of HIV DNA is contained within memory (CD57+ CD4+) T cells [57]. Research has also found that all forms of HIV DNA persist in resting CD4 cells, suggesting that there is replenishment of the reservoir, either by reactivation or by infection [21,57]. It has also been demonstrated, both in vitro and in vivo using animal models, that cytotoxic T lymphocytes can be infected by HIV-1 (Stanley et al.,1993). It is thought that the infection takes place during the peripheral interaction between CD4 and CD8 as part of an immune response, thereby allowing direct transmission of HIV-1 to the CD8 lymphocytes [27,56]. Using CD4 and CD8 naive and memory cell populations separated from PBMCs of HIV-seropositive individuals, frequencies of infected cells ranged from 30 to 670 proviral copies/10^6 CD4+ lymphocytes and from 8 to 500/106 CD8 lymphocytes [56]. The reservoir of HIV in CD4+ and CD8 lymphocytes is of clinical significance due to their important role in HIV pathogenesis, as well as their routine application in diagnostic settings as markers of HIV infection. Using qPCR targeting the HIV gag region, it was shown that memory CD577 CD4+ T cells were always more frequently infected (10–10,000 copies of gag DNA/10^5 cells) than effector CD57+CD4+ T cells [58]. The quantity of infected memory cells in this study also correlated with plasma viral load, which is a virological marker for monitoring and predicting HIV infection. However, with regard to HAND, there are limited data on correlation studies linking peripheral lymphocyte HIV DNA levels and severity of neurocognitive impairment [42,51]. Investigations using ART naïve participants with mild to moderate neurocognitive impairment, lymphocyte HIV DNA levels were shown to be significantly associated with HAND independent of plasma viral load and CD4+ count [42,54]. There is need for more data on association studies between peripheral lymphocyte HIV DNA reservoirs and HAND, since this is the preferential target of HIV infection [57]. Activated CD4+ T lymphocytes support viral replication and resting cells support

establishment of a latent reservoir [58,59]. The result of HIV replication in activated lymphocytes is the production of different forms of HIV DNA (linear, 1-LTR and 2-LTR) and resting cells predominantly constitute integrated HIV DNA, both of which have shown to have clinical consequences in the brain HAND patients [60,61]. Therefore, this cell subset can represent the true size of the reservoir with different forms of HIV DNA.

The CD14+CD16+ monocyte subset in the peripheral blood constitutes only 5–10% of peripheral blood monocytes in healthy individuals, but the percentage increases to approximately 40% in HIV infected people [67]. Both in vitro and in vivo studies demonstrated that CD14+CD16+ monocytes express high amounts of the co-receptor CCR5, which facilitates their infection. It has also been observed that cells of the monocyte lineage are not susceptible to the cytopathic effects of HIV and, once they are infected in the periphery, they produce infectious virions as they enter the CNS and when they differentiate into macrophages [27]. Monocytes circulate in the blood for up to three days before they enter tissues [27,62,66,67]. Since monocytes are only present for 1–3 days in the blood, the presence of viral DNA within these cells indicate recent infection [68]. Monocytes isolated from blood of HIV patients were shown to contain unintegrated circular viral DNA, suggesting transcriptionally active rather than latent infection [69]. However, it has been shown that blood purified monocytes contain HIV DNA over time in both treated and untreated patients [7,27,70,71].

Monocytes circulate freely and patrol blood vessels as they respond to inflammation signals in tissues, such as exposure to viral products such as gp120, which can trigger chemokine production [72]. Upregulation of chemokines lead to movement of monocytes into various tissues and this process is thought to drive trafficking of monocytes through the blood–brain barrier. Studies also observed higher levels of HIV-1 DNA in activated inflammatory monocytes (CD14+/CD16+) than in non-activated monocytes [73]. Accumulating evidence shows that the route of CNS infection appears to involve circulating activated monocytes (CAM) [73]. Increase in peripheral activated monocyte subsets correlates with HIV disease progression and severity of neurocognitive impairment [62,74]. Research has demonstrated that individuals with HAD had higher levels of circulating activated CD14 monocytes and monocytes that co-express CD69, as compared to those without HAD. The finding supports the hypothesis that activated monocytes enter the brain and subsequently trigger neurotoxin production [75]. Individuals with HAD have a higher percentage of circulating activated

monocytes/macrophages (M/MΦ), suggesting that HIV-1 DNA harboured in these reservoir cells is transported into the CNS by the same cells [73,76]. This evidence supports that accumulation of activated macrophages/microglia resulting from systemic immune activation trigger neurodegeneration in HAND [72,77]. It is clinically not feasible to sample brain tissue to determine macrophage/microglial viral DNA levels in clinical studies and routine diagnostics. Therefore, blood is the best option. Investigations using some animal models have shown that infected monocyte transmigration into the CNS appear to trigger gene expression of quiescent cells [61]. This finding raises the possibility that latent HIV harboured in peripheral monocytes may have clinical consequences upon BBB transmigration [60]. Viral DNA in blood monocytes in one study was significantly associated with eight of the nine cognitive domains that are frequently impaired in HAND [78,79]. Using magnetic resonance spectroscopy, it was demonstrated that neuronal injury and glial dysfunction was positively linked to peripheral CD14+ HIV DNA [80,81].

4. Quantitative PCR Assays to Detect Total HIV-1 DNA

There are a number of real-time PCR based assays to measure total HIV-1 DNA or cell associated DNA. These assays differ in extraction methods, PCR cycling conditions and amplification targets [82]. Some assays lack the sensitivity to quantify all the HIV-1 subtypes in the major (M) group, due to the high degree of genetic variability of the virus [83]. The genetic differences may result in DNA copy number variations due to probe and primer mismatches. The differences in PCR-based protocols for quantifying cell-associated HIV DNA has made it difficult to compare studies. Some of the confounding factors that result in non-specific variation in proviral load include input template quantity and quality, yields of the extraction process and enzymatic reactions [84]. Therefore, it is very important to prescribe the minimum requirements for a PCR-based assay to determine HIV proviral load. A requirement for quality PCR is that the extraction method produce high purity DNA that is free from haemoglobin, alcohol or high salt concentrations that may interfere with PCR amplification. Another critical requirement for determining HIV DNA proviral load in clinical samples is the assessment of the number of human cells per sample before DNA extraction [85]. The number of cells determined by flow cytometry is used to normalize HIV DNA in a sample [86]. Normalizers are used to ensure that target quantities from an equivalent amount of samples are comparable in absolute quantification, [73,87]. Data normalization, which can be done by including an endogenous gene (reference gene) in the

assay, is one of the essential key elements on the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [88].

Normalization is done to reduce non-biological variation as much as possible so that a true biological variation, explaining the phenomenon under investigation, is determined [84,89]. Housekeeping genes, such as Glyceraldehyde phosphate (GAPDH), β-globin, β-actin and Telomerase Reverse Transcriptase (TERT), determined by qPCR, were used for normalization of HIV DNA to cell equivalents in some studies [28,31,73,90]. These PCR methods are more accurate than cell counts from cell sorting. The normaliser can also be used for quality control purposes since the numbers of cell inputted affect the sensitivity, as well indicating the presence of PCR inhibitors that might alter the sensitivity of the assay [91].

Malnati et al. in 2008 [92] developed a universal HIV-1 group M-specific qPCR with a similar degree of accuracy and sensitivity across all viral subtypes. The designed primers and probe allow correct quantification of virtually all circulating HIV-1 strains. The universal assay amplifies part of the LTR-gag region that is highly conserved among all circulating group M HIV-1 subtypes [92]. The success of this group M-specific HIV-1 qPCR is based on the optimized design of primers and probes that ensure quantification of all the strains in the group. To obtain a standard for construction of a calibration curve, a fragment containing the conserved LTR-gag region was amplified from the T lymhoblastoid cell line using the optimized primers as described by Malnati. The fragment was then cloned into pCRII plasmid, from which serial dilutions were made to make standard curves. An additional qPCR was developed, in this assay, to measure the number of human cells initially present in a sample and to normalize the HIV-1 proviral DNA. This was done by measuring the copy number of a single copy human gene CCR5. The CCR5 assay permits the assessment of the sample quality as well as the number of cells. This protocol performs well if the input sample contains between 1000 and 150,000 cells per reaction. This robust method can quantify HIV-1 DNA from a crude lysate with the same degree of accuracy as with purified DNA. The advantage of a crude lysate is that it circumvents the nucleic acid extraction process, makes the assay take a shorter time and reduces the costs of the assay. The assay becomes suitable for large clinical trial cohorts. Using cell lysate, Vandergeeten et al. [91] developed a nested real-time to measure total HIV DNA from diverse subtypes. The first round of PCR was pre-quantification duplex PCR with two sets of primers, one for the HIV-1 and the second set for the normaliser CD3 gene. The second round PCR was real qPCR performed separately for

HIV quantification and for determination of cell input using the CD3 gene. The primers and probes for this assay, which target the conserved LTR-gag region, allows the precise quantification of total HIV DNA from subtypes, A, B, C, D, CRF01 A/E and CRF02_A/G, with the detection limit of three copies for all subtypes. The assay also showed that HIV DNA copies were detected with similar efficacy if the cell input ranges from 10,000 to 300,000. The assay clearly demonstrated the cell input sensitivity of HIV DNA quantification.

Recently, a simple and reproducible qPCR assay for cell-associated HIV-1 DNA was developed targeting the conserved 3' part of the integrase region of the pol gene with a sensitivity of three to five copies per million cells [82]. The 3' end of the HIV-1 pol gene has been shown to be the most highly conserved region, meaning that primers and probes targeting this region should amplify efficiently the largest numbers of samples from HIV-1 infected people without mismatches. The sensitivity of this assay was determined by testing the limiting dilution of DNA standards in accordance with the Poisson distribution. The assay was able to detect three copies of HIV DNA standards in 80–100% of the reactions. Low detection limit (LOD) of three genome copies shows that the assay is very sensitive. Inhibition of HIV DNA PCR by excess nucleic acid was prevented by the dilution of the extract to ≤170 ng/µL. The nucleic acid extract was also diluted by 1:30 for CCR5 qPCR. The control of the nucleic acid input for both HIV DNA and the CCR5 normaliser is important to avoid underestimation of proviral load through inhibition. Many other PCR based assays are used in monitoring the HIV reservoir in clinical trials and HAND studies, but they differ in many ways including target region, normaliser, sample type, sample input quantity, standards, limit of detection and cycling conditions [26,31,82]. Table 1 shows selected methods used in qPCR for HIV DNA showing different parameters, including the ANRS commercial assay, which has been evaluated for inter-laboratory reproducibility.

Assay	Normalization	Unique Features and Advantages/Disadvantages	PCR Template Loading Quantity	LOD	Reference
Whole blood ANRS *LTR* real time PCR Method	HIV-DNA copies/µg converted to copies/million leucocytes	HIV DNA extracted from re-suspended whole blood cell pellets No separation of cells Its quicker and cost serving Normalizations using cell count/cytometry is not accurate	1 µg DNA, equivalent to 150,000 cells	1 Copy	[**34**]
LTR ANRS LTR real time PCR Method	Copies/million/PBMC	First assay for estimating HIV reservoir size to be evaluated for inter-laboratory reproducibility. The assay available as a commercial	1 µg DNA, equivalent to 150,000 cells	1 Copy	[**34**]
SYBR Green gag HIV DNA PCR	Copies/hundred thousand cells	Uses SYBR green fluorescence and amplifies a 142-*gag* fragment The assay is not sensitive especially in suppressed patients	300 ng, equivalent to 1,000,000 cells	50 Copies	[**27**]
Cross-Clade Ultrasensitive nested PCR	CD3 gene copy cell equivalents	Assay is performed on cell lysate, hence circumvents laborious nucleic acid extraction Allows quantification of HIV DNA A, B, C, D, CRF01_A/E, and CRF02_A/G HIV-1 subtypes, targeting highly conserved LTR-*gag* region. Cell input equivalence is accurately quantified by CD3 gene quantitative PCR. It has a disadvantage of 2 rounds of PCR	100,000 Cells	3 Copies	[**91**]
universal real-time PCR for group-M HIV-1 DNA	CCR5 gene copy cell equivalence	HIV-1 M-specific quantitative measures the HIV-1 Proviral DNA load in group M with a similar degree of sensitivity and accuracy across subtypes. Uses cell lysate as a template Predilution required if loading quantity is above 150,000 cells	1 µg per reaction, equivalent to 150,000 cells	1 Copy	[**92**]
Novel Assay for Total cell associated HIV-1 DNA	CCR5 gene copy cell equivalence	Sensitive Quantitative real time PCR for HIV-1 Cell associated DNA (CAD) targeting 3' region of the *pol* gene. Assay involves enhanced nucleic extraction by ensuring adequate cell lysis through ultrasonic cell disruption	1.7 µg	3 Copies	[**82**]

5. Relevance of Total HIV DNA qPCR Assays to HAND

Many studies have evaluated and demonstrated the prognostic value of total HIV DNA measurement by qPCR as a marker of disease progression, independent of plasma viral load and CD4+count [27,36,93,94]. High levels of HIV DNA were associated with rapid progression to AIDS and death in treatment naïve patients [41]. A suitable HIV DNA qPCR assay for HAND should target a clinically relevant marker and should be highly specific, sensitive, reproducible, cost-effective and easy to implement in clinical settings. Both

replication-competent and defective forms of HIV DNA have been shown to be clinically relevant to neurocognitive impairment [44,60,61]. It is well understood that the different forms of HIV DNA co-exist in an infected cell, although their distribution varies depending on whether the cells are activated or latent [36,57,93,95]. Therefore, it is important to quantify all forms of HIV DNA, including the defective and unintegrated forms, and utilize them as surrogate biomarkers for HAND pathogenesis. It is also cost effective to have a qPCR that quantifies all the different forms of HIV DNA rather than separately quantifying integrated forms, 1-LTR or 2-LTR. Total HIV DNA qPCR assays can be applied to whole blood, PBMCs, monocytes or lymphocyte subsets and is easy to measure [31,34,42,96]. The assay quantifies all forms of HIV DNA that co-exist in infected cells that are involved in HIV pathogenesis [31,57]. Comparing measures such as 2LTR circles or integrated HIV DNA, total HIV DNA has the advantage of easy quantification, by standardised sensitive, real-time PCR methods [34]. It is suitable for analyzing large samples with accuracy, requires a relatively small amount of blood and is not affected by freezing thawing [96]. However, there is no commercial assay currently available for HIV DNA qPCR [41]. The above-described assays, including the ones that have been used on HAND studies, are a modification of commercial assays used in HIV RNA quantification [82,97]. Thus, an assessment of qPCR applied in HAND studies is required with regard to sensitivity and the sample types that were used in the assays.

6. HIV-1 DNA qPCR Used in HAND Studies

Application of qPCR and digital PCR to determine the association between HIV DNA levels in PBMC and its fractions, in the context of HAND, needs to consider both clinical and demographic variables of the study cohorts as well as the severity of HAND and performance characteristics of the assays. Using a very sensitive qPCR assay (LOD of 1–3 copies), it has been demonstrated that HIV DNA correlated with HAD in both HAART-naïve and HAART treated patients [73,80,81]. These studies also showed that activated monocytes HIV DNA levels in HAD patients were higher than in lymphocytes including the CD4+ cells. The sensitivity of the assay allows detection of low copies in suppressed patients and those who commence ART early in the course of HIV infection. Using a full range of neurocognitive disorders, the assay also showed that people with normal cognition had lower peripheral HIV DNA levels as compared to those with minor motor disorders and HAD [80]. Results of this study showed that the assay was sensitive enough to determine the association between circulating HIV DNA and different stages of HAND from normal cognition to the extreme

forms of HAD. The HIV DNA determined by this assay, as a proposed biomarker, has been shown to be proportional to all three levels of neurocognitive function as well as different neuropsychological deficits, making it applicable for both screening and monitoring therapeutic interventions.

The HIV DNA assay targeting the gag region has shown that monocyte HIV DNA levels correlated with all three clinical categories of HAND (ANI, MND and HAD) in HAART treatment naïve patients [11]. The study further demonstrated association between monocyte HIV DNA and glial dysfunction, neuronal injury and CSF immune activation. The important aspect of this study demonstrates the link between both neurobehavioral and neuropathological aspects of HAND and circulating HIV DNA levels. However, there was no association between PBMC HIV DNA and HAND. Different HIV DNA PCR assays including droplet digital PCR used on HAND studies produced different results depending on clinical and demographic characteristics of study participants, as well as performance characteristics of the assays [42,51,80]. Table 2 shows selected PCR assays used in HAND studies with clinical data for study participants and performance characteristics of the assays.

Table 2. HIV DNA real time PCR assays applied in HAND studies.

Reference	Target	Sample	Clinical Data	Study Outcome	Advantage/Disadvantage
[**73**]	*gag*	PBMC	HAD patients on HAART 20–39 years and 50 years and above	Quantities of HIV DNA correlated with HAD HIV was higher in activated monocytes than CD14− in two patients	Very sensitive Quantitect Sybr Green PCR assay with detection limit of 1–3 copies and applicable suppressed patients and those with low cell PBMC or cell subset counts
[**78**]	*gag*	PBMC, CD14+, CD14−	15 HAART-naïve HAD patients and 15 Non demented patients Average age, 34.1	HIV DNA in PBMC was significantly higher in HAD non demented patients HIV DNA in five HAD patients was higher in CD14/CD16 than CD4	Very sensitive Quantitect Sybr Green PCR assay with detection limit of 1–3 copies and can be applied to suppressed patients and samples with low cell count
[**31**]	*pol*	PBMC	HIV Subtype B chronic patients on cART for six months CD4+ < 350/mL Median Age 56, 74	HIV DNA was associated with HAD and not Non demented forms of HAND	Very sensitive assay with one copy as LOD, hence suitable for supressed HAND patients PCR has a wide dynamic range from three copies to 300,000 copies High efficiencies for both HIV DNA and β-actin normaliser (93.7% and 86.6% respectively) -Assay restricted to detecting Subtype B
[**42**]	LTR-*gag*	CD14− and CD14+	ART naïve participants (median age, 32) with 17 impaired and 19 unimpaired Patients had higher HIV DNA in lymphocytes than	Strong association between HIV DNA levels in lymphocytes and HAND	The target region for amplification is highly conserved and the Universal PCR assay has been customised for subtype G and CRF02_AG. However, performance of the assays not detailed

			monocytes		
[**79**]	gag	CD14+	12 HAART treated HAD and 15 non-HAD Infected with HIV-1 CRF01_AE Median Age, 32	Baseline monocyte HIV DNA correlated with HIV DNA and 48 weeks after HAART. Monocyte HIV DNA level was below detection limit in all non-HAD patients after 48 weeks	Detection limit of the assay was 10 copies/106 cells which is relatively high.
[11]	*gag*	CD14+PBMC	Treatment naïve with three clinical categories of HAND (ANI, MND, HAD) CD4+ <350/mL –CRF AE_01Mean Age, 35	No correlation between PBMC HIV DNA Before CD14+ enrichment, CD14+ was associated with HAND(ANI, MND, HAD) –CD14+ HIV DNA was associated with glial dysfunction, neuronal injury and CSF immune activation (neopterin)	Very sensitive assay with a limit of detection of one copy which is suitable for HAND suppressed patients

7. Possible Use of Digital PCR to Detect HIV DNA in HAND

The amplification signal is logarithmic and the quantification is based on an external calibration or standard curve in the above-described qPCR methods. A relatively newer technology called digital PCR (dPCR) offers simple, linear and digital quantification that is based on the number of positive and negative reactions using Poisson distribution [98]. Digital PCR uses limiting dilutions and sample partitioning into submicrolitre reactions. Each partition represents an isolated end point PCR [99]. The Poisson distribution of template molecule within these partitions is used to deduce the concentration of the target nucleic acid from frequency of negative to partitions. Digital PCR has been shown to be tolerant with sequence variations by higher robustness to primers and probe mismatches with target sequences, giving a superior advantage for HIV-1 DNA quantification and, as a result, dPCR has shown higher accuracy precision and reproducibility over qPCR.

Regarding detection and quantification of HIV proviral DNA in HAART suppressed patients, which are usually very low but relevant to HAND, dPCR can offer an alternative to qPCR-based assays [51]. Most patients who are on long term ART, the proviral load ranges from 10 to 1000 copies of HIV DNA per million cells [25,99]. The higher sensitivity of dPCR compared to qPCR will be more relevant to the determination of low quantities of HIV DNA in monocytes in the periphery, which have been shown to be predictive of HAND and thought to be a major source of virus production in the brain [67]. Although dPCR has a great potential to be used as a valid alternative to qPCR, there is need to eliminate false positive droplets, which have been reported in negative template controls [100]. The other hurdle in

the utility of dPCR for precise measurement of HIV DNA is the lack of a standardised automated method for determining threshold droplets. Threshold setting is very important to determine baseline fluorescence in order to distinguish between positive and negative droplet populations without bias [101]. There is also a need to work on the reduction of cost so that dPCR can be clinically accredited.

8. Conclusions

Classical virological and immunological parameters such as plasma viral load and CD4+ T-cell counts for HIV normally used to monitor the progression of disease cannot be used to measure and predict the onset of HAND. Most of the proposed markers of HAND are similar to the biomarkers of other neurocognitive disorders due to the similarities in the molecular mechanisms that are involved. The HIV DNA or cell-associated DNA (CAD) is detected in the peripheral blood of HAART suppressed patients and many studies have shown it to correlate with HAND. However, there is a need for an assay that is robust, sensitive and accurately quantifies HIV DNA. Total HIV DNA quantification by real-time PCR from unfractionated PBMCs is very simple, but HIV DNA from inflammatory CD16+ monocytes is linked to the pathogenesis of HAND and, therefore, more suitable as a marker. Regarding quantification of low-level HIV DNA in HAART suppressed patients, dPCR offers a superior advantage, but issues to do with standardization of threshold and cost reduction need improvement before utilization in diagnostic settings. Therefore, qPCR methods remain relevant now for clinical use since research has shown significant association between peripheral HIV DNA levels in cell compartments and severity of HAND. Additionally, evaluated commercial qPCR kits for HIV DNA load are now available. However, more data are required on T cell HIV DNA as an indicator of potential HAND-associated reservoirs in untreated patients.

Acknowledgments: Our work on investigating viral, cellular and immunological factors contributing to the development of HIV associated neurocognitive disorders is supported by the Poliomyelitis Research Foundation (PRF), National Research Foundation (NRF) and the South African Medical Research Council (SAMRC) Collaborating Centre for HIV Laboratory Research (TygHIVLab).

Author Contributions: V.R.: Conceptualization, outline, and preparation of the draft manuscript. G.B.J.: Performed critical reading and editing of the manuscript. R.H.G.: Contributed much of the content in Section of the draft manuscript, critical reading and editing of the manuscript. S.E.: Manuscript preparation, preparation of figures and final assembly of the manuscript.

Conflict of Interest: The authors declare no conflict of interest.

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Paper 2: **Ruhanya, V.**; Jacob, G.B.; Nyandoro, G.; Paul, R.H.; Joska, J.A.; Seedat, S.; Glashoff, RH.; Engelbrecht, S. Peripheral blood lymphocyte proviral DNA predicts neurocognitive impairment in clade C HIV. Journal of NeuroVirology 2020,26, 920–928.

Peripheral blood lymphocyte proviral DNA predicts neurocognitive impairment in clade C HIV

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Abstract: *It is not known if proviral DNA in the periphery corresponds to cognitive status in clade C as it does in clade B and recombinant forms. A cross-sectional study was conducted on participants investigated for HIV-associated neurocognitive impairment in South Africa. HIV-1 proviral DNA was quantified using a PCR assay targeting a highly conserved HIV-1 LTR-gag region. Fifty-four (36.7%) participants were cognitively impaired and 93 (63.3%) were not impaired. Forty-three (79.6%) of the cognitively impaired participants were female and 11 (20.4%) were male. There was no significant age difference between cognitively impaired and unimpaired participants (p = 0.42). HIV-1 DNA in cognitively impaired PLWH was significantly higher than in cognitively normal individuals (p = .016). Considering impaired participants, lymphocyte HIV-1 DNA was significantly higher in males than females (p = 0.02). There was a modest positive correlation between lymphocyte HIV-1 DNA and global deficit scores (GDS) r = 0.176; p = 0.03). The two measures of viral load, lymphocyte HIV-1 DNA copies/million and plasma RNA copies/ml, were positively correlated (r = 0.39; p < .001). After adjusting for other covariates, age, sex, treatment status, and the interactions between impairment and treatment, the multivariate regression showed association between proviral load and neurocognitive impairment; omega effect size was 0.04, p value = 0.010. The burden of HIV-1 peripheral blood lymphocyte proviral DNA corresponds to neurocognitive impairment among individuals infected with clade C disease. Therefore, therapeutic strategies to reduce the HIV-1 proviral DNA reservoir in lymphocytes may improve neurocognitive outcomes in PLWH.*

Keywords*: HIV-1 proviral DNA, HAND, Lymphocytes.*

1. Introduction

HIV-1-associated neurocognitive disorders (HAND) remain an important clinical concern. HAND affects 40–70% of HIV-1-infected patients (Williams et al. 2014; Yusuf et al. 2017), despite immune reconstitution and viral suppression because of combination antiretroviral therapy (cART) (Cysique et al. 2015). The exact mechanism of HAND pathogenesis is not known, but it is thought to be a complex interaction of factors including cellular targets, viral factors, and the immune response (Saylor et al. 2016; Carroll and Brew 2017; Ruhanya et al. 2017). Evidence from experimental models and human studies show that HAND pathogenesis involves a seeding of peripheral HIV-1 into the central nervous system (CNS) (Price et al. 2007). HIV RNA has been detected in CSF within 8 days of infection, and immune activation is associated with neuropathogenesis (Hellmuth et al. 2015; Valcour et al. 2012). Residual viremia and HIV-1 DNA reservoirs in the periphery are linked to activation-induced neuronal damage (Cysique et al. 2015; Valcour et al. 2012) as well as chronic immune activation from macrophages, glial cells, and astrocytes (Hong and Banks 2015).

Brain invasion by HIV can develop into HIV-associated encephalitis (HIVE) which is comprised of microglial nodules, activated resident microglia, multinucleated giant cells, and infiltration by blood-derived macrophages (Williams et al. 2014; Valcour et al. 2012; Hong and Banks 2015). Clinically, people with CNS HIV-1 infection present with a spectrum of cognitive symptoms referred to as HIV-associated neurocognitive disorders (HAND) (Valcour et al. 2012). The introduction of cART has significantly reduced severe forms of HAND, although more subtle clinical presentations, which can pose difficulties for detection and monitoring, are still pervasive. Therefore, HIV-specific biomarkers are needed to assist in devising diagnostic and therapeutic approaches.

Elevated levels of HIV-1 DNA in peripheral blood mononuclear cells (PBMCs) contribute to the expression of HAND (Kamat et al. 2012; Shiramizu et al. 2009). Although evidence has demonstrated a correlation between HIV-1 DNA in PBMCs and the severity of HAND, most studies have been conducted on HIV-1 subtype B, the dominant strain in North America and Europe (Valcour et al. 2010). Data are lacking regarding HAND and subtype C, which is predominant in Southern Africa. We do not know if proviral DNA in the periphery corresponds to cognitive status in clade C as it does in clade B and recombinant forms (Valcour et al. 2013). Additionally, most studies in clade B cohorts have utilized

unfractionated PBMCs and monocytes (Shiramizu et al. 2009; Kamat et al. 2012). The aim of this study was to determine whether peripheral monocyte-depleted CD14- lymphocytes correspond to HAND. We hypothesized that proviral HIV-1 DNA levels would be higher in neurocognitively impaired than in non-impaired individuals infected with HIV-1 subtype C.

Material and methods

Study design and patient samples

A cross-sectional study was conducted on a cohort of 147 HIV-1 positive individuals recruited from primary care HIV-1 clinics in Cape Town, South Africa. Participants were enrolled in a parent study focused on neuropsychological and brain imaging signatures in HIV-1 subtype C (Valcour et al. 2013; Paul et al. 2014). This study was approved by the Health Research Ethics Committee (HREC) of Stellenbosch University, Ethics Reference #: S17/02/035. The study was conducted according to South African Guidelines for Good Clinical Practice and the Medical Research council, Ethical Guidelines for Research in line with the guidelines and principles of the international declaration of Helsinki.

Neuropsychological evaluation

A battery of cognitive tests sensitive to HIV-1 was administered to all participants as described by Paul et al. 2014. Briefly, the tests were administered to assess learning, executive functions, and visuospatial and psychomotor speed. Learning was assessed as described previously (Paul et al. 2017; Valcour et al. 2013; Benedict et al. 1996). T-scores for individual cognitive tests were averaged to generate a global T-score and a global deficit score (GDS); GDS > 0.5 defined neurocognitive impairment as described elsewhere (Heaps-Woodruff et al. 2017; Jumare et al. 2017).

Cell subset separation

Whole blood samples were collected and PBMCs separated by Ficoll gradient separation (Ficoll-Histopaque, Pharmacia, Uppsala, Sweden). CD14+ monocytes were separated by magnetic cell sorting (MACS, Miltenyi Biotec GmbH, and Bergisch Gladbach, Germany) and the peripheral blood lymphocytes (CD14-) were recovered from negative fraction of the CD14+ monocytes. The total CD3+, CD4+, and CD45+ count and percentages were determined using standardized T cell subset protocols (BDMultiset) and flow cytometry analysis (BD FACS Calibur).

Quantification of proviral DNA in peripheral lymphocytes

HIV-1 DNA was extracted from monocyte depleted lymphocytes (CD14-) using the QIAamp Blood Mini extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Nine specimens without complete clinical-demographic information were excluded. The quantity and purity of extracted HIV-1 DNA was determined by NanoDrop® ND-1000, (Thermo Fisher Scientific, MA, USA) spectrophotometer readings.

Quantification of total HIV-1 DNA proviral levels were determined according to the protocol described by Malnati et al. (Malnati et al. 2008), using a quantitative real-time PCR (qPCR) that targets the conserved HIV-1 LTR-gag region. Calibration standards for quantifying proviral DNA were made using the subtype C infectious plasmid clone, pMJ4, obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, and NIH: (Ndung'u et al. 2001). Briefly, the concentration of the pMJ4 plasmid DNA in nanograms/microliter (ng/µl) was obtained using Nanodrop ND-1000 spectrophotometer. Plasmid DNA copy numbers per microliter (copies/µl) were then determined, using an online DNA copy calculator, on the assumption that the average weight of a base pair is 650 Da, using the following formula: mass × 6.023 × 1023 divided by 12,833 (length) in base pairs (bp) × 1 × 109 × 660 (l Prediger 2008). From this initial concentration (copies/µl), tenfold serial dilution stocks were made, to construct a standard curve. The qPCR for the standard curve and master mix were performed using iTaq super mix (Bio-Rad, California, USA) and HIV-1 specific primers and probes, described previously by Malnati et al. 2008.

The standards were run on a CFX 96 thermocycler using Bio-Rad CFX manager 3.1 to plot the standard curve. Proviral HIV-1 DNA copies from samples were calculated from this plot using Ct (cycle threshold) values. HIV-1 cell-associated DNA (CAD) was normalized to cell input by quantification of the CCR-5 genome copies per sample. The calibration curve for quantifying genomic DNA was constructed using the CCR5 plasmid, which was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, and NIH: pcCCR5 (Cat#3325) (Morgenstern and Land 1990). Briefly, the concentration of CCR5 plasmid DNA was obtained using the Nanodrop ND-1000 spectrophotometer and plasmid DNA copies/µl were obtained using the online copy calculator (Prediger, 2008). Sample copy numbers for genomic DNA input were then calculated from this curve, using the test sample's Ct by the Bio-Rad CFX manager version 3.1 (Bio-Rad, California, USA).

Statistical analysis

Data analyses were completed using Stata version 13.1 (StataCorp, College Station, Texas, USA). HIV DNA in lymphocytes mean differences between impaired and unimpaired were assessed using independent t test and the Cohen's d effect size was established to quantify the extend of the difference in proviral DNA. Furthermore, covariates (age, sex, treatment status, and interaction of impairment and treatment status) were adjusted for further validation of the observed effects using multiple regression modeling. Pearson correlation coefficients assessed the association between HIV-1 peripheral blood lymphocyte DNA and neurocognitive impairment. The same test was also used to assess the association between neurocognitive impairment and clinical variables, such as plasma RNA viral load, CD4+ T cell count, monocyte count, and CD4/CD8 ratio. Statistical significance was determined as p value < 0.05.

Results

Clinical and demographic characteristics

Fifty-four (36.7%) participants were cognitively impaired and 93 (63.3%) were not impaired; thus, the proportion of impaired participants was less than those not impaired ($p = 0.0017$). More females were impaired; Forty-three (79.6%) of the cognitively impaired participants were female and 11 (20.4%) were male, $p < 0.001$. There was no significant age difference between cognitively impaired and unimpaired participants ($p = 0.42$). Twenty-seven (17.8%) of the participants had initiated cART for less than 4 weeks and 125 (82.2%) had not initiated treatment, at the time of the study. The T-helper-suppressor ratio was higher in cognitively normal patients than cognitively impaired patients, although the difference was not significant ($p = 0.33$). CD14 enriched monocytes were higher in cognitively impaired participants than normal participants, although this was not statistically significant ($p = 0.21$). Table 1 summarizes the clinical and demographic characteristics of the participants in the cohort.

Table 1: Clinical and demographic characteristics of the cohort

	Neurocognitive status		
Clinical & demographic variables (Mean, SD)	***Not impaired (n = 93)***	***Impaired (n = 54)***	***p value***
Age	31.5 (5.55)	31.53 (4.70)	0.42
CD4 T-lymphocytes	243.36 (179.15)	221.56 (158.34)	0.77
CD14+	13.99 (0.62)	14.12 (0.62)	0.10
Plasma RNA viral load	96,973.29 (242238)	203,139 (545673)	0.05
CD45	1502.04 (702.03)	1515.69 (690.73)	0.45
CD4:CD8 ratio	0.31 (0.21)	0.28 (0.18)	0.33

CD14: HIV DNA qPCR

We used a sensitive qPCR with high efficiency of more than 90% for both the normalizer CCR5 and HIV-1 with all the coefficient of determination, R^2 greater than 0.99 for both assays. The limit of detection for the CCR5 assay was eight copies and that for HIV DNA assay was one copy per reaction. Figure1 shows standard curve for HIV starting from 3 to 2.5 × 104 copies using the pMJ4 standards. The average sample input DNA quantity per reaction was 220.2 ng from which an average of 88,579 CCR5 genome copies was quantified. Figure 2 shows CCR5 standard curve with quantities ranging from 8 copies to 8 × 105 copies.

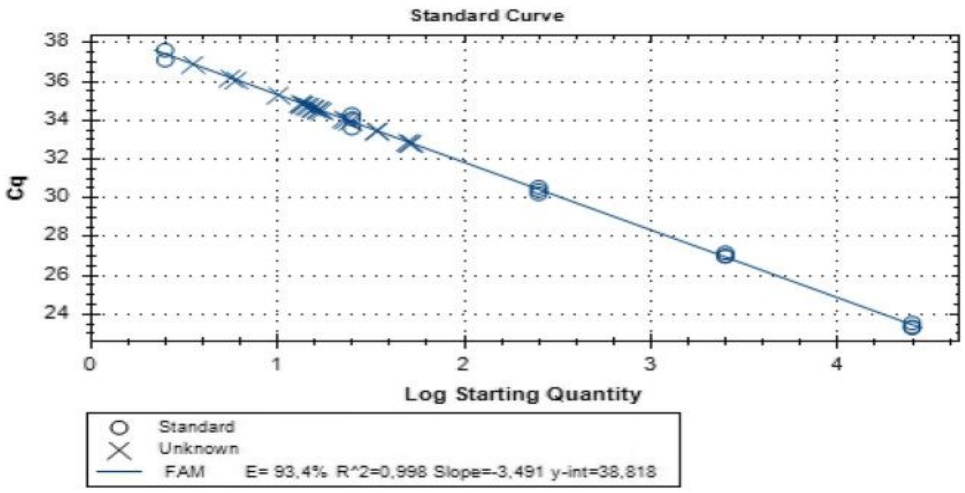
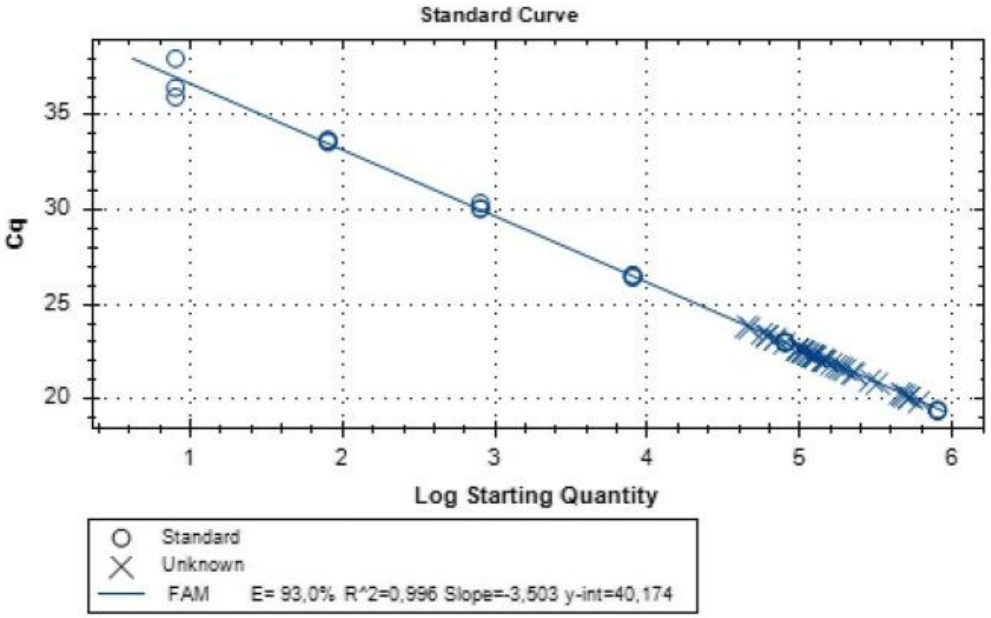


Fig 1. Standard curve used to estimate HIV-1 DNA copies in lymphocytes per reaction. The standard curve was generated by Bio-Rad CFX manager 3.1 using a tenfold dilution of the HIV-1 pMJ4 template, assayed in triplicate from 2.5 to 250,000 copies. Cq is plotted against the log of the starting quantity of template for each dilution. The calculated amplification efficiency was 97.4% with a slope of − 3.49 and the R2 value was 0.998. The y-intercept was 38.8 cycles



Standard curve to assess CCR5 genome copies in as cell number equivalence per reaction. The standard curve was generated by Bio-Rad CFX manager 3.1 using a 10-fold dilution of CCR5 plasmid template, assayed in triplicate from 8 to 800,000 copies. Cq is plotted against the log of the starting quantity of template for each dilution. The calculated amplification efficiency was 95.7% with a slope of − 3.429 and the R2 value was 1.000. The y-intercept was 40.17 cycles.

The mean peripheral blood lymphocytes HIV-1 DNA for cognitively impaired PLWH was 419.76 copies per million cells compared with 240.38 in-unimpaired cases. HIV-1 DNA in cognitively impaired PLWH was significantly higher than in cognitively normal individuals ($p = 0.016$). The Cohen's d effect size was − 0.42, lower in the unimpaired compared with impaired group with 95% confidence interval (CI): − 0.75 to − 0.08. After adjusting for other covariates, age, sex, treatment status, and the interactions between impairment and treatment, the regression omega effect size was 0.04, p value = 0.010; see Table 2.

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
Normal	93	240.3763	29.31945	282.7466	182.1454	298.6073
Impaired	54	419.7593	82.8949	609.1506	253.4932	586.0254
Combined	147	306.2721	36.19747	438.871	234.7334	377.8108
Diff		− 179.3829	73.85678		− 325.3578	− 33.40798
diff = mean(normal) - mean(impaired)						$t = -2.4288$
Ho: diff = 0			Degrees of freedom		145	
Ha: diff < 0			Ha: diff! = 0		Ha: diff > 0	
Pr(T < t) = 0.0082			Pr(T > t) = 0.0164		Pr(T > t) = 0.9918	
Effect Size			Estimate	[95% Conf. Interval]		
Cohen's d			−0 .4155383	− 0.7535504	− 0.0761216	

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
ttest hivdnacopiespermillion, by(sex)						
Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
Female	121	276.1901	32.75203	360.2723	211.3433	341.0368
Male	24	455.75	146.7344	718.849	152.2067	759.2933
Combined	145	305.9103	36.68911	441.7954	233.3916	378.4291
Diff		− 179.5599	97.92037		− 373.1184	13.99852
Diff = mean(female)—mean(male)						t = − 1.8337
Ho: diff = 0				degrees of freedom		143
Ha: diff < 0				Ha: diff! = 0		Ha: diff > 0
Pr(T < t) = 0.0344				Pr(|T| > |t|) = 0.0688		Pr(T > t) = 0.9656
Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
regress hivdnacopiespermillion age c.trt c.diag c.sex c.trt#c.diag						
Source	SS	df		MS	Number of obs =	140
					F(5, 134) = 2.81	
Model	2,644,010.25		5,528,802.051	Prob > F		= 0.0191
Residual	25,231,809.5		134,188,297.086	R-squared =		0.0948
				Adj R^2 = 0.0611		
Total	27,875,819.7		139,200,545.466	Root MSE		= 433.93
HIV DNA copies per million	Coef.	Std. Err.	t	P > t	[95% Conf. Interval]	
Age	− 7.974611	7.111015	− 1.12	0.264	− 22.03896	6.089739
trt	− 77.94466	123.3087	− 0.63	0.528	− 321.8278	165.9385
Diag	219.8801	84.31013	2.61	0.010	53.1294	386.6309
Sex	163.603	100.6832	1.62	0.107	− 35.53074	362.7368
c.trt#c.diag	− 191.853	202.5405	− 0.95	0.345	− 592.4428	208.7368
_cons	322.6054	248.1464	1.30	0.196	− 168.185	813.3958
estat esize, omega						
Effect sizes for linear models						
Source	Omega-Squared	df	[95% Conf. Interval]			
Model	0.0610753	5	0	0.1363377		
Age	0.0019048	1	0	0.0583753		
trt	0	1	0	0.0395842		
Diag	0.0412042	1	0	0.1275637		
Sex	0.0120052	1	0	0.0798124		
c.trt#c.diag	0	1	0	0.051502		

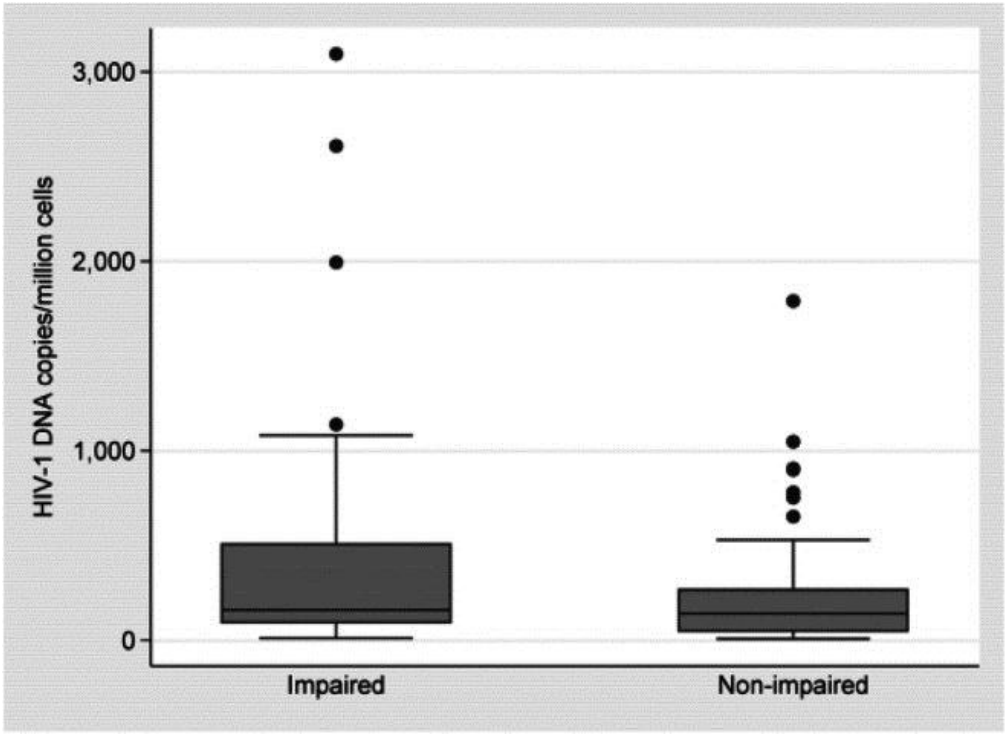


Fig 3. Box plots of HIV-1DNA copies per million cells by diagnosis showing significantly higher HIV-1 DNA in impaired participants than non-impaired participants (p = 0.016)

There was a modest positive correlation between lymphocyte HIV-1 DNA and global deficit scores (GDS) $r = 0.176$; $p = 0.03$). The two measures of viral load, lymphocyte HIV-1 DNA copies/million and plasma RNA copies/ml, were positively correlated ($r = 0.39$; $p < 0.001$). Considering impaired participants, proviral HIV-1 DNA was significantly higher in males than females ($p = 0.034$). There was no association between GDS and absolute CD4+ count or plasma viral load. However, there were significant differences in plasma viral load ($p < 0.001$) and CD4+ absolute count ($p = 0.02$) between cART naïve and participants initiating treatment. Other parameters (CD4:CD8 ratio, CD14+ cells, absolute CD45 count) did not differ by treatment status ($p = 0.28$, 0.1698, and 0.24, respectively). The normalizer, CCR5, did not differ by neurocognitive status ($p = 0.21$). We undertook further analysis to assess if there was an association between domain-specific cognitive impairments (e.g., learning, mental control, recall, and others that are used to calculate the GDS) and proviral HIV-1 DNA. We found that Hopkins Verbal Learning Test score (hvlt learning) ($p = 0.049$) and Wechsler Adult Intelligence Scale score (WAIS III symbol search) ($p = 0.035$) were significantly reduced with increased proviral HIV-1 DNA, when adjusted for sex, age, and treatment status. Compared with females, males had significantly lower Wechsler memory

scale (WMS mental control) ($p = 0.007$), WAIS III digit symbol ($p = 0.009$), and WAIS III symbol search ($p = 0.033$) test scores; see Table 3.

Table 3: The effect of proviral HIV-1 DNA on cognitive subdomains

. regress hvltlearning hivdnacopiespermillion age ib0.treated ib1.Male					
hvltlearning	Coef.	Std. Err.	t	P> t	[95% Conf. Interval]
hivdnacopiespermillion	− 0.0006601	0.0003314	− 1.99	0.049	− 0.0013159 − 4.34e-06
Age	0.0178903	0.0279508	0.64	0.523	− 0.0374236 0.0732041
Treated	0.0188188	0.3955378	0.05	0.962	− 0.7639389 .8015765
Male	− 0.2521731	0.3997102	− 0.63	0.529	− 1.043188 0.5388416
_cons	3.152604	0.9872715	3.19	0.002	1.198823 5.106385
. regress hvltrecall hivdnacopiespermillion age ib0.treated ib1.Male					
hvltrecall	Coef.	Std. Err.	t	P> t	[95% Conf. Interval]
hivdnacopiespermillion	− 0.000786	0.0003986	− 1.97	0.051	− 0.0015747 2.75e-06
age	0.0107152	0.0336163	0.32	0.750	− .0558105 0.0772409
treated	− 0.3670706	0.4757116	− 0.77	0.442	− 1.30849 .5743487
Male	.0651162	0.4807297	0.14	0.892	− .8862338 1.016466
_cons	7.304685	1.187387	6.15	0.000	4.954881 9.65449
. regress wmsmentalcontrol hivdnacopiespermillion age ib0.treated i. b1. Male					
wmsmentalcontrol	Coef.	Std. Err.	t	P > t	[95% Conf. Interval]
hivdnacopiespermillion	− 0.0020123	0.0011596	− 1.74	0.085	− 0.0043071 0.0002826
age	− 0.0521593	0.0978096	− 0.53	0.595	− 0.2457216 0.141403
treated	− 0.1953722	1.384124	− 0.14	0.888	− 2.934512 2.543768
Male	− 3.851276	1.398724	− 2.75	0.007	− 6.619311 - 1.083242
_cons		3.454805	8.37	0.000	22.08527 35.75918
. regress waisiiidigitsymbol hivdnacopiespermillion age ib0.treated i. b1Male					
waisiiidigitsymbol	Coef	Std. Err.	t	P > t	[95% Conf. Interval]
hivdnacopiespermillion	− 0.0023023	0.0025599	− 0.90	0.370	− 0.0073683 0.0027636
age	0.0760948	0.215916	0.35	0.725	− 0.3511966 0.5033861
treated	− 1.97488	3.055471	− 0.65	0.519	− 8.021569 4.071808
Male	− 8.138507	3.087703	− 2.64	0.009	− 14.24898 - 2.028034
_cons	38.19324	7.055488	5.41	0.000	24.23063 52.15584
. regress waisiiisymbolsearch hivdnacopiespermillion age ib0.treated ib1.Male					
waisiiisymbolsearch	Coef.	Std. Err.	t	P > t	[95% Conf. Interval]
hivdnacopiespermillion	− 0.0025437	0.001191	− 2.14	0.035	− 0.0049007 -0.0001868
age	− 0.0048388	0.1004548	− 0.05	0.962	− 0.2036359 0.1939583
treated	− 1.049958	1.421557	− 0.74	0.462	− 3.863176 1.763261
Male	− 3.101115	1.436552	− 2.16	0.033	− 5.944009 -0.2582203
_cons	21.3853	3.282562	6.51	0.000	14.8892 27.88139

Discussion

This study demonstrated a significant correspondence between peripheral blood lymphocyte HIV-1 DNA load and neurocognitive impairment in adults with HIV-1 subtype C infection. This is the first study in South Africa to describe positive correlation between the monocyte-depleted fraction of peripheral blood lymphocytes and neurocognitive impairment in predominantly cART naïve HIV-1 clade C-infected participants. Peripheral blood lymphocytes, primarily the CD4+ subset, are the predominant cell type harboring HIV-1 in

the blood of infected individuals (Schnittman et al. 1989; McBreen et al. 2001; Murray et al. 2014). The association of this major peripheral reservoir of HIV-1 DNA with neurocognitive impairment has significant pathological and clinical implications, particularly in the diagnosis and monitoring of the disease. Proviral DNA can be used to understand progression of HIV-associated neurocognitive impairment, particularly in subtle forms of the disease where symptomatic monitoring is difficult (Ruhanya et al. 2017). Theoretically, HIV in these cells can be targeted when devising therapeutic strategies to HIV-associated neurocognitive impairment. Infected CD4+ T lymphocytes are thought to transfect other cells, like CD8+ lymphocytes and monocytes, during the initial immune response (Gibellini et al. 2008; McBreen et al. 2001). Therefore, eradication of this reservoir might prevent the infection of peripheral monocytes, which are known to traffic HIV-1 into the brain. Infected macrophages in the brain are key factors in the development of HIV-associated neurocognitive disorders (Sasse et al. 2012).

Although the treated participants had lower lymphocyte HIV-1 proviral DNA reservoirs than treatment naïve patients, this was neither significant nor associated with improved neurocognitive performance. Previous studies found no significant reduction in proviral HIV DNA at pre- and 3-month post-treatment (Shikuma et al. 2012). Depending on the duration of cART, other findings have shown improvement in neurocognitive performance in adults on cART (Shikuma et al. 2012), but the introduction of cART has not fully eradicated neurological symptoms. Therefore, longitudinal studies are needed to examine how the reduction of peripheral lymphocyte HIV-1 DNA by cART correlates with neurocognitive performance in individuals over time. In this study, participants-initiated treatment in the preceding 4 weeks; hence, the time to observe both the reduction of peripheral HIV-1 DNA and improvement in cognitive function was probably too short. It has been observed that significant reduction in peripheral HIV-1 DNA is dependent on early initiation of cART, which was not the case in this generally immunocompromised cohort (Gianella et al. 2011; Gannon et al. 2011; Watanabe et al. 2011; Herout et al. 2016).

We used a highly sensitive real-time qPCR, which is suitable for detection of very low copies (Malnati et al. 2008). It is also suitable for quantifying proviral DNA found in a relatively large amount of genomic DNA in the sample, which could interfere with detection of HIV-1 DNA. Our normalizer, CCR5 DNA copies were not different between impaired and unimpaired participants which is expected of a good normalizer (Nguewa et al. 2008). An

appropriate normalizer enables true estimation of cells in the samples and accurate quantification of proviral HIV-1 DNA per sample, which reflect the quantity of the HIV reservoir, and does not vary between diseased and healthy patients.

Previous investigations have shown that total HIV-1 proviral DNA in PBMC subsets differed significantly between mild and severe forms of neurocognitive impairment, which show a gradation from very low quantities in asymptomatic to significantly higher quantities in severe forms (Valcour et al. 2013). Therefore, with regard to lymphocytes, additional studies are needed to evaluate the effects of the HIV-1 reservoir and the severity of neurocognitive impairment so that its utility as a biomarker in different stages of the disease is tested. To further strengthen the potential of lymphocyte HIV DNA role in the pathogenesis of HAND, there is also a need for association studies between lymphocyte proviral DNA quantity and biomarkers of neuronal injury and glial dysfunction (Valcour et al. 2013).

Comparison between cognitively impaired and normal participants showed that there was no statistical difference in the quantities of both CD4+ absolute count and plasma viral load. Therefore, in this study, the standard clinical markers used in routine diagnosis and clinical monitoring of HIV did not differentiate the two groups on the basis of global deficit scores. Previous studies have also demonstrated that viral load and CD4+ count are insensitive markers of HIV-1-associated disease (Clifford and Ances 2013). Levels of these two standard markers of HIV-1 infection observed in this study are in the same ranges with quantities observed in cART-naïve patients where CD4+ counts were low and plasma viral loads were relatively high (Valcour et al. 2010; Sánchez-Ramón et al. 2003).

Although the GDS construct of HAND is important, numerous published studies demonstrated that there is considerable heterogeneity in the pattern of cognitive impairment in people with HIV. We did a further analysis using domain-specific associations such as learning, mental control, recall, and others that are used to calculate GDS to assess the effect proviral HIV-1 DNA in relation to cognitive impairment. We found that Hopkins verbal learning test score (hvlt learning) and Wechsler Adult intelligence scale (WAIS III symbol search) were very significantly reduced with increased proviral HIV-1 DNA when adjusted for sex, age, and treatment status. This study demonstrates for the first time that monocyte-depleted lymphocyte proviral HIV-1DNA levels in subtype C patients is associated with neurocognitive status and specific neurocognitive domains in South Africa. We hypothesize that monocyte-depleted (CD14-) lymphocytes could be an important cellular subset housing

the HIV-1 DNA and play a role in global and domain-specific neurocognitive impairment. These findings also suggest differential involvement of HIV-1 DNA in some regions of the CNS that may be leading to some cognitive domains being affected more than others.

Conclusions

Our study showed that the burden of HIV-1 peripheral blood lymphocyte proviral DNA corresponds to neurocognitive impairment among individuals infected with clade C disease. Therefore, therapeutic strategies to reduce the HIV-1 proviral DNA reservoir in lymphocytes may improve neurocognitive outcomes in PLWH**.**

Acknowledgements

We gratefully acknowledge the Stellenbosch University Division of virology research team for reception sorting and archiving the blood samples for this study.

Funding information

This study was funded by the Poliomyelitis Research Foundation (PRF), National Research Foundation (NRF) and the South African Medical Research Council (SAMRC) Collaborating Centre for HIV-1 Laboratory Research.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Conflicts of Interest

The authors have no conflict of interest to declare

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CHAPTER 3

PERIPHERAL INFLAMMATORY CYTOKINE AND IMMUNE MARKERS FOR NEUROCOGNITIVE IMPAIRMENT IN HIV-1 INFECTION

There are 4 manuscripts included in this chapter. Two manuscripts of this chapter were published in peer reviewed journals. One manuscripts accepted for publication and one under review. The text format is as required by the respective journals where published.

This chapter focuses on plasma cytokine biomarkers as predictors of global neurocognitive impairment using global deficit scores. We also examined plasma as predictors of domain-specific cognitive function and their relationship with brain volumes. Finally, we determined plasma cytokine cut-off values for cognitive impairment and their clinical validity

Paper 3: Ruhanya, V.; Jacobs, G.B.; Naidoo, S.; Paul, R.H.; Joska, J.; Seedat, S.; Nyandoro, G.; Engelbrecht, S.; Glashoff, R. Impact of plasma IP-10/CXCL10 and RANTES/CCL5 levels on neurocognitive function in HIV treatment naïve patients. AIDS res. hum. retroviruses 2021, doi: 10.1089/AID.2020.0203.
The author's contributions to the published paper were as follows: Conception and design of the experiments, Performed the experiments, Data analyses and writing of manuscript

Impact of plasma IP-10/CXCL10 and RANTES/CCL5 levels on neurocognitive function in HIV treatment naïve patients.

Ruhanya, V.; Jacobs, G.B.; Naidoo, S.; Paul, R.H.; Joska, J.; Seedat, S.; Nyandoro, G.; Engelbrecht, S.; Glashoff, R.

Summary

Immune activation, which is accompanied by the production of proinflammatory cytokines, is a strong predictor of disease progression in HIV infection. Inflammation is critical in neuronal damage linked to HIV-associated neurocognitive disorders. We examined the relationship between plasma cytokine levels and deficits in neurocognitive function. Multiplex profiling by Luminex® technology was used to quantify 27 cytokines/chemokines from 139 plasma samples of people living with HIV (PLWH). The relationship of plasma cytokine markers, clinical parameters, and cognitive impairment was assessed using Spearman correlations. Partial least squares regression and variable importance in projection scores were used for further evaluation of the association. Forty-nine (35.3%) participants exhibited neurocognitive impairment based on a global deficit score (GDS) of at least 0.5 and 90 (64.7%) were classified as non-impaired. Twenty-three (16.5%) initiated on combination antiretroviral therapy for 4 weeks before cognitive assessment and 116 (83.5%) were not on treatment. We identified five proinflammatory cytokines that were significant predictors of GDS namely, IP-10 ($\beta = 0.058$; $p = .007$), RANTES ($\beta = 0.049$; $p = .005$), IL-2 ($\beta = 0.047$, $p = .006$), Eotaxin ($\beta = 0.042$, $p = .003$), and IL-7 ($\beta = 0.039$, $p = .003$). IP-10 and RANTES were the strongest predictors of GDS. Both cytokines correlated with plasma viral load and lymphocyte proviral load and were inversely correlated with CD4+ T cell counts. IP-10 and RANTES formed a separate cluster with highest proximity. Study findings describe novel associations among IP-10, RANTES, cognitive status, plasma viral load, and cell-associated viral load.

Key words: Immune activation, neuroinflamation, Luminex, GDS, clustering

Introduction

HIV associated neurocognitive disorders (HAND) are a significant challenge to HIV infected people, with prevalence ranging from 50 – 70 %1,2. The precise mechanism of HAND pathogenesis is not known, but it is thought to be a complex interaction between viral factors and the immune response and other risk factors like age, coinfection with other viruses and educational levels [3,4,5]. Studies showed that HIV infection of the central nervous system (CNS) is an offshoot of systemic infection, which provides the initial and continuous seeding of the peripheral human immunodeficiency virus in the CNS [6]. Processes that eventually lead to neurocognitive impairment are thought to emanate from activated peripheral mononuclear cells trafficking to the CNS7. Infected mononuclear cells release cytokines that contribute to the disruption of the Blood-Brain barrier (BBB), making it more permeable to proinflammatory cytokines and chemokines from the periphery[8,6]. The influx of peripheral inflammatory cytokines into the brain results in activation-induced neuroinflammation, which is a risk for HAND9,10,11.

Some soluble markers of immune activation, such as plasma tumor necrosis factor-α receptor II p175 (TNF-RII) and β2-microglobin have been shown to be predictive of HIV disease progression, with efficiency comparable to viral load and CD4+ T-cell count[12,13]. Therefore, coupling CD4+ T-cell count and viral load with estimates of immune activation could increase the predictive power of tracking HIV disease progression. Despite the success of combination antiretroviral therapy (cART), chronic immune activation, and particularly inflammation, continues to be a hallmark of HIV infection and predicts disease progression and can also lead to adverse clinical events such as HAND [14]. Research has shown that chemokines CXCL-10/IP-10, in combination with HIV-1, is neurotoxic and leads to pro-inflammatory cytokine production [15,10]. Higher levels of IP-10 produced by activated monocytes/macrophages, microglia and T-cells have been shown in HIV+ individuals with dementia15. Another chemokine CCL5/RANTES was shown to increase the replication of HIV in peripheral blood lymphocytes and high levels were also observed in inflammatory lesions of the brain and in the CSF of HIV infected patients[16,17,18]. These observations may possibly explain the role of RANTES in HAND.

There is growing interest in elucidating the role of various plasma inflammatory markers in the pathogenesis of HAND. A search from a large spectrum of candidates is necessary in order to accelerate the identification of novel cytokine signature markers associated with

clinical outcomes of neurocognitive impairment in HIV [19,20]. This study combines high-throughput Luminex based assays with different statistical tools to find the direction of maximum covariance between cytokines and identify those cytokines that contribute significantly to prediction of neurocognitive impairment, ranking and grouping the cytokines according to their predictive power. We, therefore, examined relationships between plasma inflammatory biomarkers and neurocognitive function as assessed by global T-scores which were derived from comprehensive neurocognitive testing

Materials and Methods

A cross-sectional study was conducted in 139 HIV positive individuals from primary care HIV clinics in Cape Town, South Africa. Sample size determination parameters were: 80% power, prevalence (p=15%), 95% confidence interval, 1.2 design effect, 16% attrition, and 7% margin of error. Inclusion criteria to participate in the study required the following: (i). Age ranging from 18 to 45 years with at least five years of formal education. This age range was selected to avoid age-related CNS abnormalities, (ii). HIV serostatus, determined by ELISA and then confirmed by Western blot, (iii). HIV-1 RNA for plasma viral load measured by the Abbott m2000sp and the Abbott m2000rt analysers (Abbott laboratories, Abbott Park, Ilinois, USA). Exclusion criteria included the following: any major psychiatric condition that could significantly affect cognitive status; confounding neurological disorders including multiple sclerosis and other CNS conditions; head injury with loss of consciousness greater than 30 minutes; clinical evidence of opportunistic CNS infections and current substance abuse or alcohol abuse as defined by a structured interview [21]. Data on HIV treatment status was collected as the participants were enrolled into the study. This study was approved by the Health Research Ethics Committee (HREC) of Stellenbosch University, Ethics Reference #: S17/02/035. The study was conducted according to South African Guidelines for Good Clinical Practice and the Medical Research council, Ethical Guidelines for Research in line with the guidelines and principles of the international declaration of Helsinki.

*Neuropsychological evaluatio*n

A battery of cognitive tests sensitive to deficits associated with HIV was administered to all participants as described by Paul et al., [20, 14, 21]. Briefly, the tests were administered to assess learning, executive functions/visuospatial and psychomotor speed. Learning was assessed using Hopkins Verbal Learning Test-Revised (HVLT-R), and visual learning was assessed using the Brief Visual Memory Test-Revised (BVM-R), as described previously

[22]. Executive functions and psychomotor speed were evaluated using the neuropsychological battery that has been previously described. Scores were averaged to generate a mean T-score and deficit for each of the cognitive domains to calculate a global T-score and global deficit score (GDS). A GDS was then used to categorize cases as either impaired or unimpaired [23]. GDS of 0.5 or more was regarded as neurocognitive impairment. GDS has been shown to be reliable tool for the diagnosis of HAND [24]

Cell subset separation and proviral HIV DNA quantification

Whole blood samples were collected, and peripheral blood mononuclear cells (PBMCs) were separated by Ficoll gradient separation (Ficoll - Histopaque, Pharmacia, Uppsala, Sweden). CD14+ monocytes were separated by magnetic cell sorting (MACS, Miltenyi Biotec GmbH, and Bergisch Gladbach, Germany) and the peripheral blood lymphocytes (CD14-) were recovered from negative fraction of the CD14+ monocytes. The total CD3+, CD4+ and CD45+ count and percentages were determined using standardized T-cell subset protocols (BDMultiset) and flow cytometry analysis (BD FACS Calibur). Quantification of total HIV-1 DNA proviral levels in cell subsets were determined according to the protocol described by Malnati et al [25], using a quantitative real-time PCR (qPCR) that targets the conserved HIV-1 LTR-gag region.

Plasma cytokine quantification by multiplex-bead assay

The cytokine concentrations in plasma samples were determined using a 27-plex kit, according to manufacturer's instructions (Biorad Bio-Plex Pro Human cytokine assay California, USA). Quantified cytokines/chemokines and growth factors included IL-1β, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, Eotaxin, basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP1, MIP-1α , MIP-1ß, PDGF-BB, RANTES, TNF-α and VEGF. Briefly, thawed plasma samples were incubated with antibody-coupled beads. Complexes were washed, incubated with biotinylated detection antibody, and subsequently with streptavidin-phycoerythrin, prior to assessing cytokine concentrations. Standard curves were generated using standard cytokines, with known concentration provided in the kit. Plasma cytokine levels were determined using a multiplex array reader from LuminexTM Instrumentation System (Bio-Plex Workstation from Bio-Rad, California, USA); plasma samples were run in duplicate and cytokine concentrations were calculated as the average of two independent measures using Bioplex Manager Software (California, USA).

Statistical analysis

STATA statistical package version 12.1 (StataCorp, College Station, Texas, USA, 2011) was used for data analysis. Normally distributed demographic and cytokine level variables were described using means. Variables that were skewed were analysed using non-parametric tests; the Kruskal-Wallis test was used to compare GDS median scores between males and females since they were skewed. Independent t-tests were used on normally distributed variables such as Age in years, CD4+ count/ul, CD8+ count/ul, CD4+/CD8+ ratio, CD14+ count/ul, and Plasma viral load (copies/ml) to compare cognitively impaired and unimpaired participant groups. Spearman correlation coefficients were used to assess the association between plasma cytokines and GDS as well as variables, such as plasma RNA viral load, CD4+ T-cell count, monocyte count and CD4/CD8 ratio. Variable importance in projection (VIP) scores were computed using the partial least squares regression approach using STATA Excel software. Partial least squares (PLS) approach uses multiple linear regression analysis to find the direction of maximum covariance between a dataset and class membership and prioritize features that contribute significantly to class prediction. The VIP scores were used determine cytokine importance in relation to the GDS. VIP scores above a threshold of 1 were considered important. Statistical significance was determined as a p value < 0.05.

Results

Demographic and clinical characteristics

A total of 139 participants, consisting of 115 (82.7%) females and 24 (17.3%) males, were assessed for HIV associated neurocognitive impairment. The age of participants ranged from 21 to 45 years. The median (GDS) for the cohort was 0.32. The median deficit score for males (0.5) was higher than for females (0.31), but the difference was not significant ($p = 0.0853$). Using a GDS of 0.5 or higher, 49 (35.3%) participants were designated as at least mildly impaired and 90 (64.7%) were classified as non-impaired and 23 (16.5%) initiated on cART for 4 weeks prior to cognitive assessment and 116 (83.5%) were not on treatment at the time of cognitive assessment and sample collection. There were no significant differences in cytokine levels between cART naïve and cART initiated in 26 of the 27 cytokines quantified. Only, IL-2 was significantly higher in the cART-naïve group compared to the cART group ($p = 0.0289$). Most of the participants (80%) reported completing secondary education (from Grade 8 – Grade 12), 5.8% had primary education (Grade 1 to grade 7) and 5% had tertiary education (diploma or degree). Table 1 summaries demographic and clinical

parameters of the cohort comparing impaired and non-impaired participants and Table 2 compares male and female participants.

Relationship between cytokine levels and neurocognitive impairment status

Using PLS analysis we identified 5 proinflammatory cytokines that were significant predictors of GDS before adjusting for viral suppression, IP10 ($\beta = 0.058$; $p = 0.007$), RANTES ($\beta = 0.049$; $p = 0.005$), IL2 ($\beta = 0.047$, $p = 0.006$), Eotaxin ($\beta = 0.042$, $p = 0.003$), and IL7 ($\beta = 0.039$, $p = 0.003$). The strongest associations involved IP10 ($p = 0.007$), RANTES ($p = 0.005$) and IL-2 ($p = 0.006$) respectively. These cytokines were significantly and positively associated with GDS. Table 3 shows the detail of all the 27 cytokines and correlation with GDS. However, Table 4 show that after adjusting for viral suppression, gender, and other cytokines; IP10 and RANTES were not significant markers of impairment whilst IL2 was a significant marker associated with impairment. In addition, significant protective markers were GCSF and IFN-γ.

Inverse covariation with GDS was prominent on IL-9 ($\beta = -0.056$) INF-g ($\beta = -0.052$), TNF-α ($\beta = -0.039$), IL-7 ($\beta = -0.036$), IL-17($\beta = -0.039$) and IL-13 ($\beta = -0.036$), but the associations were significant for IL-7 ($p = 0.003$) and IL-17($p = -0.003$) only. Table 5 shows association between cytokine levels and clinical markers of HIV infection. This shows that an increase in these cytokines was associated with decreased GDS. Fig 1 shows the relationship between the cytokines and GDS using PLS regression.

VIP plot ranking indicated the plasma cytokine biomarkers that were most important in relation to GDS. Again IP-10 had the highest VIP score (1.8) for predicting HAND diagnosis. They have VIP scores greater than 1 which were considered important for prediction of GDS regardless of the direction of association. Therefore, 12 cytokines IP-10, IL-9, INF-γ, RANTES, IL-2, MIP-1a, eotaxin, TNF-α, IL-17, IL-7, IL-13 and MIP-1b were the most important. Fig 2 below shows the ranking of cytokines in predicting GDS according to VIP scores.

Cluster analysis of all the 27 cytokines produced hierarchically related sets of groups of cytokines. RANTES and IP-10 came out as a cluster, with the highest Pearson proximity of greater than one. The dedrogram presented in Fig 3 has vertical lines associated with specific

cytokines that are joined by horizontal lines. A cluster with RANTES and IP-10 has long vertical lines approximately 1. 3, this shows the strength of clustering proximity.

Viral load measures, cognitive function and immune activation

Global deficit scores did not significantly correlate with standard clinical markers / measures for HIV infection such as CD4+ T and CD8+ T-cell counts, percentages and ratio, but did interestingly correlate with CD14+ monocyte count (Spearman r = 0. 180; p = 0.036). In addition, GDS correlated with IL-2 (r = 2366; p = 0.0054). IL-9 (r = 0.2; p = 0.0191). Eotaxin (r = 0.1831; p = 0.0323). IFN-γ (r = - 0.175; p = 0.010). GM-CSF (r = 0.218; p = 0.010) and RANTES (r = 0.231; p = 0.007).

Considering the two categories, proviral load was significantly higher in impaired participants (p = 0. 0058) but there was no significant difference in plasma viral load between the two groups (p = 0. 0.6205). Neither plasma viral load nor cell associated proviral load were correlated with GDS. There was a strong relationship between the two viral load measures (Spearman r = 0.414; p < 0.0001). However, both measures of viral load did not have similar correlations with markers of immune activation. Plasma viral load correlated negatively and significantly with CD4+ T-cell count (r = -0.329; p < 0.0001) and CD4%; this was not mirrored in the lymphocyte proviral load. This implies some independence between the two viral measures and their direct impact on immune status. Proviral load is associated with inactive infection and latency whereas plasma viral load is associated with productive active infection or replicating virus.

With regard to cytokines, plasma viral load was significantly correlated with 11 cytokines. Viral load was negatively correlated with 4 cytokines. IL-1β, IL-9, PDGFBB and TNF-α (p = 0.005. p = 0.0012. p = 0.0001 and p = 0.018 respectively). IL-2, IL-7, IL-8, IP-10, MIP-1β were positively and significantly associated with plasma viral load. Of these cytokines, the most significantly and positively associated with plasma viral load were IP-10 (r = 0.308; p < 0.001) and RANTES (r = 0.422; p < 0.0001).

Lymphocyte proviral load was associated with 9 cytokines in common with plasma viral load: IL-1β, IL-7, IL-8, IL-9, IP-10, PDGF-BB, MIP-1β, RANTES and TNF-α. The other 3 include IL-17, Eotaxin and IFN-γ were only associated with lymphocyte proviral load and not plasma viral load. Interestingly the cytokines which showed significant correlation with

plasma viral load lymphocyte proviral load and GDS scores included: RANTES. MIP-1β, PDGF-BB and IL-9. The only positive association across all 3 parameters (GDS, plasma viral load and lymphocyte proviral load) was RANTES with Spearman correlation coefficients of r = 0.422, 0.434 and 0.231 and p < 0.0001, < 0.0001 and 0.007 for plasma viral load, lymphocyte proviral load and GDS score respectively.

Although CD4+ T-cell count was not significantly different between impaired and unimpaired groups, CD4+ T-cell count correlated with the highest number of immune status markers and cytokines. Positive correlation was observed with CD3%, CD3 absolute count, CD8%, CD8 absolute count, CD4%, CD4:8 ratio, IL-5. IL-7, IL-9, IL-12(p70), IL-13, IL-17, Eotaxin, MCP-1, PGDF-BB, MIP-1β, TNF-α and VEGF. It correlated negatively with plasma viral load, IP-10 and RANTES. The monocyte cell count (CD14 enriched) positively correlated with GDS score, IL-2, IL-5, IL-6. IL-15, G-CSF, IP-10 and negatively correlated with CD3% and PDGF-BB. Unique to the monocyte count were positive correlations with IL-6, IL-15 and G-CSF – all 3 being myeloid/monocyte-associated cytokines.

Discussion

The objective of this study was to examine the relationship between plasma cytokine concentrations and neurocognitive impairment as measured by GDS scores. Our findings showed that the strongest associations between selected cytokines and cognitive status were IP10, RANTES, IL-2 and eotaxin, respectively. These cytokines are proinflammatory. Further analysis using VIP showed that IP-10 is the most sensitive marker of GDS. Altered expression of IP-10 /CXL10 has been associated with a number of inflammatory diseases including HIV [25]. It has been shown to positively correlate with plasma viral load in untreated HIV patients [26, 27, 28]. Elevated plasma IP-10 in primary HIV infection has been shown to be a more robust predictor of progression towards AIDS [29, 39, 31]. IP-10 positively correlated with both plasma viral load and lymphocyte-associated proviral load and inversely with plasma CD4+ T-cell count. Higher viral loads points to increased antigenic load which could worsen inflammatory response in the periphery. IP10 is known to influence T-cell generation and trafficking of CD4+ and CD8+ T- cells into the brain and has been shown to be correlated with the progression of HAND [32, 33]. Our results demonstrated a strong association between CD8+ T-cells and IP-10, supporting earlier findings on the role in T-cell proliferation and neurocognitive impairment in HIV infection [34, 35].

RANTES which was the second most important cytokine linked to GDS also correlated with plasma viral load and lymphocyte proviral load. This implies that viral replication and RANTES are linked, and these phenomena increase the risk of neurocognitive impairment. RANTES is a pro-inflammatory cytokine/chemokine which is chemotactic for lymphocytes, monocytes and other cell types and is expressed in several inflammatory diseases of the CNS and a powerful stimulus for astrocyte production of proinflammatory cytokines [36]. RANTES at high concentrations can also enhance infection through mitogen-like activation of T cells and other cells [37]. Increased levels of RANTES have been observed in brain/CSF in NeuroAIDS and evidence has shown that the cytokine promotes replication of T-tropic HIV in peripheral lymphocytes [38, 39, 40]. RANTES a key proinflammatory cytokine produced by virus-infected epithelial cells of the gut has been implicated in translocation of microflora and their products from the gastrointestinal tract into the systemic circulation [41]. Persistent epithelial gut damage and elevated plasma levels of microflora and their products likely contribute to inflammation and immune activation which has been linked to neurocognitive dysfunction in HIV infection [42].

Positive correlation between RANTES and viral load markers implicates this cytokine/chemokine with enhancement of infection [43]. It thus seems likely that RANTES facilitates infection through activation of T cells. We did not measure T cell activation but the negative association between activation cytokines such as IP-10, TNF-α and CD4+ T-cell count is indicative of elevated general T-cell activation [44, 45, 46]. The enhanced infection indicated by positive association with plasma viral load is associated with T-cell loss, shown by negative association with CD4 count.

RANTES plays an important role in sustaining CD8+ T cell responses during systemic viral infection [47, 48]. It is thought that this process produces a significant depletion of CD4+ lymphocytes and defective function of T-lymphocytes leading to dysregulation of cytokine production, resulting in immunodeficiency. RANTES itself had strong correlation with IP-10. IP-10 is thus key marker due to its relationship with RANTES, monocyte count (CD14+), CD4 count, plasma viral load and lymphocyte proviral load. The fact that IP-10 and RANTES had strong associations with GDS, measures of viral load, and formed a separate cluster indicates that we have identified a signature plasma chemokine biomarker linked to neurocognitive impairment in PLWH.

Although this study has identified a signature plasma cytokine marker associated with neurocognitive impairment in HIV, other factors such as peripheral activated or HIV infected monocyte-derived crossing the BBB may have contributed [49]. Infected microglia and astrocytes may have contributed to neurocognitive impairment. Therefore, this study is limited in that the association between this maker and neurocognitive function was not investigated. Our findings are also limited to associations of inflammatory cytokine marker to clinical and neurocognitive impairment as measured by GDS. Further studies are required to support our findings with pathological data from neuroimaging techniques which have the capacity to assess the extent of cytokine involvement in brain damage resulting in neurocognitive impairment. Our cohort was limited to predominantly woman and untreated participants. We, therefore, adjusted for gender and viral suppression on the association between cytokines and cognitive function and observed that IL-2 was still associated with neurocognitive impairement.

Conclusions

RANTES and IP-10 are important putative biomarkers of neurocognitive impairment and important indicators of viral replication and immune activation. Evidence of interdependencies between multiple proinflammatory and immune activation cytokines indicates that both cytokines are linked to the processes of viral replication and T-cell activation.

Acknowledgements

We would like to thank Cladnos Mapfumo for statistical analyses

Funding statement

This study was supported by the Poliomyelitis Research Foundation (PRF: SE. VR). National Research Foundation (NRF: SE), the South African Medical Research Council (SAMRC) Collaborating Centre for HIV-1 Laboratory Research (SE) and the new Generation of Academics Programme (nGAP), South Africa (GBJ)

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Table 1. Summary of clinic-demographic variables

Variable (mean)	Impaired (49)	Non-impaired (90)	overall	P – value
Age in years	31.5	31.8	31.00	0. 7223
CD4+ count/ul	208	209	204.00	0. 4586
CD8+ count/ul	822	787	819.00	0. 8088
CD4+/CD8+ ratio	0.22	0.26	0.25	0.4836
CD14+ count/ul	1.2×10^6	1.1×10^6	1.2×10^6	0.2046
Plasma viral load (copies/ml)	1.87×10^4	1.4×10^4	1.5×10^4	0.6205

Table 2. Summary of gender stratified demographic and clinical parameters.

Parameter	Male	female	P - value
Age (t-test, mean)	33.3	31.4	0.1089
GDS (kruskal-wallis, median)	0.5	0.31	0.0853
CD4+ count/ul (t-test, mean)	168	209	0.1481
CD8+ count/ul (t-test, mean)	673.5	822	0.3118
CD4+/CD8+ ratio (t-test, mean)	0.205	0.260	0.6316
CD14+ count (t-test, mean)	1600000	1100000	**0.0228**
Plasma viral load copies/ml (t-test, mean)	47677	13197	0.0676

Table 3: Coefficients for 27 predictor cytokine variables against GDS outcome

Variable	Mean levels pg/ml	Coefficient(β)	Std. deviation	Lower bound (95%)	Upper bound (95%)	P value
IL-1β	2.62	-0.013	0.017	-0.047	0.021	0.684
IL-1α	167.23	-0.001	0.024	-0.049	0.047	0.912
IL-2	4.32	0.047	0.031	-0.015	0.110	0.006
IL-4	3.31	-0.014	0.019	-0.052	0.024	0.668
IL-5	12.83	-0.030	0.014	-0.057	-0.003	0.194
IL-6	3.82	0.006	0.013	-0.020	0.032	0.637
IL-7	12.68	-0.036	0.016	-0.068	-0.005	0.003
IL-8	15.27	0.024	0.023	-0.022	0.070	0.871
IL-9	412.36	-0.056	0.027	-0.110	-0.003	0.563
IL-10	3.33	-0.013	0.016	-0.045	0.019	0.070
IL-12p70	2.97	-0.021	0.017	-0.055	0.013	0.678
IL-13	1.85	-0.036	0.019	-0.073	0.001	0.211
IL-15	60.28	-0.006	0.021	-0.048	0.036	0.899
IL-17	23.85	-0.039	0.021	-0.080	0.002	0.033
Eotaxin	40.29	0.042	0.035	-0.027	0.110	0.003
FGF basic	36.70	-0.018	0.018	-0.053	0.018	0.319
GCSF	51.54	-0.012	0.012	-0.036	0.013	0.092
GMC-SF	1.17	0.004	0.019	-0.032	0.041	0.402

INN-γ	13.64	-0.052	0.016	-0.083	-0.020	0.213
IP-10	491.21	0.058	0.034	-0.008	0.125	0.007
MCP-1	23.75	0.020	0.025	-0.030	0.070	0.542
MIP-1α	2.60	0.042	0.052	-0.060	0.145	0.202
PDGF-BB	1018.04	-0.018	0.021	-0.059	0.024	0.403
MIP-1β	21164.10	-0.035	0.018	-0.072	0.001	0.997
RANTES	1051.21	0.049	0.027	-0.005	0.103	0.005
TNF-α	284.48	-0.039	0.022	-0.083	0.005	0.898
VEGF	57.68	-0.010	0.015	-0.040	0.021	0.061

Table 4: Coefficients for 27 predictor cytokine GDS outcome controlled for viral load and gender

GDS	IRR	Std. Err.	z	p value	95% Conf.	Interval
Viral suppression	1,35	0,349	1,17	0,244	0,81	2,24
Males	1,62	0,376	2,08	0,038	1,03	2,55
IL-1β	0,98	0,038	-0,48	0,634	0,91	1,06
IL-1α	1,00	0,000	-0,45	0,652	1,00	1,00
IL-2	1,11	0,036	3,35	0,001	1,05	1,19
IL-4	0,94	0,058	-1,02	0,307	0,83	1,06
IL-5	0,99	0,022	-0,38	0,705	0,95	1,04
IL-6	1,00	0,017	-0,22	0,829	0,96	1,03
IL-7	1,00	0,009	0,14	0,886	0,98	1,02
IL-8	1,00	0,000	1,13	0,258	1,00	1,00
IL-9	1,06	0,035	1,7	0,089	0,99	1,13
IL-10	1,01	0,016	0,77	0,443	0,98	1,04
IL-12p70	0,99	0,082	-0,07	0,948	0,85	1,17
IL-13	1,00	0,002	-1,33	0,185	0,99	1,00
IL-15	0,99	0,009	-0,88	0,379	0,97	1,01
IL-17	1,00	0,003	1,75	0,080	1,00	1,01
Eotaxin	1,00	0,005	-0,14	0,885	0,99	1,01
FGF basic	1,00	0,000	-2,23	0,025	1,00	1,00
GCSF	1,05	0,036	1,57	0,117	0,99	1,13
GMC-SF	0,96	0,012	-2,95	0,003	0,94	0,99
INN-γ	1,00	0,000	1	0,319	1,00	1,00
IP-10	1,00	0,005	-0,22	0,825	0,99	1,01
MCP-1	1,00	0,003	1,15	0,249	1,00	1,01
MIP-1α	1,00	0,000	1,11	0,269	1,00	1,00
PDGF-BB	1,00	0,000	-1,44	0,151	1,00	1,00
MIP-1β	1,00	0,000	-0,5	0,619	1,00	1,00
RANTES	1,00	0,000	0	0,997	1,00	1,00
TNF-α	1,00	0,000	-0,45	0,655	1,00	1,00
VEGF	0,40	0,140	-2,62	0,009	0,20	0,79

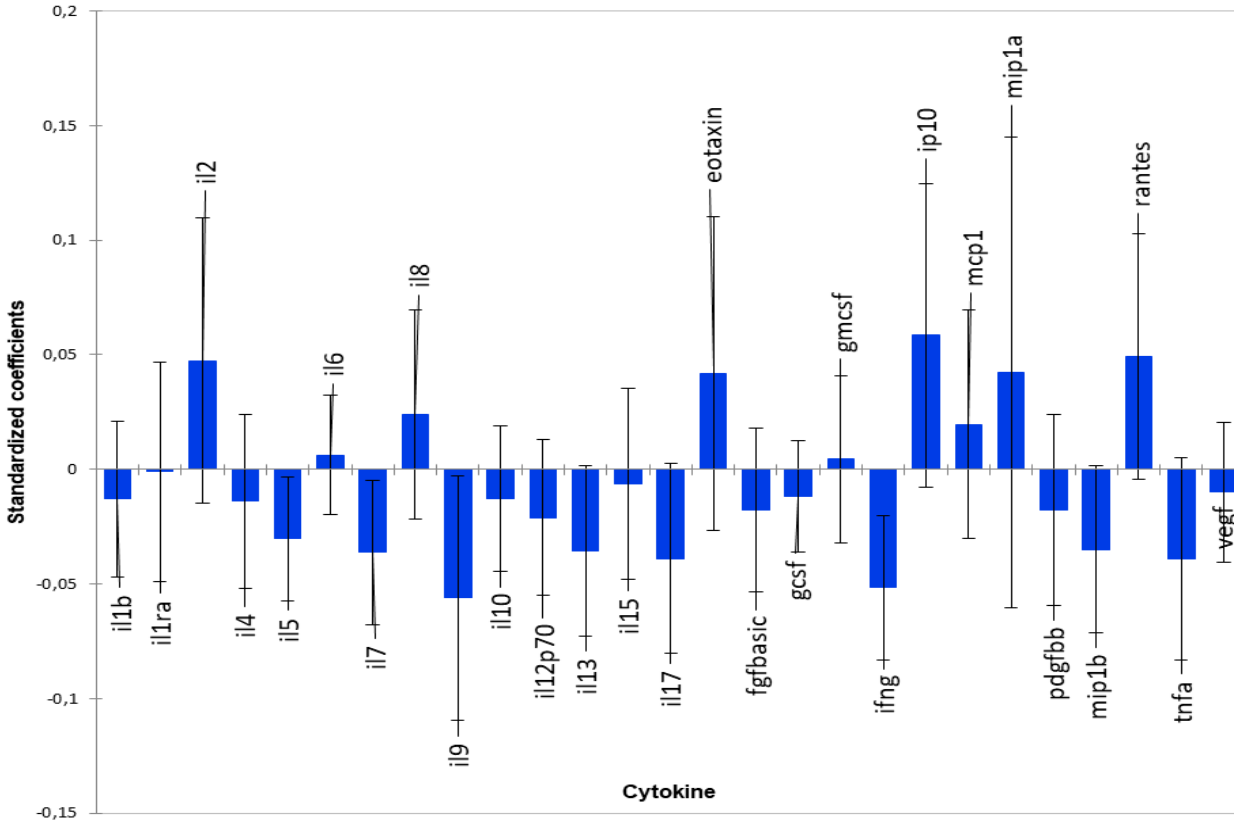

Fig 1. *Graphical representation of the magnitude and direction of the effect of plasma cytokines on global deficit score using PLS-R standardized coefficients and 95% confidence intervals. IP-10, RANTES, Eotaxin and IL-2, MCP-1, and MIP-1a were prominent cytokines associated with higher GDS. Il-9, INF-γ, and TNF-α, Il-17, Il-13 and MIP-1β are associated with lower GDS.*

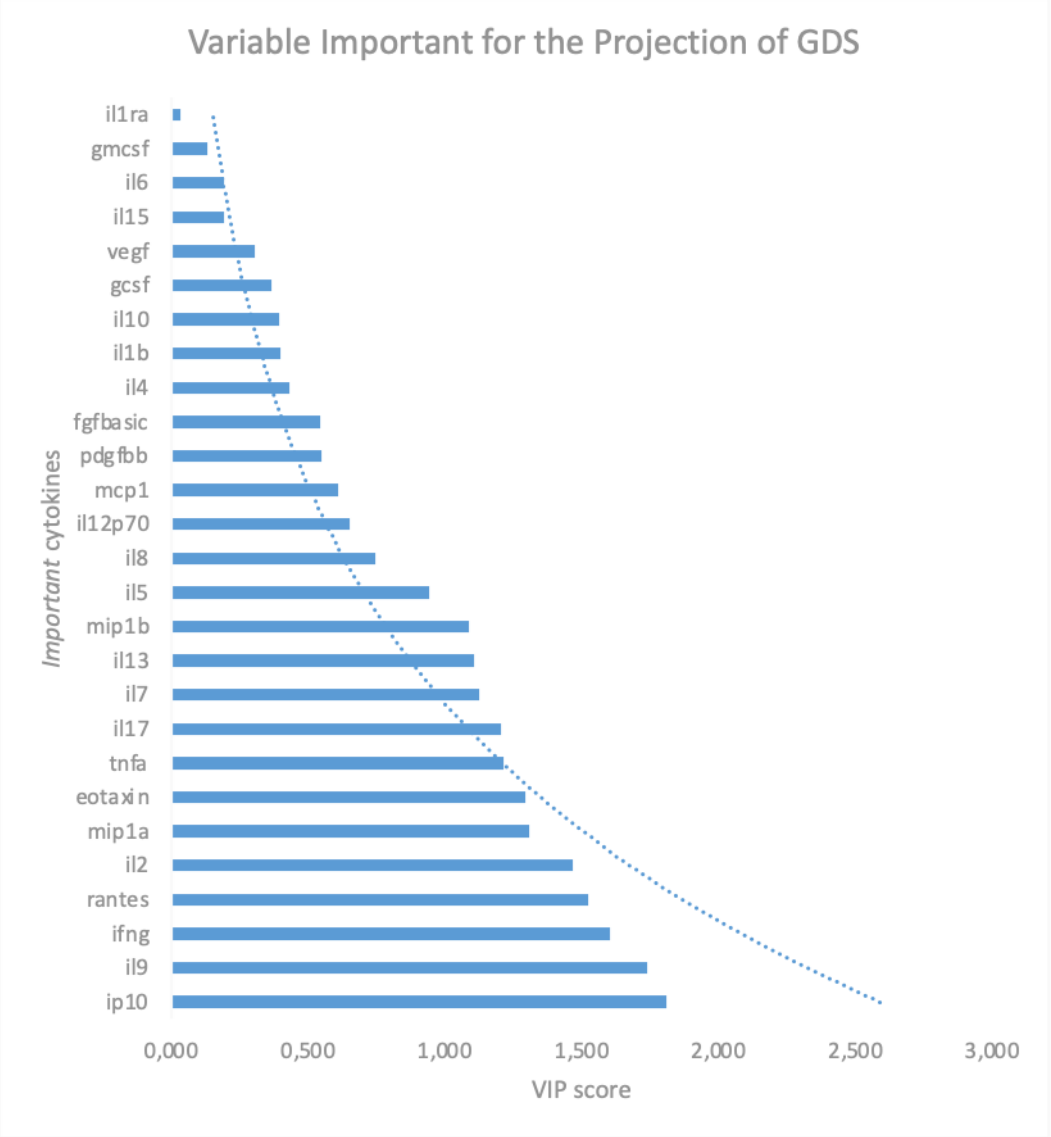

***Fig 2**: VIP Plot ranking 27 cytokines with 12 cytokines IP-10, IL-9, INF-γ, RANTES, IL-2, MIP-1a, eotaxin, TNF-α, IL-17, IL-7, IL-13 and MIP-1b having a VIP score above 1, IP-10 had the highest VIP score indicating that it had the highest influence on GDS.*

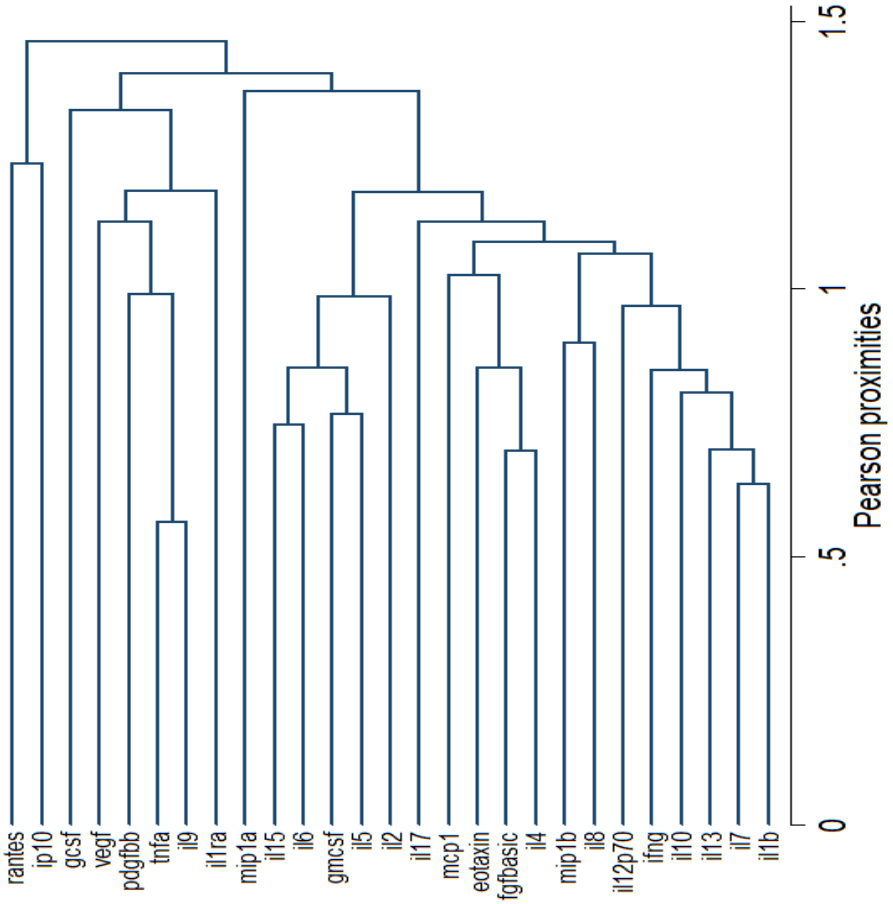

Fig 3*. Hierarchical clustering of cytokines showing RANTES and IP-10 with highest Pearson proximities, making a cluster of their own*

Paper 4: Ruhanya, V.; Jacobs, G.B.; Paul, R.H, Joska, J.; Seedat, S.; Nyandoro, G.; Engelbrecht, S.; Glashoff, R. Plasma cytokine levels as predictors of global and domain-Specific Human immunodeficiency virus-associated neurocognitive impairment in treatment-naive individuals. Journal of Interferon & Cytokine Research 2021,41,doi.org/10.1089/jir.2020.0251.

The author contribution to the published paper were as follows: Conception and design of the experiments, Performed the experiments, Data analyses and writing of manuscript

Plasma Cytokine Levels As Predictors of Global and Domain Specific Human Immunodeficiency Virus-Associated Neurocognitive Impairment in Treatment-Naive Individuals

Ruhanya, V.; Jacobs, G.B.; Paul, R.H, Joska, J.; Seedat, S.; Nyandoro, G.; Engelbrecht, S.; Glashoff, R.

Summary

Central nervous system dysfunction, associated with human immunodeficiency virus (HIV) infection, remains a significant clinical concern, affecting at least 50% of infected people. Imbalances in cytokine expression levels have been linked to HIV-associated neurocognitive disorders. The aim of this study was to evaluate plasma cytokine levels as predictor neurocognitive impairment in HIV infection using a multiplex profiling kit. Stepwise regression model was used to identify cytokine biomarkers of overall and domain-specific cognitive performance. Higher interleukin (IL)-2 ($\beta = 0.04$; $P = 0.001$) and eotaxin ($\beta = 0.01$; $P = 0.017$) were predictors of global neurocognitive, whereas higher IL-5 ($\beta = 0.005$; $P = 0.007$) was negative predictor of global cognitive deficit. IL-2 was a negative predictor of most cognitive domain functions, including recall ($\beta = 0.24$; $P = 0.005$), recognition ($\beta = 0.04$; $P = 0.026$), mental control ($\beta = 0.38$; $P = 0.005$), symbol search ($\beta = -0.55$; $P = 0.001$), and digital symbol ($\beta = -0.79$; $P = 0.019$). IL-6 was associated with 3 impaired domains, mental processing ($\beta = -0.468$; $P = 0.027$), recognition ($\beta = -0.044$; $P = 0.012$), and learning ($\beta = 0.02668$; $P = 0.020$) These results show that plasma cytokines/chemokines may serve as markers of neurocognitive impairment in HIV infection.

Key Words: *GDS, multiplex-bead assay, neuroinflammation, biomarkers, HIV infection*

Introduction

Despite combination antiretroviral therapy (cART) changing HIV infection from a devastating lethal disease to a chronic manageable one with a life expectancy close to population norms[1,2,3], people living with HIV(PLWH) continue to experience cognitive, motor and psychological disorders[4]. Collectively, these central nervous system (CNS) manifestations of HIV infection are known as HIV associated neurocognitive disorders (HAND)[5-7]. More than 50% of HIV infected people have some form of HAND[8,9]. The most severe form of HAND, which was prevalent in the pre-cART era, called HIV-associated dementia (HAD), is characterized by extreme difficulty in maintaining attention, and delayed speed of processing information and mental flexibility[10,11]. Subtle cognitive impairment, which involve minor motor function impairment and behavioral abnormalities are more common in the cART era[12-15].

The neuropathogenesis of HAND is not fully understood but evidence from experimental animal models and human cross sectional and autopsy studies indicate that it is a complex and multifaceted interaction between viral factors, host cellular targets and the immune response[16,17]. It is thought that brain micro-environmental changes, due to an overwhelming inflammatory response and neuronal excitotoxicity induced by viral proteins or proinflammatory cytokines play a significant role. Chemokines have been shown to affect neuronal signaling with subsequent disturbance of glial and neuronal functions[18,19,20]. Inflammation has also been shown to affect brain areas associated with memory, such as hippocampus, entorhinal and temporal cortices[21,22]. Therefore, host immune responses are pivotal in HIV-associated neuropathology leading to HAND.

Assessment of HAND largely relies on neuropsychometric analysis for both diagnosis and clinical monitoring progression of the disease[23,24.] These neurocognitive tests have a challenge in detecting and monitoring more subtle presentations. There is need for strong predictive blood markers related to the immune response associated with HAND to augment the diagnosis and monitoring of the disease.

Immune activation, as indicated by inflammatory cytokines, was observed in advanced treatment naive HIV-infected patients with HAND[25,26]. The inflammatory response is the main mediator of neuronal damage in HIV-related neurodegenerative disease[27]. Increased levels of circulating pro-inflammatory cytokines, emanating from several tissues has led to

HIV infection being considered a chronic inflammatory disease in PLWH. Unbridled viral replication and inflammation in untreated neuroHIV drives severe neurocognitive impairment (NCI) and HIV-D, while in treated disease, persistence of viral particles, together with pre-existing legacy effects continue to cause low grade inflammation[28,29]. Such evidence provides the rationale for assessing levels of inflammatory cytokines as essential to evaluate the risk of neurocognitive impairment in HIV infection.

The primary objective of the current study was to understand the significance of immune activation, particularly pro-inflammatory cytokines, and chemokines on neurocognitive impairment in a predominantly viremic untreated cohort.

Materials and Methods

A cross-sectional study was conducted on a cohort of 139 HIV positive Xhosa-speaking individuals recruited from primary care HIV clinics in Cape Town, South Africa, that was being investigated for HAND. Inclusion criteria to participate in the study required the following: 1. Age ranging from 18 and 45 years with at least five years of formal education. This age was selected to avoid age-related CNS abnormalities. 2. Positive HIV serostatus, determined by ELISA and then confirmed by Western blot. 3. HIV-1 RNA for plasma viral load measured by the Abbott m2000sp and the Abbott m2000rt analyzers (Abbott laboratories, Abbott Park, IL, USA). Exclusion criteria included the following: any major psychiatric condition that could significantly affect cognitive status; confounding neurological disorders including multiple sclerosis and other CNS conditions; head injury with loss of consciousness greater than 30 min; clinical evidence of opportunistic CNS infections and current substance abuse or alcohol abuse as defined by structure of interview. This study was approved by the Health Research Ethics Committee (HREC) of Stellenbosch University, Ethics Reference #: S17/02/035. The study was conducted according to South African Guidelines for Good Clinical Practice and the Medical Research council, Ethical Guidelines for Research in line with the guidelines and principles of the international declaration of Helsinki.

Neuropsychological Evaluation

A battery of cognitive tests sensitive to deficits associated with HIV was administered to all participants as described earlier[29]. Briefly, the tests were administered to assess cognitive functions namely, learning, executive functions/visuospatial and psychomotor speed. Learning was assessed using the Hopkins Verbal Learning Test-Revised (HVLT-R), and

visual learning was assessed using the Brief Visual Memory Test-Revised (BVMT-R). Executive functions and psychomotor speed were evaluated using the tools described earlier24,30. Performances were averaged to generate mean T-score and deficit for each of the cognitive domains to calculate a global T-score and global deficit score (GDS). A GDS of 0.5 or more was regarded as neurocognitive impairment[30]. Global deficit score was then used to categorize the cases as either impaired or unimpaired.

Plasma cytokine quantification by multiplex-bead assay

Cytokine/chemokine concentrations in plasma samples were determined using Biorad Bio-Plex ProTM Human cytokine assay, a 27-plex kit according to manufacturer's instructions. The panel was chosen because it detects the most often researched and biologically relevant cytokines in a single cell. Quantified cytokines included IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, Eotaxin, FGF- basic, G-CSF, GM-CSF, IFN-γ, interferon γ-induced protein 10 (IP-10), MCP-1, MIP-1α, MIP-1ß, PDGF-BB, regulated upon Activation, Normal T Cell, Expressed and Secreted (RANTES), TNF-α and VEGF. Briefly, plasma samples were incubated with antibody-coupled beads. Complexes were washed, incubated with biotinylated detection antibody, and subsequently with streptavidin-phycoerythrin, prior to assessing titers of cytokine concentration. Standard curves were generated together with samples using standard cytokines provided in the kit. Plasma cytokine levels were determined using a multiplex array reader from LuminexTM Instrumentation System. (Bio-Plex Workstation from Bio-Rad). Plasma samples were run in duplicate and cytokine concentrations were calculated as the average of two independent measures using Bioplex Manager Software (California, USA).

Statistical analysis

Stata statistical package version 12.1 (StataCorp, College Station, Texas, USA, 2011) was used for analyzing data. Variables were described using means and medians. Stepwise regression model was used to assess plasma cytokine levels and clinico-demographic parameters as predictors of global and domain-specific neurocognitive impairment. Statistical significance was determined as p value < 0.05.

Results

Demographic and clinical characteristics

Data from 139 participants, consisting of 115 (82.7%) females and 24 (17.3%) males, were available for analyses. The age of the participants ranged from 21 to 46 years. The median GDS for the cohort was 0.32. The median GDS for males (0.5) was higher than females (0.31), but the difference was not significant ($p = 0.0853$). Using GDS of 0.5 or higher 49 (35.3%) participants were categorized as impaired and 90 (64.7%) were classified as normal. Among the participants, 23 (16.5%) had initiated cART within 4 weeks and the rest were treatment naive. Most of the participants, 80% had some level of secondary education, 5.8% had reached Grade 7 (7th year of formal schooling) and 5% had at least Grade 12 level. There was a significantly higher GDS in participants who had received secondary education than those without ($p = 0.0008$). Examination of cognitive function by gender shows that WMS mental control scores were higher in males than females ($p = 0.0030$), but WAISIII symbol search scores were higher in females than males ($p = 0.0079$). Both color naming on the Stroop test and semantic fluency using fruit-vegetables fluency test were higher in females than males ($p = 0.0314$ and $p = 0.0201$ respectively). Table 1 summarizes demographic and clinical parameters of the cohort, comparing male and female participants.

Cytokine levels as a function of neurocognitive status

Using GDS categories, Interferon gamma (IFN-γ) was significantly higher in non-impaired than neurocognitively impaired patients ($p = 0.0068$). Monocyte (MCP-1 or CCL2) was higher in cART-initiated individuals, than those which were not ($p = 0.05$). However, IL-2 was significantly elevated in the group which did not initiate cART than the ones initiating cART ($p = 0.029$). This is indicative of viral replication-associated T cell proliferation and activation.

Using stepwise multiple regression model, it was observed that 4 cytokines; namely, Eotaxin, IL-2, MIP-1 (or CCL4) and IL-5 were predictors of worse cognitive performance ($p = 0.017$, $p = 0.001$, $p = 0.059$ and $p = 0.007$). Table 2 below shows GDS as function of cytokine concentration

Impact of cytokines on measures of visuospatial memory

Higher plasma IL-2 associated with worse performance on the learning ($\beta = -0.542$; $p = 0.006$), recall ($\beta = -0.24$; $p = 0.004$), and recognition ($\beta = 0.04$; $p = 0.026$) scores on the BVMT-R. IL-8 (CXCL8) ($\beta = -0.036$; $p = 0.019$) and GM-CSF ($\beta = -0.12$; $p = 0.028$) associated with worse BVMT-R learning. IL-5 showed a significant positive association with

learning (β = 0.03; p = 0.04) and recall (β = 0.027; p = 0.0.05) as well as the total measures of visuospatial memory domain (β = 0.068; p = 0.031). Table 3 summarizes cytokine predictors of BVM test scores. MCP-1 was predictive of impaired recognition ability (β = -0.011; p = 0.005).

Impact of Cytokines on Verbal Learning abilities using HVLT-R

Higher plasma IL-2 associated with worse of impaired recall (β = - 0.084; p = 0.049). Eotaxin (β = 0.006; p = 0.020) was another cytokine predictive of impaired delayed recall domain although less so than IL-2. IL-6 (β = -0.0447; p = 0.012) and MIP1-α (β = -0.020; p < 0.0001), were associated lower recognition and learning respectively. Only GM-CSF had a significant positive association with HVLT-recognition (β = 0.076; p = 0.031).

Besides impaired recall and impaired recognition, IL-2 was also a strong predictor of impaired mental processing (β = - 0.377; 0.005) using Wechsler Memory Scale (WMS). Although with a lower magnitude, MCP-1 (β = -0.053; p = 0.017 also associated with impaired mental processing. IL-2 (β = -0.791; p = 0.019) was again strongly associated with low processing speed and visual perception using Wechsler Adult Intelligence Scale (WAISIII) digital symbol search test. Although the magnitude was lower than IL-2, IL-6 was a negative predictor of mental processing speed and visual perception (β = -0.469; p = 0.027). Table 5 summarizes cytokine the associations

Discussion

The aim of this study was to evaluate the relationship between plasma cytokine levels and neurocognitive function, determined by GDS and domain specific cognitive test scores. Findings from this cross-sectional study showed that cytokines differed on their effect on the overall cognitive function. Some cytokines, such as IL-2 and Eotaxin, were positive predictors of cognitive impairment, whilst MIP-1α and IL-5 were negative predictors.

HIV causes a profound disruption in the cytokine network from the earliest points of HIV infection, shortly after first detection of systemic virus[30]. This dysregulation of cytokines, especially IL-2, increases viral replication due to its activation and promoting T-cell proliferation, including the latent pool of HIV infection[31]. IL-2 also upregulates expression of CCR5 on the same cells, which may increase virus expression on latently infected cells. Virus-induced systemic immune activation and inflammatory processes have been linked to brain dysfunction in people with HIV infection[32]. Inflammation in the periphery can emanate

from circulating viral proteins, such as p24 antigen, Nef, tat and Vpr[33,34] or be related to cellular damage/toxicity as well gut leakage. All these stimuli activate innate inflammatory cells via pattern recognition receptor recognition. It is thought that in the scenario of CNS penetration of HIV and its products, highly activated monocytes and T-cells from peripheral blood initiate a cascade of neuroinflammation resulting in astrocytosis and microgliosis[34]. Activated and HIV-infected peripheral monocytes infiltrating the BBB have been linked to HAND[35].

Eotaxin, a chemokine produced by a variety of cells including endothelial cells, stimulates migration of Th2 cells and macrophages. It attracts lymphocytes through chemokine receptors CCR2, CCR3 and CCR5, but it has highest affinity for CCR3[36]. It has been demonstrated that elevation of eotaxin in plasma suppressed neurogenesis and neurocognitive function and can contribute to cognitive decline by directly inhibiting adult hippocampal neurogenesis[37]. The chemokine accesses the CNS by hematogenous spread through the BBB leading to activation of the brain cells and consequently activation-induced neurodamage[38.] Higher levels of plasma eotaxin have also been linked to worse virological outcome in primary HIV infection[39.] Since eotaxin is related to the expression of chemokine receptors, CCR3 and CCR5[40], this could influence the efficiency of HIV replication, thereby increasing antigenic load, which results in increased systemic immune activation. Based on this evidence we speculate that higher levels of eotaxin promote biological processes that lead to immune-mediated neurocognitive impairment in PLWH. Major chemokine receptors for HIV infection CCR5, CCR3 and CXCR4 in human brain and have been detected in microglia, astrocytes and neurons[41].

MIP-1α and other β-chemokine are the natural ligands for CCR5[41,42] and it has been suggested that CD8+ T-lymphocytes use HIV-suppressive chemokines like MIP-1α as one of the mechanisms to control HIV infection[43], by competitive inhibition of HIV attachment to target cells. In this study, increase in plasma concentration of MIP-1α was associated with lower GDS. This could imply that higher levels plasma was associated with higher cognitive function. Earlier studies have also indicated that production of HIV-suppressive chemokines correlated with disease stage[44,45]. An association between overproduction of β-chemokine by activated CD4+ T-cells and resistance to in vitro HIV infection has been demonstrated[46] and has been associated with decreased risk of progression of in infected people. We suggest that MIP-1α-mediated anti-HIV immune response, which plays a role in virus control, may

contribute to suppression of neurocognitive impairment. However, the results of this study do not rule out the possibility of other cytokines or chemokines that are anti-HIV replication[47]. We also suggest that anti-HIV immune response reduces systemic activation and CD4+ T-cells and other peripheral blood lymphocytes, which have been linked to HIV associated neurocognitive impairment. MIP-1α is increased in active infection, which is what we observed in this study, but the association with neurocognitive function is novel. However, in treated HIV indviduals with undetectable viral load, the associations may not hold.

Although weak, the inverse correlation between IL-5 and GDS indicates that elevated IL-5 plasma levels had a positive impact on cognitive function. One of the many established roles of IL-5 is to trigger humoral immune response, by inducing B-cell proliferation and priming the same cells to secrete immunoglobulins[48]. The cytokine regulates antibody mediated immune response which is associated with clearance of extracellular antigens/pathogens4[9,50]. HIV neutralising antibodies are involved in free viral clearance and blocking new infections and they accelerate destruction of infected cells. This process reduces systemic immune activation and its impact on activation- induced neurocognitive impairment.

Considering domain-specific neurocognitive function, IL-2 was associated with at least 6 number of impaired cognitive domains including, visuospatial memory. Brain centres linked to visual memory include the posterior parietal cortex while spatial memory involves different parts of the brain including the hippocampus. This implies that the cytokine affected functional integrity of brain centers controlling these domains. However, our findings need to be supported by physiological and anatomical data from neuroimaging techniques which have the capacity to assess the localization of extent of brain involvement.

The interleukin was shown to induce neuropsychiatric changes in cancer patients under IL-2 immunotherapy[51,] and recently, evidence showed that IL-2 disrupts BBB and alters brain microcirculation, which is important in the development of chronic neurological disorders[52]. Possibly, impairment of BBB, increases infiltration of activated cells from the peripheral blood that is likely to amplify the inflammatory response, which is detrimental to neurocognitive function. It could also imply that resident brain macrophages responded to elevated proinflammatory markers in the peripheral blood due to viral components and cytokines which accessed the brain through a ‘leaky’ BBB. The hippocampus, which is mainly associated with memory, is part of the brain primarily affected by inflammation[53]. These findings also suggest that uncontrolled peripheral cytokine production like IL-2 is

potentially damaging to the CNS resulting leading to impairment of a number of cognitive domains in untreated indviduals. These associations will probably not hold in treated HIV with undetectable VL. What is useful though is that on patients presenting at clinic prior to treatment, the marker could be used to assess risk of neurocognitive impairment in the absence of comprehensive cognitive testing[54].

MCP-1 was linked to lower executive capacity and memory. This could imply that elevated plasma MCP-1 could be involved in processes that interfere with the function of dorsolateral prefrontal cortex (DLPFC), which is responsible for executive functions like working memory, cognitive flexibility, and abstract reasoning. It has been shown earlier that MCP-1 plays a role in BBB disruption by directly altering its permeability[55]. MCP-1 was shown to be overproduced in people with HIV encephalitis (HIVE) and accumulates in the brains of people with HIV associated dementia (HAD)[56]. Infected/activated peripheral monocytes and lymphocytes of this viremic cohort could release viral products which may have resulted in increased production and release of MCP-1 into the brain[57,58]. Possibly this could result in impairment of these cognitive domains[59]. However, besides the possible contribution of plasma MCP-1 to impairment of cognitive domains observed in this study, other factors such as HIV infected resident monocyte-derived cells and astrocytes may have contributed to impairment of cognitive domains[60].

IL-6 plays an important role in immune activation in HIV infection[61,62]. HIV infected people have shown elevated systemic IL-6 which has been shown to be predictive of HIV disease progression and mortality[63,64,65]. Elevated IL-6 was associated with impaired function in 5 cognitive domains. The findings indicate that peripheral IL-6 levels play a role neuropsychological dysfunction. Increased peripheral IL-6 in HIV infected people with psychological stress has been reported earlier[66]. Since this cohort was viremic and largely untreated, HIV particles and gene products may have contributed to production of inflammatory cytokines, including IL-6[67]. Aberrant signaling by IL-6 and other inflammatory cytokines could have resulted in CNS pathological abnormalities, leading to impairment of cognitive domains observed in this study. In fact, IL-6 mediated inflammation is known to cause gliosis and dendritic damage in patients with other neurological diseases like Parkinson's disease (PD) and Alzheimer's Disease (AD) as well as HIV associated dementia[68]. IL-6 is often used as a key cytokine marker of generalized inflammation in many disease scenarios. The finding of multiple association with cognitive domain dysfunction,

strengthens the concept that inflammation, which is highly elevated in untreated HIV, is associated with neurocognitive impairment.

Conclusions

The study shows that there is a link between plasma cytokine concentrations and neurocognitive function in untreated HIV infection, with IL-2, being a strong predictor of both global and domain-specific neurocognitive dysfunction. However, our findings need to be supported by neuroimaging data to locate pathologies in brain centers thought to be affected by the cytokines.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Author Contributions

Study conception and design: VR, SE, RP, RG. Acquisition of data: VR, RP, JJ, SS. Analysis, and interpretation of data: VR, SE, RG and GJ. Drafting of manuscript and Critical revision: all authors

Funding

This study was supported by the Poliomyelitis Research Foundation (PRF), National Research Foundation (NRF) and the South African Medical Research Council (SAMRC) Collaborating Centre for HIV-1 Laboratory Research.

Acknowledgments

Authors would like to thank Cladnos Mapfumo for statistical analysis.

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Table 1. Summary of clinical parameters.

Parameter	Male	Female	P - value
Age	33.3	31.4	0.1089
GDS	0.5	0.31	0.0853
CD4+ count/ul	168	209	0.1481
CD8+ count/ul	673.5	822	0.3118
CD4+/CD8+ ratio	0.205	0.260	0.6316
CD14+ count	1600000	1100000	**0.0228**
Plasma viral load	47677	13197	0.0676

Table 2: Cytokine predictors of global deficit scores

	Coefficient	**p**	**Adjusted R^2**	**P**
GDS				
Eotaxin	0.0012984	0.017	0.1002	0.0012
IL-2	0.0410804	0.001		
MIP-1a	-2.00E07	0.059		
IL-5	-0.0053929	0.007		

Adjusted R value is for the whole model fit

Table 3: Summary of cytokine predictors of BVM test scores

	Coefficient	P	Adjusted R	P
BVM total				
FGF-basic	0.0510443	0.058		
IL-2	-0.542087	0.006	0.0686	0.0130
IL-4	-0.5360093	0.067		
IL-5	0.0684189	0.031		
IL-9	0 .0000767	0.048		
BVMT learning				
IL-8	-0.0364666	0.019		
MCP1	0.0205714	0.011	0.1103	0.0010
GMSCF	-0.1180607	0.028		
VEGF	-0.0000323	0.075		
IL-5	0.0289232	0.004		
BVMT recall				
fgfbasic	0.0203731	0.084	0.0436	0.0415
IL-5	0.0271751	0.050		
IL-2	-0.239156	0.005		
IL-4	-0.2124688	0.098		
BVMT recognition				
eotaxin	0 .0018645	0.075		
IFN-γ	0.0104616	0.063	0.0521	0.0250
IL-2	-0.0401725	0.026		
MCP1	-0.0104553	0.005		

Table 4: cytokine predictors of cognitive domains performance by HVLT

	Coefficient	P	Adjusted R	P
HVLT total				
PDGFB-BB	-0.000127	0.082	0.0627	0.0183
MIP-1α	-0.0296123	0.029		
IP10	-0.0005507	0.071		
GMCSF	-0.1571083	0.080		
Il-5	0.036201	0.052		
HVLT learning				
Il-6	-0.0266481	0.087	0.0141	0.0875
HVLT recall				
Eotaxin	-0.0059091	0.020		
PDGFB-BB	0.0102415	0.071	0.0383	0.0414
Il-2	-0.084408	0.049		
HVLT recognition				
GMCSF	0.0769581	0.031		
IL-6	-0.0447247	0.012	0.1451	0.0001
IL-13	-0.1281681	0.060		
MIP1-α	-0.0203566	0.000		
IL-7	0.018444	0.096		
RCF 3min				
Il-17	-0.7795159	0.001		
IFN-γ	-2.013528	0.006	0.3386	0.0000
Eotaxin	-0.2740568	0.002		
RANTES	-0.0014615	0.010		
IL-5	0.6702811	0.019		
IL-13	12.1144	0.000		
mcp1	1.92493	0.000		
IL-8	1.55125	0.001		
IP-10	-0.0096874	0.047		
IL-10	-4.51021	0.006		
RCF 30min				
IL-13	-0.826766	0.001		
IFN-γ	0.2076652	0.000	0.0942	0.0010
IL-2	-0.2605575	0.047		

Table 5: Cytokine predictors of Memory and adult Intelligence

	Coefficient	P	*Adjusted R^2*	p
WMS mental processing				
MCP1	-0.0535014	0.017		
IFN-γ	0.0802129	0.053	0.0636	0.0080
IL-2	-0.3776405	0.005		
WAISIII digital				
IL-15	0.0295265	0.069		
MIP1-α	0.0900107	0.033	0.1947	0.0001
IL-2	-0.7919105	0.019		
IL-4	-1.985095	0.007		
FGF-basic	0.1003554	0.027		
IL-6	-0.4693132	0.027		
RANTES	0.0001989	0.069		
PDGFBB	-0.0000524	0.048		
IL-9	0.0005237	0.000		
Eotaxin	0.0537155	0.053		
IL-12p70	0.5412007	0.001		
GMCF	0.547567	0.094		
WMS spatial Span F				
IL-7	0.0136184	0.068		
PDGFBB	3.40e-06	0.040	0.0589	0.0234
IL-10	0.0398772	0.010		
IP10	-0.0002278	0.081		
IL-5	0.012973	0.037		
WMS spatial Span B				
IL-8	-0.0337712	0.012	0.0458	0.0157
FGF-basic	0.0118742	0.017		
WMS spatial Span total				
IL-10	-0.1271757	0.075		
IL-12p70	-0.0686556	0.078	0.0613	0.0199
1L-2	-0.1357036	0.050		
IL-13	0.2797473	0.047		
IL-5	0.0391422	0.002		

Paper 5: **Ruhanya**, V.; Jacobs, G.B.; Paul, R.; Joska, J.; Seedat, S.; Nyandoro, G.; Engelbrecht, S.; Glashoff, R.H. Proinflammatory Cytokines and Chemokines are Related to MRI Measures of White and Gray Matter in HIV Infection. Pathogens Preprints 2021, 2021050501 (doi: 10.20944/preprints202105. 0501.v1).

The author contribution to the published paper were as follows: Conception and design of the experiments, Performed the experiments, Data analyses and writing of manuscript.

Proinflammatory Cytokines and Chemokines are Related to MRI Measures of White and Gray Matter in HIV Infection.

Ruhanya, V.; Jacobs, G.B.; Paul, R.; Joska, J.; Seedat, S.; Nyandoro, G.; Engelbrecht, S.; Glashoff, R.H. Proinflammatory Cytokines and Chemokines are Related to MRI Measures of White and Gray Matter in HIV Infection.

Summary

HIV is accompanied by production of proinflammatory cytokines which are regarded as critical in neuronal damage, leading to brain dysfunction, hence the need to identify cytokine biomarkers sensitive to brain damage. Methods: We applied MRI volumetric neuroimaging and high throughput Luminex based immunoassays to examine the relationship between cortical white matter, subcortical gray matter and total gray matter brain volumes and plasma cytokines in HIV indviduals using generalised linear models and Partial least square regression model. Results: Higher plasma inflammatory cytokines CCL5/RANTES and MCP-1 were significantly associated with lower cortical white matter volume. Higher IL-6 was associated with both lower subcortical gray matter and lower total gray matter, whereas higher IL-8 and GM-CSF were associated with lower total gray matter only. Higher VEGF, PDGF-BB and IL-9 were associated with higher cortical white matter volumes. After standardisation and adjusting for clinical and demographic variables, IL-6, IL-8, MCP-1 remained associated with lower volumes of the three brain regions whereas IL-9, VEGF and PDGF-BB were associated with higher volumes. Conclusions: Association proinflammatory cytokines RANTES, MCP-1 and IL-6 with lower brain volumes could imply possible involvement in neurodegerative processes in HIV infection while IL-9, VEGF and PDGF may have a neuroprotective or neurotrophic role.

Keywords*: inflammation; Luminex; volumetric neuroimaging, cognitive impairment*

Introduction

Despite the immense contribution by combination antiretroviral therapy (cART) to reduce morbidity in people infected with HIV, people living with HIV (PLWH) continue to develop a spectrum of cognitive, motor and psychological manifestations, clinically referred to as HIV associated neurocognitive disorders (HAND). This condition affects more than 50% of HIV infected people [1-3]. The pathology of HAND is not completely understood, but it is thought to be a complex interaction of virus, inflammation and the host immune response. Pathological evidence of brain invasion by HIV includes neural loss, dendritic damage, astrogliosis, microgliosis and nucleated giant cell formation [4]. HIV is thought to penetrate the central nervous system (CNS) through trafficking of cell-free virus by transcytosis across the blood brain barrier (BBB) and also through the disrupted BBB caused by HIV infection. It is believed that BBB disruption is enhanced by peripheral proinflammatory environment, which is induced by HIV [5]. Transport of cytokines across the BBB has been demonstrated for IL-1α, IL-1β, IL-6 and tumor necrosis alpha (TNF-α) [6]. This passage of blood-born cytokines to the brain can potentially affect brain integrity and function, with cytokines altering the integrity of the BBB and compromising the ability to regulate trafficking of immune cells [7-8]. Therefore, peripheral inflammatory cytokines may be putative mediators of neuroinflammation in HIV infection. A link between peripheral inflammation and increased cytokine production in the CNS has been demonstrated in HIV infection [9-10]. It has been suggested that the major impact of peripheral inflammatory cytokines on the CNS may possibly be through cytokine-mediated production of prostaglandins in the brain endothelium [11].

Plasma cytokine markers are easy to measure and could be clinically relevant to assess HIV associated neuropathological changes [12]. In fact, systemic immune activation and inflammation has been linked to HAND in several studies [13-14]. Greater changes in inflammation-associated neuronal loss have been observed in HAND patients [15]. Multiplex analytic technologies in proteomics can be used to accelerate the identification of cytokine biomarkers associated with clinical outcomes in HIV associated neurocognitive impairment [16]. Luminex based high throughput assays can be used been to identify biomarker signatures of immune activation in HIV infection [17].

Recently, magnetic resonance imaging (MRI) techniques have shown greater potential in understanding the mechanisms underlying HIV associated neurocognitive impairment [18].

MRI imaging technologies which are non-invasive can generate objective brain measurements that are able to determine the extent of neurodamage associated with HIV infection. Fully automated MRI technologies have been developed for automated segmentation of the brain [19-21] in which specific brain regions of interest are assessed for abnormal structural changes in HIV infected people [22]. The advantage of segmentation-derived brain volumetric measures are non-invasiveness and high throughput, and both have led to widespread use to investigate brain changes in HIV infection [23-28]. It has been observed that volumetric changes correlate with neuropsychological testing and clinical measures in HIV associated cognitive impairment and brain gray and white matter atrophy has been reported in people living with HIV (PLWH) [29]. Primary brain regions that have been shown to be affected by neuroinflammation are the hippocampus and entorhinal and temporal cortices [30]. Structural imaging of HIV patients have shown impact on grey matter structures and subcortical regions [31] and cerebral atrophy with ventricular enlargement [32-33]

This study applies volumetric neuroimaging and high throughput Luminex based immunoassays to examine the relationship between specific brain regions and plasma cytokine levels to assess the inter-relatedness of brain changes in HIV and concomitant peripheral immune activation in treatment naïve patients. We hypothesized that higher levels of pro-inflammatory cytokines are associated with lower volumes of specific brain regions.

2. Results

Considering the three regions of interest (ROI), the unstandardized regression coefficients and associated p-values for cytokines significantly associated with brain volumes using generalized linear model (GLM) are shown in Table 1. Regarding cortical white matter, only RANTES and MCP-1 were related to lower white cortical volume. The rest were related to higher cortical white matter volume including IL-7, IL-9 and VEGF. Considering subcortical gray matter, IL-1α was significantly associated with higher volume, whereas IL-6 was associated with lower volume. Lower total gray matter volume was associated with higher levels of 9 cytokines/chemokines including, IL-6, IL-8, MCP-1 and RANTES. Only TNF-α and IL-1α were associated with higher total gray matter volume.

Table 1: Relationship between cytokines levels and brain volumes

Cytokine	Coef.	SE.	Z	P -value	95% CI	
			Cortical white matter volume			
IL-1β	.0020477	.0009955	2.06	0.040	.0000966	.0039988
IL1α	4.44e-06	1.67e-06	2.66	0.008	1.17e-06	7.70e-06
IL7	.0010158	.000482	2.11	0.035	.000071	.0019606
IL9	1.42e-06	6.33e-07	2.24	0.025	1.80e-07	2.66e-06
MCP-1	-.0009288	.0003753	-2.47	0.013	-.0016643	-.0001933
PDGFBB	3.57e-07	1.42e-07	2.52	0.012	7.89e-08	6.36e-07
RANTES	-1.52e-06	4.82e-07	-3.16	0.002	-2.47e-06	-5.77e-07
VEGF	1.57e-06	4.93e-07	3.19	0.001	6.07e-07	2.54e-06
			Subcortical gray volume			
IL1α	7.88e-06	2.47e-06	3.19	0.001	3.04e-06	.0000127
IL6	-.0021455	.000831	-2.58	0.010	-.0037743	-.0005167
			Total gray volume			
IL1α	3.16e-06	1.57e-06	2.01	0.045	7.61e-08	6.25e-06
IL6	-.0020823	.0005579	-3.73	0.0001	-.0031758	-.0009888
IL8	-.0014577	.0006186	-2.36	0.018	-.0026701	-.0002453
IL10	-.0035407	.0013919	-2.54	0.011	-.0062688	-.0008126
IL12p70	-.0020964	.0008961	-2.34	0.019	-.0038527	-.0003402
IL15	-.0001525	.0000626	-2.44	0.015	-.0002751	-.0000298
GCSF	-.003109	.0010898	-2.85	0.004	-.0052449	-.0009732
GMCSF	-.0059812	.0031477	-1.90	0.057	-.0121505	.0001881
MCP1	-.0024353	.0008448	-2.88	0.004	-.0040912	-.0007795
RANTES	-2.54e-06	1.04e-06	-2.44	0.015	-4.57e-06	-5.02e-07
TNF-α	5.11e-06	2.52e-06	2.03	0.042	1.75e-07	- .00001

After adjusting for age, sex, plasma viral load and CD4+T absolute count only IL-9 (p = 0,045) remained significantly associated with higher cortical white matter volume. MCP-1 (p = 0.047) and GCSF (p = 0.025) were related to lower cortical white matter volume after adjusting for the same clinical and demographic parameters. With regards to subcortical gray matter two cytokines, IL-6 (p = 0.008) and IL-8 (p = 0.042) remained significantly associated lower subcortical. The same cytokines, including MCP-1 (p = 0.032) remained associated with lower total gray matter volume.

2.1. White matter volume as a function of plasma cytokine volumes

Using PLS-R modelling, 13 cytokines/chemokines including RANTES, MCP-1, IP-10, IL-6, IFN-γ Eotaxin, GM-CSF, IL-13 and IL-8 were associated with lower cortical white matter volume as shown as indicated by their relationship with standardised coefficients in Fig 1. Among these pro-inflammatory cytokine, MCP-1, RANTES, GM-CSF, and IL-6 had strongest negative association as shown by the magnitude and direction of bars on the graphs showing standardised coefficients of cortical volume as a function of cytokines in **Fig 1.**

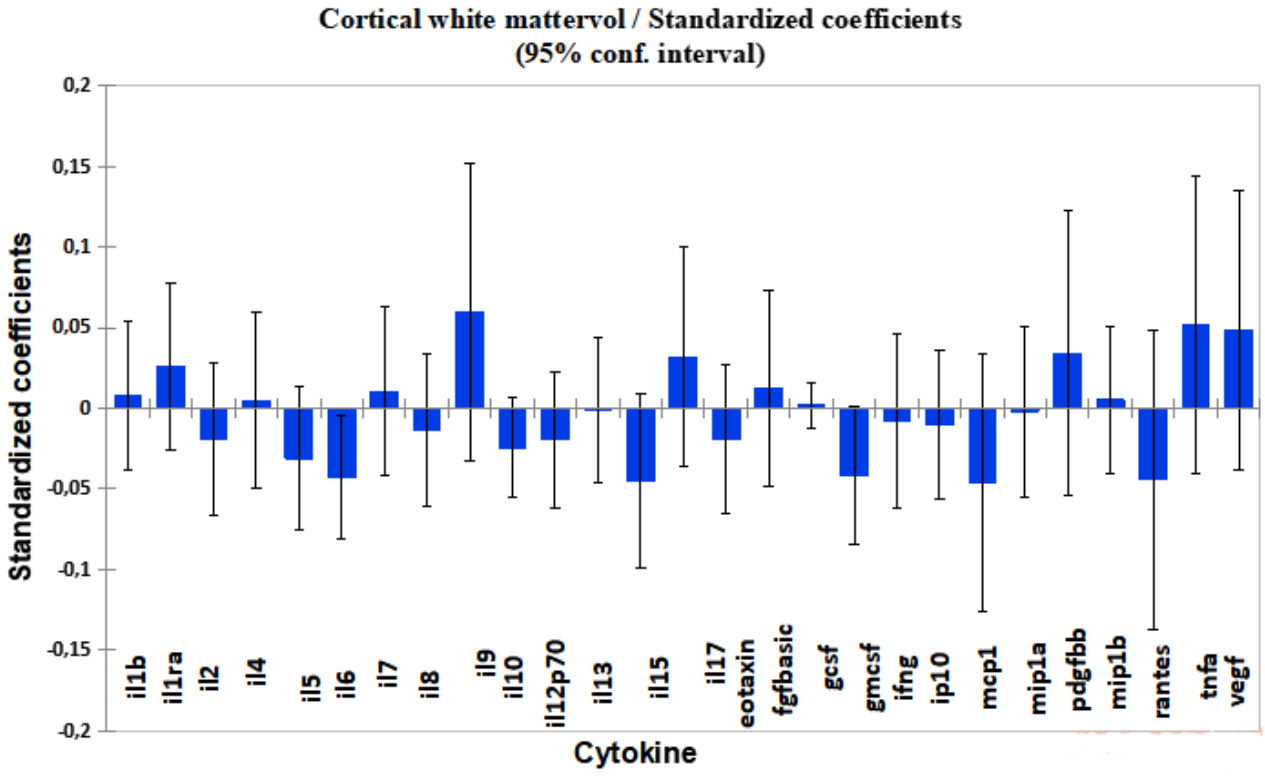

Fig 1. Graphical representation of the magnitude and direction of plasma cytokines on cortical white matter volume using PLS-R standardized coefficients and 95% confidence intervals. RANTES (- 0.044; -0.137 – 0.049), MCP-1 (- 0.046; - 0.136 – 0.34), IL-6 (-0.043; - 0.082 - (- 0.005)) and GM-CSF(- 0.042; - 0.082 – 0.001) were prominent cytokines in the reduction of cortex volume (table 3).

However, using multiple generalized linear models to determine significance of the association only MIP-1β (β = - 0.005; p = 0.029) was significant. The other cytokines IL-1α, IL-7, IL-9, PDGF-BB, TNF-α, FGF-basic. IL-9 had the strongest positive association with cortical white matter volume as shown in fig 1. However, multiple generalized regression models did not show statistical significance of the association as shown in Table 2.

Table 2: Standardized coefficients for cortical white matter volume in multiple GLM

Variable	Coefficient	Std. deviation	Lower bound (95%)	Upper bound (95%)	P value
IL-1β	0.008	0.023	-0.038	0.054	0.147
IL-1α	0.026	0.026	-0.026	0.078	0.627
IL-2	-0.019	0.024	-0.067	0.028	0.545
IL-4	0.005	0.027	-0.049	0.059	0.815
IL-5	-0.031	0.022	-0.076	0.013	0.723
IL-6	-0.043	0.019	-0.082	0.005	0.281
IL-7	0.010	0.026	-0.042	0.063	0.794
IL-8	-0.014	0.024	-0.061	0.034	0.694
IL-9	0.059	0.047	-0.033	0.152	0.099
IL-10	-0.024	0.015	-0.055	0.006	0.24
IL-β12p70	-0.020	0.021	-0.062	0.023	0.856
IL-13	-0.001	0.023	-0.047	0.044	0.955
IL-15	-0.045	0.027	-0.099	0.009	0.822
IL-17	0.032	0.034	-0.036	0.100	0.693
Eotaxin	-0.019	0.023	-0.066	0.027	0.429
FGF-basic	0.013	0.031	-0.048	0.073	0.648
GCSF	0.001	0.007	-0.013	0.014	0.255
GMCSF	-0.042	0.022	-0.084	0.001	0.633
IFN-γ	-0.008	0.027	-0.062	0.046	0.898
IP-10	-0.010	0.023	-0.056	0.036	0.967
MCP-1	-0.046	0.040	-0.126	0.034	0.13
MIP-1α	-0.001	0.027	-0.054	0.052	0.49
PDGF-BB	0.034	0.045	-0.055	0.123	0.688
MIP-1β	0.005	0.023	-0.040	0.051	0.029
RANTES	-0.044	0.047	-0.137	0.049	0.694
TNF-α	0.052	0.046	-0.041	0.144	0.381
VEGF	0.048	0.044	-0.038	0.135	0.155

2.1.2. Subcortical gray matter volume as a function of cytokine levels

The relationship of plasma cytokines with subcortical gray matter volume followed the same pattern as the one observed in cortical white matter. Although regression coefficients were smaller in subcortical gray matter than white matter as shown in Fig 2, they were statistically significant. For example, proinflammatory cytokines IL-2 (β = -0.008; p = 0.012), IL-6 (β = 0.0.017; p = 0.002) and GM-CSF (β = -0.016; p = 0.03) were significantly related lower volumes as shown in table 3. Cytokines showing positive with subcortical gray matter volume included IL-7, IL-9, FGF-basic and VEGF but the association was not significant.

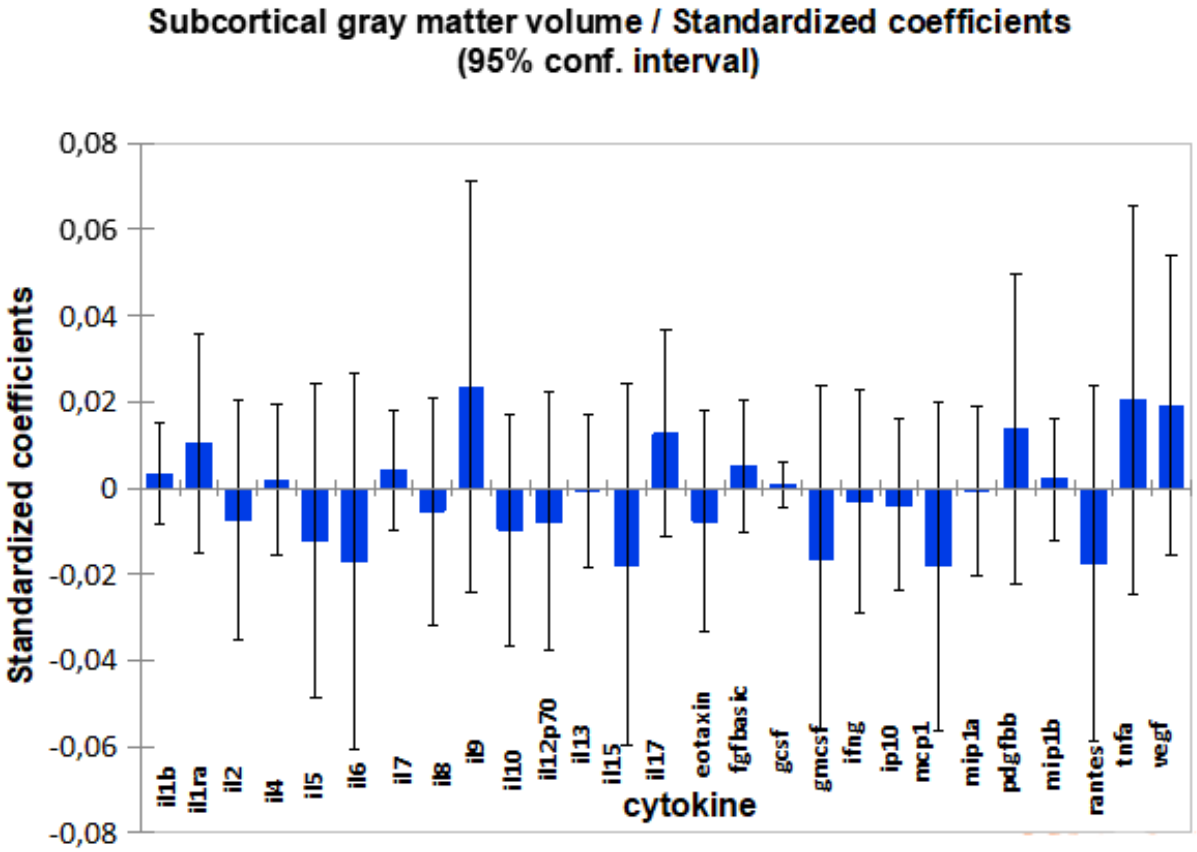

Fig 2. Graphical representation of the magnitude and direction of plasma cytokines impact on subcortical gray matter volume using PLS-R standardized coefficients and 95% confidence intervals. RANTES (- 0.17; - 0.059 – 0.024), MCP-1 (-0,018; -0,056 - 0,020), IL-6 (-0,017; -0,061 - 0,026) and GM-CSF (-0,016; -0,057 - 0,024) were outstanding in their association with lower gray matter.

Table 3: Standardized coefficients for subcortical gray matter volume in multiple GLM

Variable	Coefficient	Std. deviation	Lower bound (95%)	Upper bound (95%)	P value
IL-1β	0.003	0.006	-0.009	0.015	0.046
IL-1α	0.010	0.013	-0.015	0.036	0.304
IL-2	-0.008	0.014	-0.035	0.020	0.012
IL-4	0.002	0.009	-0.016	0.019	0.543
IL-5	-0.012	0.018	-0.049	0.024	0.216
IL-6	-0.017	0.022	-0.061	0.026	0.002
IL-7	0.004	0.007	-0.010	0.018	0.014
IL-8	-0.005	0.013	-0.032	0.021	0.018
IL-9	0.023	0.024	-0.024	0.071	0.419
IL-10	-0.010	0.014	-0.037	0.017	0.89
IL-12p70	-0.008	0.015	-0.038	0.022	0.81
IL-13	-0.001	0.009	-0.018	0.017	0.05
IL-15	-0.018	0.021	-0.060	0.024	0.821
IL-17	0.013	0.012	-0.011	0.037	0.87
Eotaxin	-0.008	0.013	-0.034	0.018	0.064
FGFbasic	0.005	0.008	-0.010	0.020	0.039
GCSF	0.000	0.003	-0.005	0.006	0.388
GMCSF	-0.016	0.020	-0.057	0.024	0.03
IFN-γ	-0.003	0.013	-0.029	0.023	0.927
IP-10	-0.004	0.010	-0.024	0.016	0.072
MCP-1	-0.018	0.019	-0.056	0.020	0.481
MIP-1α	0.000	0.010	-0.020	0.019	0.249
PDGF-BB	0.014	0.018	-0.022	0.050	0.988
MIP-1β	0.002	0.007	-0.012	0.016	0.761
RANTES	-0.017	0.021	-0.059	0.024	0.655
TNF-α	0.020	0.023	-0.025	0.065	0.357
VEGF	0.019	0.017	-0.016	0.054	0.705

Total gray matter volume as a function of plasma cytokines

Seven proinflammatory cytokines, chemokines namley, RANTES, MCP-1, GM-CSF, IL-6, IL-8, IL-15 and were associated with lower gray matter volume, shown in Fig 3. However, only IL-6(β = -0.041; p = 0.019), IL-8 (β = -0.013; p = 0.023) and Eotaxin (β = 0.019; p = 0.035) were statistically significant as shown in Table 4.

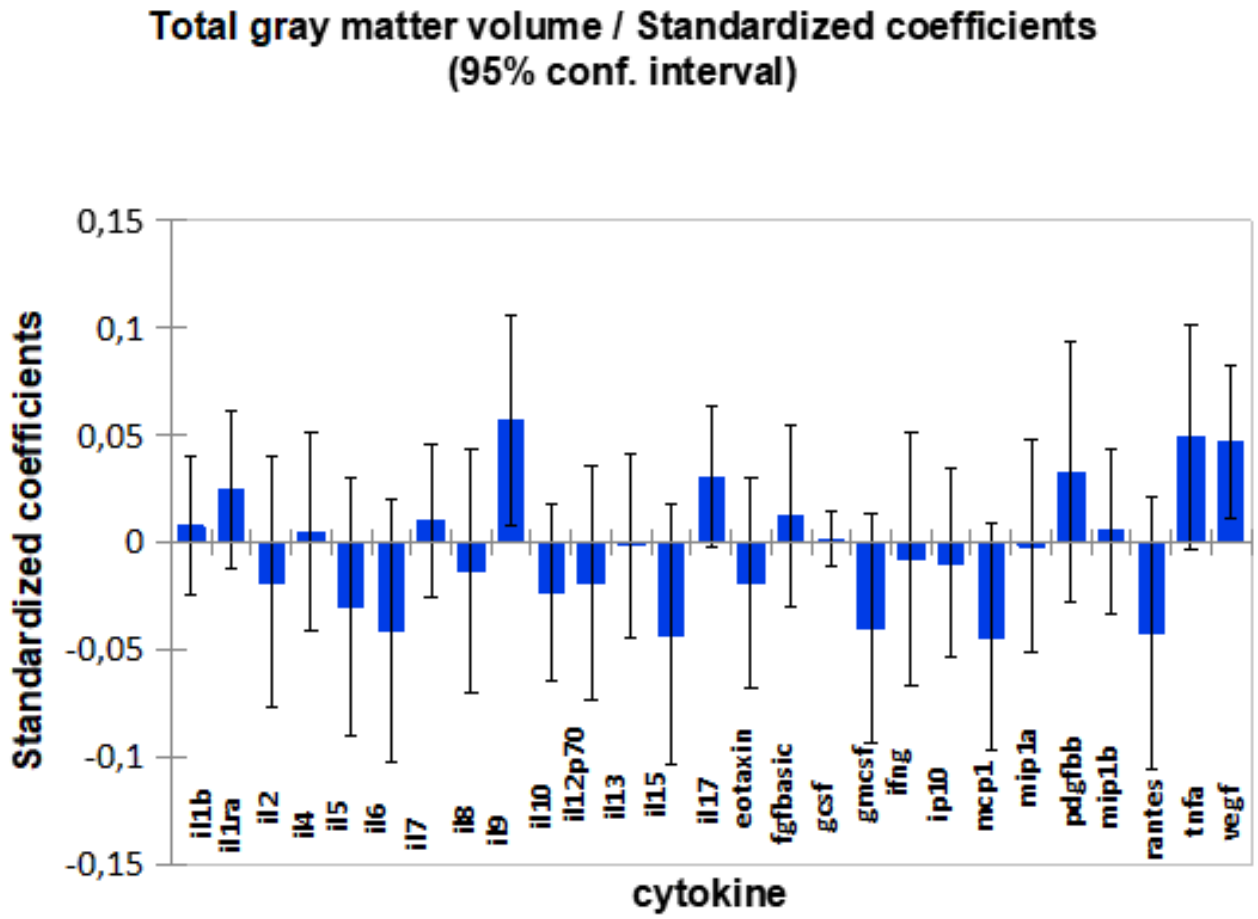

Fig 3. *Graphical representation of the magnitude and direction of plasma cytokines on total gray matter volume using PLS-R standardized coefficients. RANTES (-0,042; -0,106 – 0,021), MCP1 (-0,044; -0,097 - 0,009), IL-6 (-0,041; -0,103 - 0,020) and gmcsf (-0,040; -0,093 - 0,013) were outstanding cytokines related to lower total gray matter volume.*

Table 4: Standardized coefficients for total gray matter vol in a multiple GLM

Variable	Coefficient	Std. deviation	Lower bound (95%)	Upper bound (95%)	P value
IL-1β	0.008	0.016	-0.025	0.040	0.176
IL-1α	0.025	0.019	-0.012	0.062	0.894
IL-2	-0.018	0.029	-0.077	0.040	0.605
IL-4	0.005	0.023	-0.042	0.051	0.730
IL-5	-0.030	0.030	-0.091	0.031	0.444
IL-6	-0.041	0.031	-0.103	0.020	0.019
IL-7	0.010	0.018	-0.025	0.045	0.144
IL-8	-0.013	0.028	-0.070	0.044	0.023
IL-9	0.057	0.025	0.008	0.106	0.826
IL-10	-0.023	0.021	-0.065	0.018	0.349
IL-12p70	-0.019	0.027	-0.073	0.035	0.778
IL-13	-0.001	0.021	-0.044	0.041	0.425
IL-15	-0.043	0.031	-0.104	0.017	0.592
IL-17	0.031	0.016	-0.002	0.063	0.979
Eotaxin	-0.019	0.025	-0.068	0.031	0.035
FGF-basic	0.012	0.021	-0.030	0.054	0.651
GCSF	0.001	0.007	-0.012	0.014	0.297
GM-CSF	-0.040	0.027	-0.093	0.013	0.054
IFN-γ	-0.008	0.029	-0.066	0.051	0.626
IP-10	-0.009	0.022	-0.053	0.035	0.255
MCP-1	-0.044	0.027	-0.097	0.009	0.075
MIP-1a	-0.001	0.025	-0.050	0.049	0.672
PDGF-BB	0.033	0.030	-0.028	0.093	0.532
MIP-1b	0.005	0.019	-0.033	0.044	0.216
RANTES	-0.042	0.032	-0.106	0.021	0.860
TNF-a	0.050	0.026	-0.003	0.102	0.347
VEGF	0.046	0.018	0.011	0.082	0.147

3. Discussion

Brain volume loss is widespread in neurological disease; hence it can be a useful measure of CNS damage and a marker of clinical disease progression and cortical volume loss has been used as a marker to detect overall structural changes during disease process. We examined the relationship between plasma cytokine concentration and cortical white matter and gray matter volumes in untreated HIV infection. Our findings showed that RANTES was the strongest predictor of low white matter and gray matter volumes followed by IL-2, eotaxin, IL-8, MCP-1 and IL-6 and GMSCF respectively. All the cytokines are proinflammatory cytokines and chemokines. Brain volume reduction is thought to be enhanced by proinflammatory environment, which is partly caused by blood derived activated monocytes/macrophages and their associated inflammatory cytokines [34]. Structural brain imaging of patients suspected of HIV associated neurocognitive impairment has revealed a profound impact on grey matter, subcortical regions, and cerebral atrophy [34]

HIV-infected patients have elevated levels of systemic inflammatory markers, due to persistent viral replication, release of progeny virus and viral proteins from infected cells with

clear effects on white matter microstructure [35-36]. Brain structural studies in HIV infection have shown decreased cortical white matter volume [37] and subcortical gray matter with executive deficits [38]. Results of this study clearly demonstrated inverse association on the brain volumes and peripheral inflammation implying that there is a link between systemic levels and HIV associated brain volume alterations. Chemokine-mediated inflammation is known to cause gliosis and dendritic damage in HIV [39] and cortical thinning on MRI was shown to be a sensitive index of declining neurological and immune function in AIDS patients [40] Even mild dendritic loss may lead to behavioral alterations in HIV-associated minor cognitive motor disorder. Our previous studies have shown that plasma proinflammatory cytokines/cytokines, particularly RANTES, IP-10 and IL-2 were strong correlates of HIV- associated cognitive impairment [41].

We have demonstrated that the detected changes in cortical white mater and total gray matter volumes of HIV-infected patients was associated plasma RANTES concentration, but with variation between the brain centres. HIV-related neurocognitive impairment is associated with cerebral atrophy [42]. Therefore, neuroimaging and measures of systemic inflammation like RANTES has a potential be used to diagnose HIV-related CNS injury. In fact, higher levels of RANTES have been observed in brain lesions of HIV patients and have been expressed in several inflammatory diseases of the CNS [43-44].

The monocyte chemoattractant protein-1 (MCP-1/CCL2) showed a negative correlation with both cortical white matter and total grey matter volumes but had greater impact in cortical white matter than total grey matter. The link between MCP-1 and neurodamage was shown in HIV encephalitis (HIVE) and increased in brains of AIDS patients with HIV associated dementia (HAD) [45-46]. It has been shown to play a major role in blood brain barrier disruption (Stamatovic et al., 2005)] and higher levels in CSF were associated with glial dysfunction [47]. This study which has combined quantitative neuroimaging and proteomic technologies has shown that plasma MCP-1 has impact on the degree of brain injury in HIV infection. Higher plasma MCP-1 level has been associated with greater severity and faster cognitive decline in HIV [48]. Therefore, plasma MCP-1 might reflect the risk and progression HIV associated neurodamage. Ongoing brain injury may be subclinical for long periods in HIV infection but the use of inflammatory biomarkers like MCP-1and MRI technologies can quantify the degree of neurological involvement. The advantage of such

approach is that MRI is non-invasive and plasma samples for cytokines are easy to access and the biomarker is to measure.

IL-6 mediated inflammation is known to cause higher incidence of gliosis and dendritic damage to patients with neurological diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) [49-50]. The observed association between elevated plasma IL-6 levels and significantly lower cortical white matter, and grey matter volumes in HIV infection suggests that related processes in PD and AD pathology are involved. Using PLS-R, IL-6 had the greatest impact on cortical white volume reduction followed by total gray matter and subcortical grey matter was the least. However, only the IL-6 associated reduction of subcortical grey matter and total grey matter were significant. This implies that cortical grey matter and white matter loss may be partly linked to IL-6 mediated inflammation, but the magnitude is different. Volumes of cortical grey matter had also been shown to be significantly negatively correlated with IL-6 in Schizophrenia [51]. Higher levels of IL-6 in older adults have been cross-sectionally and longitudinally associated with cortical thinning, cognitive impairment, as well as increased dementia risk [52-53]. These findings vindicate our observations on the link between the cytokine and neurological conditions. However, the consequence of the increase IL-6 on neuronal and glial health and the integrity of cortical volume in the context of HAND need to be further investigated to accumulate sufficient data for use in clinical assessments.

Contrary to the relationship between IL-6, RANTES, MCP-1, IL-2, eotaxin, IL-8, and GM-CSF other cytokines such as IL-9, IL-1α, vascular endothelial growth factor (VEGF) were significantly associated with higher brain volumes. IL-9 is pleiotropic cytokine produced by a variety of cells [54]. It also preferentially promotes CD4+ T cell proliferation and affects both B cell development and function. It has the capacity to actively induce resolution of inflammation [55]. We suggest that association between higher IL-9 and larger brain volumes could be related to the anti-inflammatory role of the cytokine which might have interfered with or inhibited or reduced inflammation-induced neurodamage. It could also have influenced humoral immunity through its effects on B cell development and function. This cytokine might play an important in brain tissue regeneration and repair in the brain in HIV infection [56]. IL-9 mediated interference with inflammation and tissue repair could be further investigated to elucidate the neuroprotective mechanisms in HIV infection and exploring strategies of IL-9 immunotherapy in HIV associated neurocognitive impairment.

The VEGF, produced by many cells is both a potent angiogenic factor and mitogen which has been demonstrated to directly stimulate tumor cells to induce apoptosis resistance [57]. We observed that higher levels of the growth factor were associated with increased brain volumes. The growth factor has been shown to have neurotrophic effect and enhances survival of neurones in some brain regions [58] and higher levels have been detected in HIV associated CNS diseases [59-61]. Another growth factor related to VEGF, platelet-derived growth factor PDGF-BB was also associated with increased cortical white mater and grey matter volumes. The growth factor has demonstrated promotion of neuronal proliferation and reversal of neurotoxicity mediated by HIV-1 Tat [62]. The growth factor is also involved in astroglial scar formation which confines inflammation to the lesion core and protects the neuronal tissue [63]. Although both VEGF and PDGFBB positive influence on brain volumes, PDGFBB influence was less than that of VEGF. We suggest that the results can guide further clinical studies on the neuroprotective role of both PDGF-BB and VEGF. However, it would be interesting to determine the neuropsychological correlates of plasma VEGF and PDGF-BB to assess how these growth factors impact on the neurobehavioural aspects of HIV. Neuropsychological correlates of VEGF would enable us to confirm that the observed links between higher plasma levels of the growth factors and higher cortical white matter and grey matter volumes were a result of a biological process.

Although TNF-α was associated with all increased brain volumes, cortical white matter, subcortical grey matter and total grey matter, the association was only significant in total grey matter. TNF-α is an inflammatory cytokine whose neurotoxic role in HIV associated neurocognitive disorders is well documented [64-67]. The implications of our findings were contrary to the neurodegenerative role of TNF-α as it was associated with increased brain volumes. Our findings seemed to portray a neuroprotective or neurotrophic role of TNF-α in HIV infection. The neuroprotective role of TNF-α have been demonstrated in experimental models [67] and humans with Alzheimer and vascular dementia [6-69]. Therefore, the neuroprotective or neurodegenerative effects of the cytokine depends on other factors that determine the final effect of the cytokine on the CNS. Detrimental or beneficial effect of cytokine depends on level and time of expression among other factors, and this could determine the final effect on CNS. We suggest future studies could perform threshold analysis for direct association studies between the cytokine and brain volumes to determine which levels are protective or neurotoxic.

4. Materials and Methods

4.1. Study participants

A cross-sectional study was conducted on a cohort of 139 HIV positive Xhosa-speaking individuals recruited from primary care HIV clinics in Cape Town, South Africa, that was being investigated for HAND. Inclusion criteria to participate in the study required the following: 1. Age ranging from 18 and 45 years with at least five years of formal education. This age was selected to avoid age-related CNS abnormalities. 2. HIV serostatus, determined by ELISA and then confirmed by Western blot. 3. HIV-1 RNA for plasma viral load measured by the Abbott m2000sp and the Abbott m2000rt analysers (Abbott laboratories, Abbott Park, IL, USA). Exclusion criteria included the following: any major psychiatric condition that could significantly affect cognitive status; confounding neurological disorders including multiple sclerosis and other CNS conditions; head injury with loss of consciousness greater than 30 min; clinical evidence of opportunistic CNS infections and current substance abuse or alcohol abuse as defined by structure interview [70].This study was approved by the Health Research Ethics Committee (HREC) of Stellenbosch University,Ethics Reference #: S17/02/035.

4.2. Plasma cytokine quantification by multiplex assay

Cytokine concentration plasma samples was performed using a 27-plex Biorad Pro Human cytokine assay kit as described earlier (Ruhanya et al., 2021). Quantified cytokines included IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, Eotaxin, basic FG, G-CSF, GM-CSF, IFN-γ, IP-10, MCP1, MIP-1α, MIP-1ß, PDGF-BB, RANTES, TNF-α and GM-CSF, VEGF. Briefly, plasma samples were incubated with antibody-coupled beads. Complexes were washed, incubated with biotinylated detection antibody, and subsequently with streptavidin-phycoerythrin, prior to assessing cytokine concentrations. Standard curves were run together with samples using standard cytokines provided in the kit. Plasma cytokine levels were determined using a multiplex array reader from LuminexTM Instrumentation System (Bio-Plex Workstation from Bio-Rad); plasma samples were run in duplicate and cytokine concentrations were calculated as the average of two independent measures using Bioplex Manager Software (California USA).

4.3. Structural neuroimaging and volumetric analysis

Acquisition of images and volumetric analysis of specific brain regions of interest (ROI) were performed according to the methods described earlier [72-73]. Briefly high field MRI was used to acquire T1 weighted 3-dimensional magnetization-prepared rapid acquisition gradient echo (MPRAGE) images which provide contrast for segmenting, gray matter, white matter, and CSF. The data obtained from MPRAGE was used for volumetric quantification of ROI using Freesurfer software suite (v5.1) (Martinos Center, Harvard University, Boston, MA, USA. http: //surfer.nmr.mgh.harvard.edu). Briefly, MP-RAGE scans were transformed into a template space with the skull stripped and the brain segmented into white matter, gray matter, and ventricles. The brain was further segmented into subcortical and cortical ROIs. Previous studies identify that these ROIs are impacted by HIV neuropathogenesis [74-77].

4.4. Statistical analysis

Stata statistical package version 12.1 (StataCorp, College Station, Texas, USA, 2011) was used for all statistical analysis. Generalized linear modelling was used to determine the association between brain regions of interest and plasma cytokine levels. Partial least squares (PLS) approach which uses multiple linear regression analysis to find the direction of maximum covariance between plasma cytokine levels and volumes of ROI, was used (Kamat et al., 2012). Statistical significance was determined as p value < 0.05.

5. Conclusions

The observation that some proinflammatory cytokines were associated with increased cortical white matter and grey matter volumes suggest that these cytokines such as RANTES, MCP-1 and IL-6 are involved in neurodegenerative pathological processes in HIV infection. The other group of cytokines including IL-9, VEGF and PDGF-BB which were associated with increased cortical white matter and grey matter volumes suggest that these cytokines have neuroprotective or neurotrophic role against inflammation mediated neuronal damage. We suggest that those cytokines which were strongly associated with reduced brain volumes like RANTES are potential biomarkers for HAND whilst those associated with increased brain volumes could be explored in search for immunotherapeutic approaches to HAND. However, that data need to be collected in longitudinal studies in order observe to changes in both cytokine levels and imaging data over time. We also suggest that threshold analysis be done to determine the cut off values associated with each clinical outcome.

Author Contributions:

Study conception and design: VR, SE, RP. Acquisition of data: VR, RP, JJ, SS, GN. Analysis, and interpretation of data: VR, GN, SE, RG, GJ. Drafting of manuscript and Critical revision: all authors.

Funding: This study was supported by the Poliomyelitis Research Foundation (PRF) and the South African Medical Research Council (SAMRC) Collaborating Centre for HIV-1 Laboratory Research.

Institutional Review Board Statement: This study was approved by the Health Research Ethics Committee (HREC) of Stellenbosch University, Ethics Reference #: S17/02/035.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest: Declare conflicts of interest or state "The authors declare no conflict of interest.

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Paper 6: Ruhanya, V.; Jacobs, G.B.; Paul, R.; Joska, J.; Seedat, S.; Nyandoro, G.; Engelbrecht, S.; Glashoff, R.H. Plasma cytokine biomarker cut-off values for HIV-associated neurocognitve impairment in adults. Viral Immunology: http://mc.manuscriptcentral.com/viral(Viral Immunology: http://mc.manuscriptcentral.com/viral (Accepted for publication – Under production))

The author contribution to the published papers were as follows: Conception and design of the experiments, Performed the experiments, Data analyses and writing of manuscript.

Plasma cytokine biomarker cut-off values for HIV-associated neurocognitve impairment in adults.

Ruhanya, V.; Jacobs, G.B.; Paul, R.; Joska, J.; Seedat, S.; Nyandoro, G.; Engelbrecht, S.; Glashoff, R.H. Plasma cytokine biomarker cut-off values for HIV-associated neurocognitve impairment in adults.

Summary

Diagnosing HIV associated neurocognitive impairment in most high-burden, but resource-constrained settings is difficult due to the unavailability of specialist neurologists and neuropsychologists in primary health care centers. New tests that are easy to perform, based on virological and host immune response biomarkers may be valuable in the diagnosis of HAND. The receiver operator characteristics (ROC) curve analysis was used to investigate the diagnostic accuracy of threshold/cut-off concentrations for peripheral lymphocyte proviral load and plasma biomarkers as diagnostic candidates for neurocognitive impairment in 133 HIV infected individuals using Global Deficit Scores as a clinical gold standard. Forty-five (33.83%) of the participants had HIV associated neurocognitive impairment, with 17.29% being mildly impaired 16.54% moderately impaired. IL-2 had the best performance as a diagnostic tool for neurocognitive impairment with sensitivity of 67% and specificity of 52% while the lowest performance was IL-6 with 65% sensitivity and 39% specificity. MIP-1α had the highest precision for the cut-off value as indicated by the narrow 95% CI (2.23 – 3.27) followed by IL-2 with 95% CI (3.02 – 5.12). RANTES had least precision as shown by the widest 95% CI (135 – 9487.61). For clinical markers of HIV diagnosis and monitoring, lymphocyte proviral load cut-off value of 145 genome copies/million cells had the highest accuracy with 60% sensitivity and 51% specificity. Plasma viral load had imperfect balance of 46% sensitivity and 78% specificity. The study demonstrated low to medium diagnostic accuracies for plasma cytokine biomarker cut-off values for defining neurocognitive impairment in PLWH.

Introduction

Studies have shown that HAND pathogenesis is determined by a complex interplay between cellular targets, viral factors and the immune response (7). HIV entry into the brain and infection of susceptible cells such as macrophages, microglia and astrocytes result in uncontrolled release of cellular neurotoxins and proinflammatory cytokines. Active replication of HIV in the brain releases viral proteins such as gp120, Tat, Nef and Vpr which function as neurotoxins. Together with inflammatory cytokines, viral proteins promote neuronal apoptosis and cell death, inhibition of axonal growth, neural dysfunction, astrocyte necrosis, and increased permeability of the blood brain barrier (BBB) (13, 18). Hence there is a diagnostic and disease-tracking potential for cytokines in HIV associated neurocognitive impairment.

Detailed clinical assessments are the gold standards for detecting neurocognitive impairment and have clearly defined the scope and severity of HAND (22, 24). Diagnosis of cognitive impairment is performed by HIV neurologists and neuropsychologists (31) after exclusion of other causes. Diagnosing the disease in most high-burden, but resource-constrained settings is difficult due to the unavailability of these specialists in primary health care centers. New tests that are easy to use, based on host immune response biomarkers may be valuable in aiding the prognostic diagnosis of HAND. Currently, there is no single laboratory test able to reveal the underlying neuropathology of HAND, and which can aid in the diagnosis in clinical settings There is need for balance or complementarity between behavioural, clinical and biological phenotypes to confirm the diagnosis of HAND. Biomarkers also become useful in the era of ART where psychometric performance testing is challenging in identifying patients with more subtle neurocognitive deficits (5).

How cytokine levels impact cognition in HIV infection is not well understood, however plasma concentrations have been linked to cognitive impairment in other neurological diseases like multiple sclerosis and Alzheimer's disease where inflammation is thought to worsen the underlying disease process (6). Can we use plasma cytokine levels to predict the clinical outcome of HAND? It has been demonstrated that inflammatory cytokine levels were significantly higher in patients with HAND and were directly correlated with proviral HIV DNA (1). In the past 25 years, studies have identified several inflammatory pathways that are associated with HIV neuropathogenesis and have established the neuroinflammatory nature of HIV infection in the CNS (1, 11, 35). Evidence of CNS-migrating immune cells and

inflammatory cytokines produced by them has also been demonstrated in HIV-associated neuroinflammation (14). Since plasma concentrations of some cytokines has been shown to be predictive of disease progression, they have the potential to be used as biomarkers in prognosis and clinical management (27). However, the fundamental criteria for use of biomarkers in clinical practice are accuracy and optimal cutoff point selection. In this case diagnostic accuracy is used to define how close the result of the cytokine biomarker measurement (index test) is to the true clinical disease situation (9). This study utilizes high through put Luminex assays and statistical approaches to determine diagnostic accuracy of cytokine cut-off values as well as virological and cellular cut-off values for HIV associated neurocognitive impairment.

Methods

A cross-sectional study was conducted on 133 HIV positive Xhosa-speaking individuals recruited from primary care HIV clinics in Cape Town, South Africa. Inclusion criteria to participate in the study required the following: 1. Age ranging from 18 to 45 years with at least five years of formal education. This age range was selected to avoid age-related CNS abnormalities. 2. HIV serostatus, determined by ELISA and then confirmed by Western blot. 3. HIV-1 RNA for plasma viral load measured by the Abbott m2000sp and the Abbott m2000rt analyzer. Exclusion criteria included any major psychiatric condition that could significantly affect cognitive status, clinical evidence of opportunistic CNS infections (toxoplasmosis, progressive multifocal, leukoencephalopathy, neoplasms); current substance, abuse or alcohol abuse as defined by structured interview. (26, 28, 30). This study was approved by the Health Research Ethics Committee (HREC) of Stellenbosch University, Ethics Reference #: S17/02/035. The study was conducted according to South African Guidelines for Good Clinical Practice and the Medical Research council, Ethical Guidelines for Research in line with the guidelines and principles of the international declaration of Helsinki.

Neuropsychological evaluation

A battery of cognitive tests sensitive to deficits associated with HIV was administered to all participants as described by Paul et al., 2014 (26). Scores for individual cognitive tests were averaged to generate a mean T-score and deficit for each of the cognitive domains to calculate a global T-score and global deficit score (GDS). GDS was then used to categorize cases as either impaired or unimpaired (15). GDS of 0.5 or more was regarded as

neurocognitive impairment. GDS < 0.5 considered unimpaired, mildly impaired 0.5 ≤ GDS < 2, moderately impaired 2 ≤ GDS < 3.5 and severely impaired GDS ≥ 3.5. The categorization/classification for neurocognitive impairment, using GDS was determined in the context of established measures of HIV associated neuropsychological impairment called, the Frascati criteria (8,7).

Plasma cytokine quantification by multiplex-bead assay

The cytokine concentrations in plasma samples were determined using a 27-plex kit as describe earlier in our laboratory (29).Quantified cytokines included IL-1β, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, Eotaxin, basic FG, G-CSF, GM-CSF, IFN-γ, IP-10, MCP1, MIP-1α , MIP-1ß, PDGF-BB, RANTES, TNF-α and GM-CSF , VEGF.

Statistical analysis

STATA statistical package version 12.1 (StataCorp, College Station, Texas, USA) was used for data analysis. The receiver operator characteristics (ROC) curve analysis procedure was used to assess the diagnostic accuracy of individual biomarkers for neurocognitive impairment in HIV infection. Optimal cut-off values and associated sensitivities and specificities; in view of statistical assurance of internal validity, consistency and reliability two statistical approaches were deployed, empirical estimation (21) and Youden's index (10, 33). The Liu method maximizes the product of the sensitivity and specificity; the Youden method maximizes the sum; and the nearest to (0,1) method finds the cutpoint on the ROC curve closest to (0,1) (the point with perfect sensitivity and specificity). Non-parametric ROC analyses were done to determine the area under the roc curve (AUC) and the optimal cutoff point, as defined by Zweig & Campbell (36). Cutoff points for maximum efficiency and based on ROC curve were also computed.

3. Results

3.1 Cohort participants' neurocognitive profile

The participants had a range of neurocognitive deficits. The majority 88 (66.17%) were unimpaired and 45 (33.83%) were impaired. In the impaired group, 23 (17.29%) were mildly impaired/asymptomatic neurocognitive impairment and 22 (16.54%) were moderately impaired and no participants were severely impaired. Fig 1 shows distribution of participants according to severity of neurocognitive impairment.

3.1. Cytokine biomarker cut-off/ threshold values using the empirical method

The reference diagnosis for HIV associated neurocognitive impairment was deficit in neurocognitive function measured by global deficit scores. Cytokine cut-off/threshold levels were then considered as novel diagnosis/ classification markers for neurocognitive impairment. Cut-off values for different cytokines differed as did their specificity and sensitivity as shown in Table 1. The GM-CSF had the lowest cut-off value at 1.17 pg/ml and RANTES had the highest cut-off value of 4676.25 pg/ml. IL-2 had the best performance as a diagnostic tool for neurocognitive impairment with sensitivity, Se (%) of 67% and specificity, Sp (%) of 52%, while the lowest performance was IL-6 with 65% sensitivity and 39% specificity. Of the biomarkers displaying the most accurate cut-off points, MIP-1α had the highest precision for the cut-off value as indicated by the narrow 95% CI (2.23 – 3.27), followed by IL-2 with 95% CI (3.02 – 5.12). RANTES had least precision as shown by the widest 95% CI (135 – 9487.61). Figure 2 shows the graphical presentation of cytokine cut-off values and associated specificities and sensitivities

3.1.2. Virological and cellular biomarker cut-off or threshold values

Proviral load had the highest accuracy as indicated by the balanced sensitivity and specificity with a cut-off value of 145 copies per million cells. However, the cut-off values were not statistically significant ($p = 0.13$). The CD8 absolute count cut-off value of 819 cells/µl was significant ($p = 0.000$), with sensitivity, specificity, and ROC area under curve (AUC) all above 50% as shown in table 2 below.

All the cellular and virological biomarker had ROC area under curve (AUC) of above 50% indicating a higher chance for the cut-off value to distinguish between impaired and unimpaired participants. Plasma viral load had the largest ROC AUC of 62%. (Fig 3) shows viral and cellular cut-off values and associated specificities and sensitivities

3.2. Cytokine biomarker cut-off/ threshold values using Youden Method

Using the Youden method, the cut-off values and accuracy for the cytokine biomarkers were slightly higher than the empirical method but the difference was not significant. However, this analysis determined additional measures like efficiency, Youden index (J), likelihood ratios and predictive values for sample prevalence as shown in table 3. Although IL-2 had the highest accuracy, MIP-1 had highest Youden Index, efficiency, as well as AUC. Using this

analysis variability measures of accuracy (sensitivity and specificity) for different cut-off values adjusted for sample prevalence (Wilson) were determined. IL-2 which had the most accurate cut-off point estimation had an adjusted sensitivity of 66.7% (95% CI: 52.5 to 78.3) and specificity was 52.3% (95% CI: 42.0 to 62.4). Eotaxin which had a perfect balance between two measures of accuracy for the cut-off value had sensitivity of 60.4% and specificity of 58.0% (95% CI: 47.5 to 67.7). IP-10 and GM-CSF had cut-off values with the least accuracy with Sensitivity of 45.8% (95% CI: 32.6 to 59.7), specificity of 63.6% (95% CI: 53.2 to 72.9) and Sensitivity of 45.8% (95% CI: 32.6 to 59.7), specificity of 70.5% (95% CI: 60.2 to 79.0) respectively. In both Empirical and Youden methods there were no significant differences between the measures of accuracy in the cut-off values. Table 3 shows cut-off values and their associated diagnostic accuracies using Youden method.

Discussion

Clinical diagnosis of HIV-associated Neurocognitive Disorder (HAND) typically requires a battery of lengthy neurocognitive assessments. Although GDS has demonstrated excellent accuracy for diagnosis of neurocognitive impairment compared to clinical ratings, it has a limitation of multicolinearity with clinical ratings because they are generated from the same neuropsychometric scores (25). These tests are also time consuming, requring face-to-face interaction between assessor and patient. There is need for independent neuropathological biomarkers to avoid this problem. Interpretation of these biomarkers would also be less intensive than GDS. We evaluated 10 plasma inflammatory cytokine markers cut-off values and peripheral markers of HIV infection, for their diagnostic accuracy using GDS as a clinical reference diagnostic standard.

Considering virological and immunological parameters, peripheral monocyte depleted (CD14-) HIV proviral load demonstrated the highest accuracy. The test had an optimal cut-off /threshold value of 145 genome copies per million cells with a balance between sensitivity 60% and specificity of 51%. Ideally, a good diagnostic test should have both high sensitivity and high specificity (32) but it may not always be the case due to disease spectrum and patient variability. We identified a low discriminative power of lymphocyte proviral DNA as a diagnostic test for HAND (AUC = 0.56). Our findings have extended the current understanding by exploring the possibility of using intracellular reservoir as a diagnostic tool for neurocognitive impairment in HIV infection. A previous study conducted using monocyte (CD14+) proviral DNA determined the optimal cut-off value for neurocognitive impairment

to be 45 copies per million cells with 86% sensitivity and 70% specificity (31). Both our study and Valcour et al., were conducted in treatment naive patients and GDS was used as reference standard for diagnosis of HAND. However, the disease spectra which directly influences diagnostic accuracy was different in both studies and therefore it is difficult to compare the diagnostic accuracy of the two tests in discriminating HAND patients. The seemingly low diagnostic accuracy shown in our study may have emanated from high number of non-impaired and predominantly low to mild disease zone of the spectrum. Diagnostic accuracy of an index test is not constant but varies across clinical contexts, disease spectra and even patient subgroups (20). Although monocyte (CD14+) derived HIV DNA role in HAND pathogenesis is well established, lymphocytes including CD4+ T-cells represent the largest peripheral reservoir of HIV DNA (12). and shown to be a predictor of cognitive impairment in HIV infection (15, 29). We also believe that therapeutic strategies targeting the largest reservoir would improve clinical outcomes of HAND and therefore may potentially be used in predicting progression or remission of the disease.

Although plasma viral load threshold value had mild overall, discriminative power (AUC = 0.62) it had a very low sensitivity of 46% implying that its ability to correctly identify those participants with neurocognitive impairment was not good. Despite low sensitivity, plasma viral load diagnostic cut-off value was highly specific (78%) and therefore can be important in diagnosis, particularly in cases where treatment has not been initiated. Further studies may be required with higher sensitivity so that balanced trade-off between sensitivity and specificity for plasma viral load cut-off point suitable for diagnosis is obtained (23).

Cytokines have been implicated in many diseases caused by inflammatory processes including Alzheimer's diseases, where neurological outcomes were found to be associated with abnormal cytokine profiles (2). However, a limited number of studies have investigated clinical cytokine cut-offs to support diagnosis (19). We determined clinical diagnostic cut-off values for 10 plasma proinflammatory cytokines that have shown potential to be predictive of HIV associated neurocognitive impairment (29). We noted that diagnostic accuracy of the 10 cytokine biomarkers varied from moderate to low levels. Clinical validity of biomarkers as screening or diagnostic tools is measured on how well they are able to distinguish between those with neurocognitive impairment from those without (16). Our findings indicated that the ability of the 10 cytokines to correctly identify (sensitivity) those participants with neurocognitive impairment ranged from 46% to 67% and the ability to identify truly non-

impaired (specificity) participants ranged from 39% to 70%. Our study is one of a few exploratory studies that determined cut-off values for cytokines as a prognosis for HIV associated neurocognitive impairment.

IL-2 had an optimal cut-off value with a balanced trade-off between sensitivity and specificity for discriminating between impaired and non-impaired participants. The sensitivity of its cut-off value to separate impaired from non-impaired though moderately high, was above the rest of cytokine biomarkers examined. Cytokine levels vary greatly among individuals and their release and effects vary by activating signals, specific cell targets, physiological environment for example serum or plasma state and fitness levels (4, 23). This variability affects both cut-off values and diagnostic accuracy thereof. In our accuracy study we compared the results of an index test (cytokine cut-off value and reference test (GDS). The discrepancy between the two is assumed to arise from error in the index test (cytokine biomarker cut-off). In this case it is assumed that GDS is 100% accurate as a clinical reference standard, which rarely holds true in practice. Previous studies showed GDS sensitivity ranging from 60% to 83% and specificity ranging from 77% to 92% (3, 25) compared to clinical ratings. Therefore, the accuracy values of cytokine biomarkers may have been underestimated, since it can never be deemed to perform better than the reference standard GDS. We recommend additional studies be conducted on diagnostic accuracy of cytokine biomarkers comparing with both GDS and clinical ratings as well as neuroimaging findings.

Interestingly, two markers RANTES and IP-10 that demonstrated a strong association with neurocognitive impairment in HIV infection (28) had low sensitivity of 46% for both and 72% and 64% specificity respectively. Sensitivity and specificity do not depend on the prevalence and thus are considered measures of intrinsic measures of diagnostic accuracy (36). A closer look at the diagnostic cut-off values of IP-10 and RANTES showed that there was high variability as shown by wide 95% confidence intervals of the cut-off values. Sensitivity and specificity for a quantitative classifier are dependent on the variability of the cut-off value chosen and the spectrum of disease (21). In our study, the clinical reference standard categories ranged from non-impaired, mildly impaired to moderately impaired (7). This could have also introduced spectrum bias (24) since there was no severe forms of neurocognitive impairment in our cohort. Differences in disease severity may lead to

different estimates of diagnostic performance of an assay. Therefore, future studies should attempt to include a broader spectrum of cognitive impairment categories.

Cytokine release, concentrations and subsequent effects on target anatomical sites vary based upon activating signals like stress, fitness level nutrition and environment (35). Although our recruitment controlled for opportunistic infections which could influence the clinical diagnosis of cognitive impairment and cytokine levels, other variables not investigated in the current study might have impacted on cut-off values and related diagnostic accuracies. In addition, diagnostic parameters obtained in this study pertain to treatment-naive indviduals. Therefore, they cannot be extrapolated to indviduals on combination antiretroviral therapy (cART). In order to consider potential utility of cytokine cut-off values in defining cognitive impairment HIV infection, future studies need to be conducted in treated indviduals as well.

Besides sensitivity and specificity, other basic statistical and fundamental techniques used in diagnostic testing and medical decision making, like predictive values, likelihood ratios, Youden Index, ROC, AUC were used in this study. A comparison of the empirical method and the non-parametric methods showed that the cut-off values for cytokines and associated diagnostic accuracies were almost the same. This indicates that the values were true based on the disease spectrum of neurocognitive impairment and variability in patient cytokine levels. In clinical practice, the clinician/neuropsychologist would want to know, how good the test is at predicting neurocognitive impairment. Therefore, we determined the proportion of participants with a positive cytokine result who had neurocognitive impairment (positive predictive function). Positive predictive values for all the cut-off values were very low, indicating that the tests had low predictive power. Negative predictive values were moderate, ranging from 67% to 74% indicating that the cut-off values were better at identifying participants without neurocognitive impairment. However, predictive values depend on the prevalence of disease (25) and therefore cannot be generalized to other settings with different disease prevalences, especially if the prevalences do not reflect population prevalences.

Conclusions

The study determined low to medium diagnostic accuracies for plasma cytokine biomarker cut-off values for defining neurocognitive impairment in people living with HIV (PLWH). IL-2, and lymphocyte proviral load had the highest diagnostic discriminatory ability. Establishing an algorithm combining the biomarkers could improve diagnostic accuracy.

Further investigations are needed to generate more data on diagnostic performance of these biomarkers, including in ART-treated individuals. Obtaining sensitive and specific tests for cognitive impairment in HIV infection using cytokines is significant because they easy measure. Standard methods are costly as they require highly specialised physicians who are not affordable for clinical diagnosis and monitoring of the disorder resource limiting settings.

Acknowledgements

We would like to Cladnos Mapfumo for statistical analyses

Authors' contributions

Study conception and design: RG. VR. SE. RP. Acquisition of data: VR. SN. RP. JJ. SS. Analysis and interpretation of data: VR. GN. SE. RG. GJ. Drafting of manuscript and Critical revision: all authors

Authors' disclosure statement

The authors declare no competing of interest.

Funding

This study was supported by the Poliomyelitis Research Foundation (PRF: SE. VR). National Research Foundation (NRF: SE) and the South African Medical Research Council (SAMRC) Collaborating Centre for HIV-1 Laboratory Research (SE).

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Figure Legends

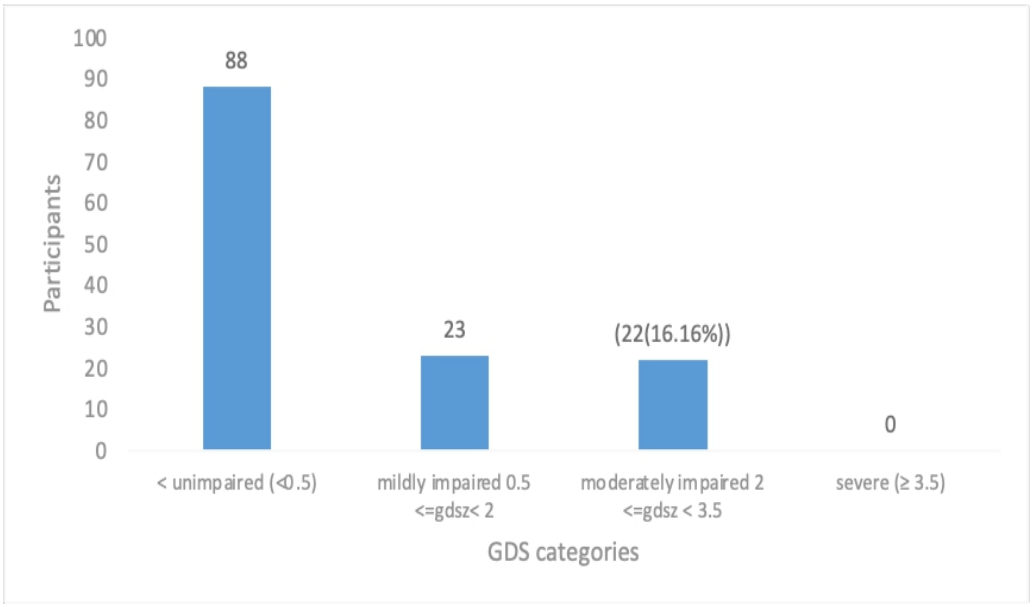
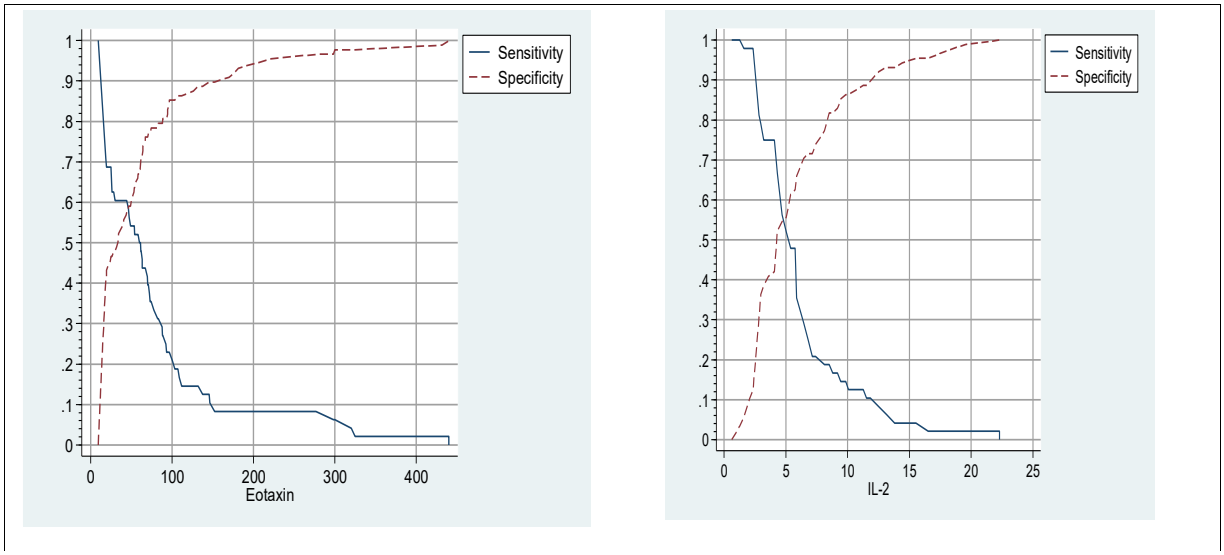


Fig1. Spectrum of cognitive impairment in study participants ranging from unimpaired to mildly impaired.



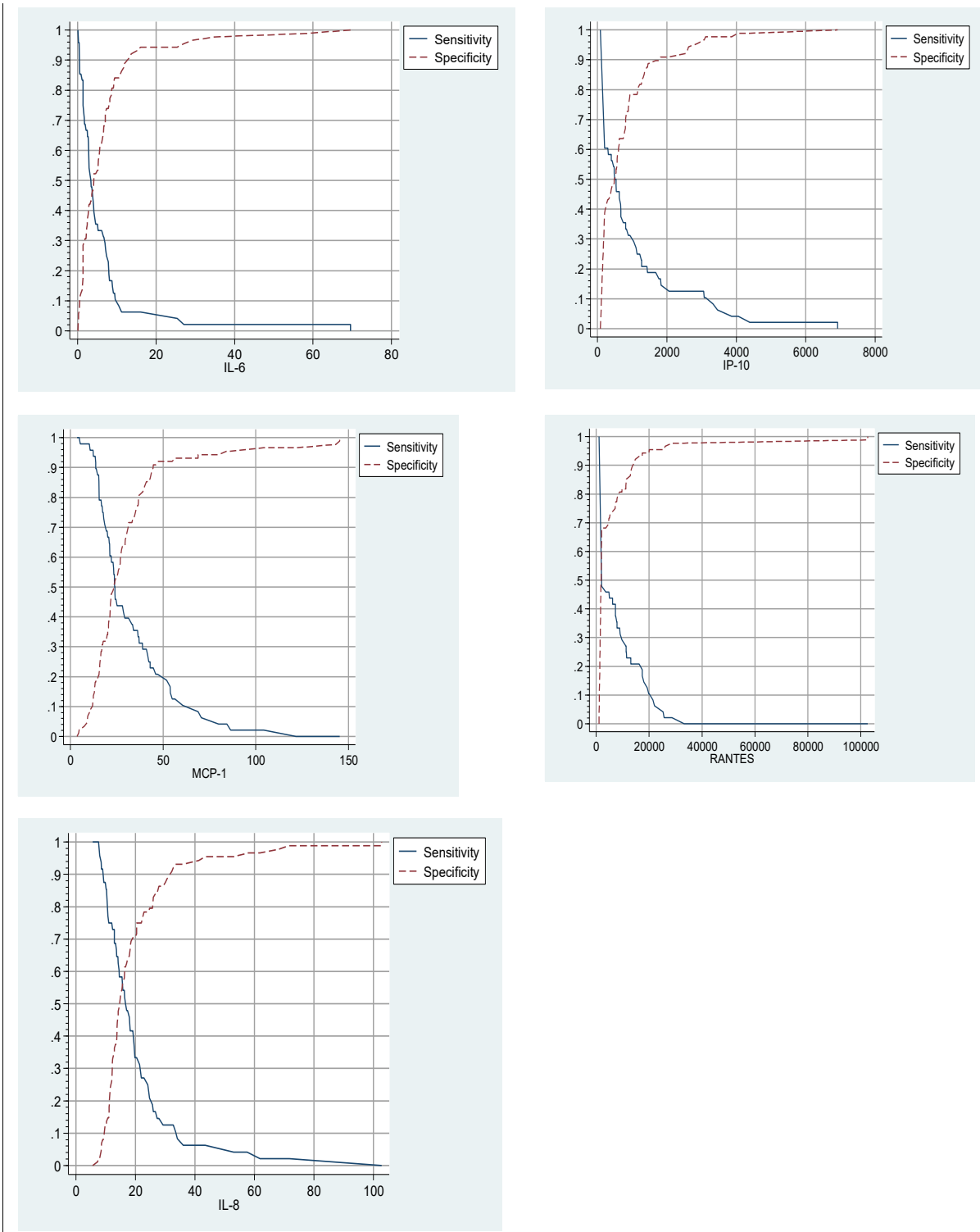


Fig 2. Graphical presentation of optimal cytokine cut-off values and associated sensitivities and specificities

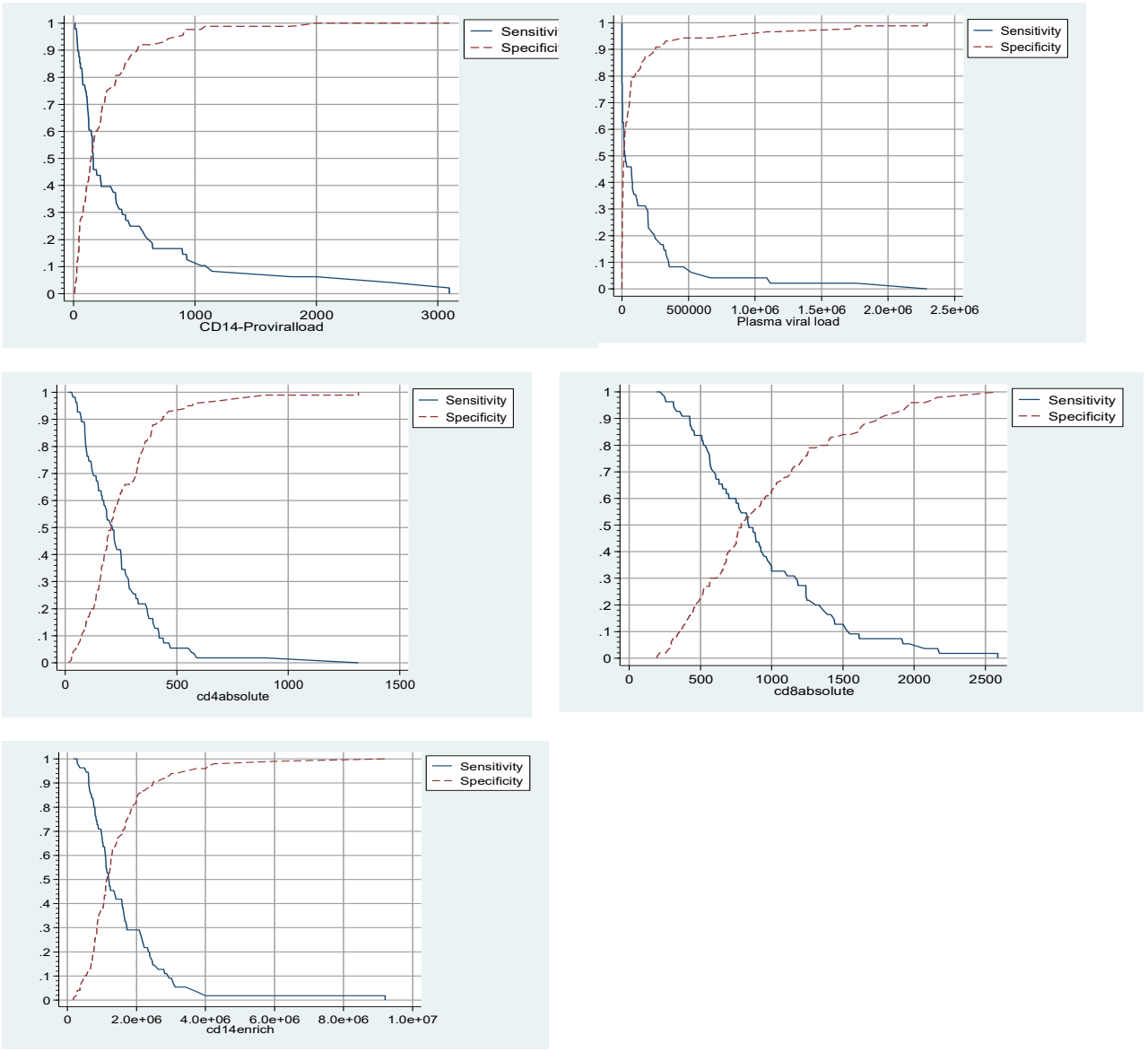


Fig 3. Optimal cut-off values for viral and cellular biomarkers for HIV associated neurocognitive impairment and associated sensitivity and specificity

Table 1: Accuracy of different cytokine cut-off values for neurocognitive impairment

cytokine	Cut-off value	95% CI	Se (%)	Sp (%)	ROC	p-value
IL-2	4.07	3.02 – 5.12	67%	52	59	0.000
IL-8	16.26	13.06 – 19.46	52%	61	57	0.000
MIP-1α	2.75	2.23 – 3.27	54	67	61	0.000
MCP-1	21.85	9.01 – 34.69	60	48	54	0.000
Eotaxin	43.53	14.67 – 72.40	60	58	59	0.003
RANTES	4676.25	135 – 9487.61	46	72	59	0.057
IP-10	627	270.80 – 983.19	46	64	55	0.001
G-CSF	38.13	42.21 – 72.05	65	47	56	0.028
GM-CSF	1.17	0.901- 1.438	46	70	58	0.000
IL-6	2.55	-1.228 - 6.328	65	39	0.52	0.186

Table 2. Viral and cellular cut-off values

Biomarker	Cut-off value	Se (%)	Sp (%)	ROC	95% CI	p-value
CD14– Proviral load	145	60	51	0.56	-42.99- 332.99	0.13
Plasma viral load	68258	46	78	0.62	24478.67- 112037.3	0.002
Monocyte count	1310000	45	64	0.55	630459 - 1989541	0.000
CD4 absolute count	217	49	56	0.53	659.62 - 978.37	0.000
CD8 absolute count	819	55	53	0.54	630459 - 1989541	0.000

Table 3. Analysis of cut-off values, the Youden method

Cytokine	Cut-off	Se (%)	Sp (%)	AUC	Eff (%)	Youden J (%)	LR+	1/LR-	PV+ (%)	PV-(%)
IL-2	4.32	66.7	52.3	0.57	57.4	18.9	1.40	1.57	43.2	74.2
IL-8	16.35	52.1	61.4	0.54	58.1	13.4	1.35	1.28	42.4	70.1
Eotaxin	44.48	60.4	58.0	0.59	58.8	18.4	1.437	1.464	43.9	72.9
MCP-1	22.06	60.4	47.7	0.56	52.2	8.14	1.156	1.206	38.7	68.9
RANTES	2102.45	47.9	68.2	0.59	61.0	16.1	1.506	1.309	45.1	70.6
IP-10	629.24	45.8	63.6	0.53	57.4	9.47	1.26	1.175	40.7	68.3
MIP-1α	2.83	54.2	67.0	0.59	62.5	21.2	1.644	1.463	47.3	72.8
gcsf	39.63	64.6	46.6	0.50	52.9	11.2	1.209	1.316	39.7	70.7
gmcsf	1.25	45.8	70.5	0.56	61.8	16.3	1.551	1.301	45.8	70.5
IL-6	2.67	64.6	38.6	0.47	47.8	3.22	1.052	1.091	36.5	66.7

CHAPTER 4

DIVERSITY AND HIV-ASSOCIATED NEUROCOGNITIVE IMPAIRMENT

Two manuscripts included in this chapter, submitted for publication in the Journal of Neurovirology.

The text format is as required by the respective journals where submitted.

This chapter uses in-depth bioinformatic analyses to identify genetic markers of neurocognitive impairment on HIV-1 subtype C tat and vpr genes. We also investigated on how these markers impact on plasma viral load, proviral load and plasma cytokines

Paper 7: HIV-1 subtype C Tat exon-1 amino acid residue 24K is a signature for neurocognitive impairment

Ruhanya, V.; Jacobs, G.B.; Paul, R.; Joska, J.; Seedat, S.; Nyandoro, G.; Glashoff, R.H.; Engelbrecht, S. HIV-1 subtype C Tat exon-1 amino acid residue 24K is a signature for neurocognitive impairment.

The author contribution to the published papers were: Contributed towards conceptualisation, design of the experiments, performed the laboratory experiments, data analysis and writing of manuscript.

HIV-1 subtype C Tat exon-1 amino acid residue 24K is a signature for neurocognitive impairment.

Ruhanya, V.; Jacobs, G.B.; Paul, R.; Joska, J.; Seedat, S.; Nyandoro, G.; Glashoff, R.H.; Engelbrecht, S. HIV-1 subtype C Tat exon-1 amino acid residue 24K is a signature for neurocognitive impairment

Summary

Variation and differential selection pressure on Tat genes have been shown to alter the biological function of the protein, resulting in pathological consequences in a number of organs including the brain. We evaluated the impact of genetic variation and selection pressure on 147 HIV-1 subtype C Tat exon1 sequences from monocyte-depleted peripheral lymphocytes on clinical diagnosis of neurocognitive impairment. Genetic analyses identified two signature amino acid residues, Lysine at codon 24, (24K) with a frequency of 43.4% and Arginine at codon 29 (29R) with a frequency of 34.0% in individuals with HIV associated neurocognitive impairment. The analyses also revealed two signature residues, Asparagine, 24N(31.9%) and Histidine, 29H (21.3%) in indviduals without neurocognitive impairment. Both codons, 24 and 29 were associated with high entropy but only codon 29 was under positive selection. The presence of Signature K24 increased by 2.08 times the risk of neurocognitive impairment, 3.15 times higher proviral load and 69% lower absolute CD4-T cell count compared those without the signature. The results support a linkage between HIV-1 C Tat N24K polymorphism, proviral load, immunosuppression, and neurocognitive impairment. The signature may induce more neurotoxic effects, which contributes to establishment and severity of HIV-associated neurocognitive impairment.

Key words: *HAND, VESPA, entropy, Lysine, Arginine, Asparagine, Histidine*

1.0. Introduction

Tat (trans-acting activator of transcription) protein is a 101 amino acid protein which plays an important role in HIV-1 replication through its interaction with the long terminal repeat (LTR) promoter (Jeang et al. 1999; Roy et al. 2015). Tat interacts with RNA polymerase II (RNAPII) to elongate viral RNA (Rossenkhan et al. 2013). It enhances latently infected CD4+ T cells to actively produce virus, recruiting transcription factors to the 5' LTR to produce full length mRNA. Tat protein can be secreted by infected cells, can be taken up by uninfected bystander cells (Frankel and Pabo 1998), alters cellular gene expression and is an important mediator of HIV-1 disease progression including HIV-associated neurocognitive disorders (HAND) which affects 42.6% of infected people globally (Wang et al. 2020).

While the precise mechanism of the pathogenesis of HIV associated neurocognitive disorders (HAND) is not completely elucidated, Tat contribute to the pathogenic process through various ways (Dahiya et al. 2015; Maubert et al. 2015; Clifford 2017; Spector et al. 2019). The tat protein plays an important role in neuropathogenesis by recruiting HIV infected peripheral lymphocytes into the CNS where the virus induces neurotoxicity; synaptic loss; and oxidase stress and host proinflammatory gene expression (Spector et al. 1991; Albini et al.1998; Kimet al. 2008; Agrawal. 2012; Pu et al. 2003; Buonaguro et al.1992).

Studies have shown that the differential neuropathogenesis observed in HAND may be related to genetic variations / mutations and biological activity of tat protein (Li et al. 2012; Ronsard et al. 2014). Some studies have shown that specific tat amino acid variations have a role in promoting tat-mediated apoptosis on neural cells and modulating the expression of endothelial surface proteins, tight junctions, and oxidase stress pathways in the brain Kruman et al. 1998; Bagashev et al. 2013;Williams et al. 2019). Recently, it has been demonstrated that the presence of the R57 residue allows Tat to be taken up more efficiently by uninfected brain microglial cells (Ruiz et al. 2019). The same study also found that the axonal/dendritic integrity is significantly compromised in co-cultures treated with conditioned microglial media with Tat-R57 compared to Tat-S57 variants.

It is well established that cysteine-rich domain of tat protein governs the transactivation functions of Tat. It has also been shown that in HIV-1 subtype C Tat, a Serine (S) residue replaces a cysteine (S) at position 31 and subtype B contains Cysteine (Choi et al. 2012). This (C31S) variation affects the biological function of Tat including its neurotoxic potential (Antinori et al. 2007; Heaton et al. 2011). While laboratory studies consistently demonstrated

reduced neuropathogenesis in relation to the C31S polymorphism (Nath et al. 2000; Mishra et al. 2008; Rao et al. 2014), there is little evidence from clinical cohorts that the genetic mutation significantly diminishes the expression of HAND (Paul et al. 2014). The discrepancy between laboratory and clinical cohort data emphasizes the importance of exploring the entire tat exon 1 for signatures that may affect the neurological clinical outcomes of HIV infection.

Unlike viral essential enzymes, such as *protease* and *reverse transcriptase*, *tat* undergoes continuous substitutions due to host selection pressure (Allen et al. 2000). Tat variation may occur at any amino acid residue spanning the entire protein. It is therefore important to investigate diversity and variations on Tat which may have an impact on neurocognitive impairment . This study investigates the possible links between the *Tat* exon1 gene variations and clinical diagnosis of neurocognitive function in HIV subtype C. We also evaluated how variation in Tat impacted clinical markers of HIV infection like plasma viral load, proviral load and inflammatory cytokines.

2.0. Methods

A cross-sectional study was conducted on a cohort of 147 HIV-1 positive individuals recruited from primary care HIV-1 clinics in Cape Town, South Africa. Participants were enrolled in a parent study that focused on neuropsychological and brain imaging signatures in HIV-1 subtype C as described earlier (Paul et al. 2014; Paul et al. 2017; Heaps-Woodruff et al. 2017; Ruhanya et al. 2020, 2021).The majority of participants were anti-retroviral treatment (ART) naïve; with a smaller subset having enrolled within one month. Briefly, the tests were administered to assess learning, executive functions, and visuospatial and psychomotor speed. Learning was assessed. T-scores for individual cognitive tests were averaged to generate a global T-score and a global deficit score (GDS); GDS > 0.5 defined neurocognitive impairment as described elsewhere (Heaps-Woodruff et al. 2017; Jumare et al. 2017). This study was approved by the Health Research Ethics Committee (HREC) of Stellenbosch University, Ethics Reference #: S17/02/035. The study was conducted according to South African Guidelines for Good Clinical Practice and the Medical Research council, Ethical Guidelines for Research in line with the guidelines and principles of the international declaration of Helsinki.

2.1. Cell subset separation

PBMCs were separated by Ficoll gradient separation (Ficoll - Histopaque, Pharmacia, Uppsala, Sweden). Plasma fraction was stored at -80^{0}C and later used for cytokine assays. Monocytes (CD14+) were separated by magnetic cell sorting (MACS, Miltenyi Biotec GmbH, and Bergisch Gladbach, Germany) and the peripheral blood lymphocytes (CD14-) were recovered from negative fraction of the CD14+ monocytes. Monocyte depleted (CD14-) lymphocytes were used to extract proviral HIV DNA using the QIAamp Blood Mini extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity and purity of extracted HIV-1 DNA was determined by NanoDrop® ND-1000, (Thermo Fisher Scientific, MA, USA).

2.2. Proviral DNA extraction and quantification from peripheral lymphocytes

HIV-1 DNA was extracted from lymphocytes using the QIAamp Blood Mini extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity and purity of extracted HIV-1 DNA was determined by NanoDrop® ND-1000, (Thermo Fisher Scientific, MA, USA) spectrophotometer. The proviral load assay was done as described earlier (Malnati et al. 2008; Ho et al. 2013), using a quantitative real-time PCR (qPCR) that targets the conserved HIV-1 LTR-gag region (Ruhanya et al. 2017).

2.3. PCR Amplification and sequencing of Tat Exon 1

Tat exon 1 was amplified from proviral DNA with Promega GoTaq® Flexi Kit, according to manufacturer's instructions (Promega Corporation, Madison, USA). A fragment of 1451 bp was amplified in the (pre-nested), first round of PCR using external primersVif-1 5'-GGGTTTATTACAGGGACAGCAGAG-3' (HXB2 Position: 4900 -4923) and CATH4R 5'-GTACCCCATAATAGACTGTGACC-3' (HXB2 Position: 6329-6351) as forward and reverse primers, respectively. The PCR vials were incubated at 94 ^{o}C for 2min initial denaturation before the amplification. The amplification was performed for 40 cycles, each cycle consisting of melting at 94 ^{o}C for 30s, annealing at 60^{o}C for 30s, and extension at 68 ^{o}C for 1min, followed by final extension at 68^{o}C for 10 min. Two microliters of the first-round PCR products were transferred to the second-round PCR to amplify a fragment of 953 base pair fragment containing *vpr- tat* exon 1 using primers Vif-1F 5'-GGAATTTGGGTCATGGAGTCTCCATA-3'(HXB2 Position: 5276→5301) and Tat1_OR 5'-CTCATTGCCACTGTCTTCTGC-3' (HXB2 Position: 6209 – 6229) as forward and reverse primers, respectively. The cycling conditions were identical to the first round of

amplification. 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, California USA).

The PCR products were cleaned with the Nucleospin PCR Clean-up kit, according to manufacturer's instructions (Machery-Nagel, Düren, Germany). The sequencing of the purified PCR products was done using the internal primers Vif-1F and Tat1_OR with the Applied Biosystems BigDye® Terminator v3.1 Cycle Sequencing Kit, according to the manufacturer's instructions (ThermoFisher Scientific, 2016) and run on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were read and assembled into contigs using Sequencher v 5.2.4 (Gene Codes Corporation, Ann Arbor, MI, USA).

2.4. Sequence and phylogenetic analysis

All sequences were checked for quality assurance using the HIV-1 Sequence Quality Analysis tool (http://www.hiv.lanl.gov/content/sequence/QC/index.html) before further analyses. These tools include finding the most similar database sequence and possible recombinant sequences using the recombinant identification program (RIP). Subtypes were also obtained using Context-based Modeling for Expeditious Typing (COMET) (Struck et al. 2014) https://comet.lih.lu/

The latest HIV-1 subtype reference sequence dataset was obtained from the LANL database (http://www.hiv.lanl.gov) and aligned with 147 tat sequences using MAFFT as implemented in Geneious Prime 2020.1.2 (http://geneious.com). Sequences were then codon-aligned and manually checked for insertions and deletions. The Maximum likelihood (ML) method was used to infer phylogenetic trees with MEGA 6(Tamura et al. 2013) using the best substitution model for the dataset with 1000 bootstrap replicates.

2.5. Variability analysis of HIV-1 subtype C amino acid sequences

Each position in a sequence alignment may be empirically and theoretically predictive of biological function. We used an entropy calculator which offers a graphic interface that allows conservation inference to compare variability in the two sets of aligned sequences (https://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy.html). Briefly, the frequency of each amino acid residue is calculated for each position and the sum of the products of each frequency gives the entropy value for each position. The entropy (H) reaches its minimum

value when the same amino acid residue or base is present at one position, while the maximum is reached when all the amino acids/nucleotide bases are present. H ranges from zero when only one residue is present at that position to 4.322, when all 20 amino acid residues are equally represented in that position (Litwin et al. 1992).

2.6. Variability analysis of Vpr amino acid sequences

Information at each position in the alignment has been found to be empirically and theoretically predictive of biological function (Pristisanac et al. 2019). We used the entropy calculator software which offers a graphic interface that allows conservation inference. Since we were comparing two alignments, impaired and non-impaired, Entropy-two was used to compare variability in the two sets of aligned sequences, impaired and non-impaired sequences (https://www.hiv.lanl.gov/content/sequence/ ENTROPY/ entropy.html). We also used Protein variability sever (PVS)(http://imed.med.ucm.es/PVS/pvs-help.html). which calculated sequence variability within impaired alignment and non-impaired alignment separately using Shannon entropy as a variability metric. Briefly, the frequency of each amino acid residue is calculated for each position and the sum of the products of each frequency gives the entropy value for each position. The entropy (H) reaches its minimum value when the same amino acid residue or base is present at one position, while the maximum is reached when all the amino acids/nucleotide bases are present. H ranges from zero when only one residue is present at that position to 4.322, when all 20 amino acid residues are equally represented in that position (Liwtin and Jores 1992).

2.7. Amino Acid Signature pattern analysis

HIV-1 tat exon 1 subtype C sequences were separated into cognitively impaired and non-impaired groups, after exclusion of all other subtypes. We used signature pattern analysis with the Viral Epidemiological Signature Pattern Analysis (VESPA) tool to detect amino acid substitutions that may be unique to the query (impaired) group relative to the background (non-impaired) group (https://www.hiv.lanl.gov/content/sequence/VESPA/vespa.html). 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 et al. 1992). Signature pattern analysis purposefully uses the most common amino acid at a position, rather than merely describing positions that exhibit differences in amino acid distributions (Huang et al. 2012)

2.7. Evaluation of the selection pressure

Analyzing the rate of immediate non-synonymous (amino acid-changing) and synonymous (silent) changes in amino acid sequences, gives important information on how proteins evolve (Massingham et al. 2005). In particular, the ratio of the rates of non-synonymous fixation has been used to measure the level of selection pressure on proteins. We used Datamonkey, (http://www.datamonkey.org) a web-based suite of phylogenetic analysis tools for evolution (Weaver et al. 2018) to measure selection pressure on HIV-1 subtype C Tat exon 1 amino acid sequence alignments from participants with neurocognitive impairment and non-impaired participants. The rationale was to determine which sites/amino on Tat exon 1 were under positive selection in HIV associated neurocognitive impairment. Those sites/amino acids with significant access of non-synonymous over synonymous were used as evidence for adaptive evolution in either impaired or non-impaired participants. Positive selection pressure analysis was performed on the tat exon 1 gene site-by-site codon using two methods, single-likelihood ancestor counting (SLAC) (Pond et al. 2005; Delport et al. 2010) and the Mixed Effects Model of Evolution (MEME) (Murrell et al. 2012) .

Briefly, SLAC uses a combination of maximum likelihood (ML) and counting approaches to confer the mean ratio (ω) of nonsynonymous changes (dN) and the synonymous substantiations per synonymous (dS) per site in a corresponding alignment. It involves reconstruction of ancestral sequences using a single most likely ancestor. If $\omega > 1$, the site is said to have evolved under positive selection. MEME combines constant effects at site level and random effects at branch level and can identify instances of both episodic and pervasive positive selection at the level of an individual site. We used MEME to determine whether there were more sites under positive selection than the sites identified with SLAC.

Statistical analysis

We examined the association of Tat exon 1 signature amino acids and neurocognitive impairment as measured by the GDS, clinical parameters using generalized linear modelling (GLM) to determine the adjusted relative risk ratios. The dependent variables in the model were neurocognitive impairment, measures of viral load, CD4 cell count and the independent variables were amino acid variations, clinical parameters, and demographic factors. The tests were performed at 5% significance level in Stata version 13.

3.0. Results

3.1. Demographic and clinical characteristics of the cohort

The demographic and clinical characteristics of the cohort (N=155) are summarized in Table 1. The age of the participants ranged from 21 to 46 years. The mean GDS score for the cohort was 0.32 (SD). The mean GDS was higher for males (0.5) than females (0.31) but the difference was not significant (P = 0.085). Using a GDS of 0.5 or higher, 53 (36.1 %) participants were classified as impaired; all others were classified as cognitively normal.

Table 1. Demographics and clinical parameters

	Non-Impaired, n= 94	**Impaired, n=53**	**P value**
Mean Age (years) (SD)	31,6 (5,45)	31,8 (5,17)	0,783
Mean GDS score (SD)	0,2 (0,15)	1,0 (0,50)	< 0,001***
Plasma viral load	118638,9 (340017,80)	153365,1 (306985,50)	0,557
Proviral load	231,2 (275.87)	432,6 (693,15)	0,012**
CD4+ absolute count	246,2 (181,79)	233,6 (160,90)	0,667
THS ratio	0,3 (0,21)	0,3 (0,18)	0,420
CD14+ count	1385657,0 (941235,80)	1613455,0 (1331136,00)	0,218

SD Standard deviation

3.2. Subtyping and phylogenetic inference

One hundred and forty-seven sequences were obtained. RIP and COMET identified all sequences as subtype C, except for RP236 (subtype G) and RP050 and RP202 (subtype A1). RIP could not determine the subtype of RP094. COMET identified RP094 as A1 and suggested to check for CRF02_AG. The most similar sequence in the database was identified as sequence AJ251057|SN|02_AG|95.

Phylogenetic analysis can be problematic with sequences less than 500 bp in length. The length of the tat gene is 216 bp. The ML phylogenetic tree is shown in **Figure 1**. The general time reverse model with discrete gamma distribution (GTR+G+I) was the best model with the lowest Bayesian Information Criterion (BIC) score (21503.81469). From a total of 147 sequences, 143 (97.2%) were inferred as subtype C. Sequence RP050 was an outlier to subtype A1 and RP202 an outlier to subtype C. Both sequences were identified as A1 by the online tools. One sequence R263 clustered with subtype G, confirming the results of both

online tools. RP094 clustered within subtype A2, but with a long branch. These four sequences were removed from the dataset before further analysis.

3.3. Comparison of Tat Variability between impaired and non-impaired participants

Using the Shannon variability metric, two positions on the sequence alignment from participants with neurocognitive impairment were considered variable since their Shannon entropy (H) values were greater than 2 ($H \geq 2$). These were position 24 (lysine, 24K) with entropy of 2.238 and position 29 (arginine, 29R) with entropy of 2.671. These two residues were identified as signature amino acid residues (SAR) for neurocognitive impairment using VESPA. Considering the alignment from the non-impaired participants, the same positions were variable, position 24 (Asparagine, 24N); H = 2.607 and position 29 (Histidine, 29H); H = 3.079. The distribution of Shannon variability in the consensus sequence of all participants is shown in **Figure 2**.

Using Entropy-TWO, we identified two positions which were conserved in the impaired group as compared to the non-impaired group. Amino acids Proline (P) at position 3 (3P), p = 0.04) and Arginine at position 52 (52R), (p = 0.02) were conserved in the impaired group. Only alanine at position 58, 58A (p = 0.05) was conserved in the non-impaired group.

3.4. Signature pattern analysis of *tat* exon 1 sequences

Signature pattern analysis identified two signature amino acid residues that were uniquely associated with the impaired group, namely Lysine at codon 24 (24K) with frequency of 43.4% and Arginine (R) at codon 29 (29 R) with a frequency 34%. Asparagine (N) at position 24 (24 N) with a frequency of 31.9% and Histidine (H) at position 29 (29 H) with a frequency of 21.3% were signature amino acids in the non-impaired neurocognitive function (**Table 2).** Both residues are in the second domain (22-37 residues) which is the cysteine-rich region. Notably, we found N24K and H29R substitutions in neurocognitive impairment in HIV-1 type C infection.

Table 2. Signature amino acids and their frequencies

Cognitive impairment signature amino acids	**K**	**R**
Frequency among the impaired set	0.434	0.340
Frequency among the non-impaired set	0.319	0.191
Non-impaired amino acids	**N**	**H**
Frequency among the impaired set	0.264	0.170
Frequency among the non-impaired set	0.319	0.213
Alignment position	**24**	**29**

Using SLAC, positive selection was inferred at five positions, 4, 21, 58, 68, and 69 in the impaired alignment. Four positions, 29, 36, 67, and 70 were only selected in the non-impaired group as shown in **Table 3** and **Table 4.** Residue 29H which, identified as a signature in the non-impaired group, was inferred for positive selection. Using MEME, 9 sites were inferred for episodic selection in the impaired group, with 5 of them, 4, 21, 58, 68 and 69 common between SLAC and MEME. The other four sites, 29, 36, 39 and 57 were inferred for positive selection using MEME only. Residue 29R inferred for episodic positive selection was also identified as a signature amino acid in the impaired using VESPA. In the non-impaired group, 13 sites were under episodic positive/diversifying selection. Again, signature amino acid 29H was inferred as under positive selection.

Table 3. Sites under positive selection in the tat exon 1 for the impaired group using SLAC

Site	**Residue**	**frequency**	**Synonymous changes**	**Non-synonymous changes**	**P-value**
4	Valine	77.2	0	4.309	0.038
21	Proline	54.7	0	5.492	0.012
58	Alanine	69.8	0	3.499	0.059
68	Proline	56.6	0	598.560	0.008
69	Isoleucine	50.9	0	5.668	0.031

Table 4. Sites under positive selection in tat exon 1 for the non-impaired group using SLAC

Site	Residue	frequency	Synonymous changes	Non-synonymous changes	P-value
4	Valine	69.1	0	5.630	0.016
21	Proline	52.1	0.999	6.999	0.019
29	*Histidine	21.3	1.342	3.191	0.005
36	Valine	90.4	1.002	549.990	0.054
58	Alanine	56.4	0	7.499	0.002
67	Asparagine	75.5	0	61.493	0.021
68	Proline	56.4	0	1.292	0. < 0001
69	Isoleucine	61.7	1.394	109.498	0.007
70	Serine	83.0	1.001	7.932	0.012

**Signature amino acid identified by VESPA which is under positive selection.*

3.5. Impact of Signature Amino acids on cognitive function and clinical parameters

Comparing the two signature amino acid residues, 24K was associated with significantly higher GDS of 0.5862 than 24N with GDS of 0.4013 (p = 0.0490). Although 29R was a signature in sequences for cognitively impaired patients, there was no significant GDS difference with 29H which was a signature on sequence alignment from non-impaired participants (p = 0.4877). Results also showed that 24K was linked to significantly higher mean proviral load (399.3243 DNA copies/10^6 cells) than 24N with mean proviral load of 176.9 DNA copies/10^6 cells (p = 0.0190). Regarding tat amino acid position 29 there was no significant differences in proviral load between signature 29R and 29H (P = 0.4014). **Figure 3** are graphical presentations comparing the impact of the signature amino acids and systemic markers of HIV and neurocognitive function.

3.5.1. Tat Signature amino acids as risk factors for neurocognitive Deficit and markers of HIV infection

The presence of K24 on Tat exon 1 was associated with higher GDS (IRR = 2, 08; p = 0.00). The signature was also associated with increased monocyte depleted (CD14-) proviral load (IRR = 3.15; p < 0.0001). The amino acid residue also corresponded to low absolute CD4+ T cell count (0, 69; p = 0.02) and could possibly be as a signature for HIV disease progression as shown in **Table 5.**

Table 5: Risk of tat 24K on cognitive function, viral and immune markers

Parameter	IRR	Standard Error	95% CI	p value
GDS	2.08	0.53	1.26 - 3.43	< 0.0001
Plasma viral load	0.74	0.53	0.18 - 3.05	0.67
CD4+ Absolute count	0.69	0.11	0.51 - 0.94	0.02
Proviral load	3.15	1.19	1.50 - 6.61	< 0.0001

4. 0. Discussion

Due to its important role in HIV replication, infection and interaction with host immunity, Tat undergoes continuous substitutions due to host selection pressure (Krishnan et al. 2015; Romani et al. 2010) . In this study, we investigated how genetic variation and selection pressure in HIV-1 subtype C Tat is linked to HIV associated neurocognitive impairment. Consequently, we identified two signature amino acid residues (SAR), Lysine at codon 24 (24K) and Arginine (R) at codon 29 (29 R) which were unique to the sequence alignment from participants with neurocognitive impairment. We have also identified two SAR, 24N and 29H that were unique to the non-impaired group. All the signatures were in the second cysteine- rich domain (residues 22 to 37) which is highly conserved. Therefore, any amino acid change in this domain is likely to affect the phenotypic characteristics of tat (He et al. 2013; Teto et al. 2016). We suggest that these signatures contribute to biological processes that result in these two clear clinical consequences of HIV infection.

The study also revealed higher risk of neurocognitive impairment and immunosuppression associated with the presence of 24 K residue on Tat exon 1. The presence of 24K residue in cognitively impaired people is indicative of a link between HAND and tat sequence amino acid variation. It has been shown that Tat-induced neurotoxicity may be influenced by specific protein signatures present in Tat-B (Williams et al. 2019). As previously indicated in subtype B, we also believe that polymorphism at Tat-C residue 24, is associated with mechanisms of HIV associated neurocognitive impairment. Previous studies have shown Tat-C to contain polymorphisms, such as Serine at residue 31(C31S), Serine substitution at residue 57(R57S) and Glutamate at residue 63 (Q63E), which are involved in neurological outcomes (Ruiz et al. 2019).

We believe that we have identified an additional polymorphism at residue 24 (N24K) which distinguishes impaired patients from non-impaired. This signature may contribute to biological processes resulting in the clinical differences observed in these participants. Previous studies linking Tat polymorphism like (C31S) in laboratory studies did not produce

corresponding results in clinical settings (Paul et al. 2014). Besides higher GDS, N24K polymorphism was associated with clinical biomarkers like, higher proviral load and lower absolute CD4+ T cell count that are used in monitoring disease progression and therapeutic interventions. The findings imply that the polymorphism lead to changes in viral replication and establishment of HIV latency as well as cellular consequences that compromise systemic immune status (Ajasin et al. 2020). Using cytokine data obtained from the same cohort in our previous study (Ruhanya et al. 2021), this polymorphism/signature was associated with higher plasma inflammatory cytokines RANTES /CCL5 and IP-10/CXCL10. These pro-inflammatory cytokines have been linked to neurocognitive HIV associated impairment in many studies (Yuan et al. 2013; Bandera et al. 2019, Borrajo et al. 2021; Burlacu et al. 2020). In addition, the N24K polymorphism substitutions occurred in a region that has been identified as immunodominant in subtype C Tat (Novitsky et al. 2002). This study has shown that genetic differences at HIV-1 C Tat residue 24 impacts on markers of clinical markers and systemic immune activation that has been shown to influence BBB endothelial tissue behavior which may affect the integrity of the barrier allowing entry of virus into the CNS(10). It is thought that variation within the protein may increase the severity of neurocognitive impairment by impairing BBB. In addition, tat peptides covering the cysteine rich region were able to potentially influence monocyte migration. We suspect that the presence of this signature may play a role in BBB impairment and transmigration of infected monocytes and lymphocytes into the brain. Therefore, functional studies need to investigate impact of 24K on the observations made in this study as well as on the integrity of BBB.

that can induce cell transcellular signaling by binding to some cell surface receptors, which have been shown to promote pro-inflammation beyond levels induced by viral replication. It also causes trafficking of T cells, particularly CD4+ and CD8+, which might be infected by HIV into the brain. This process increases antigenic load in the CNS, promoting inflammation- induced neuron damage. The 24K signature amino acid residue (SAR) was also a marker of monocyte-depleted (CD14-) proviral HIV DNA load which has been shown to be predictive of HAND (Jumare et al. 2017; Ruhanya et al. 2020). This signature might play a role in processes leading to latency and establishment of an HIV reservoir, which are a pool of cells that harbor transcriptionally inactive, but replication-competent HIV-1 proviruses (Ho et al. 2013). It had been previously demonstrated that Tat plays a role in the establishment of viral latency(Reza et al. 2003; Mousseau et al. 2015; Kamori et al. 2009), with some polymorphisms demonstrating impaired transactivation activity and were

significantly enriched in the latently infected CD4+ T cells (Yukl et al. 2009). Our study has revealed that there is high risk of increased proviral load in peripheral (CD14-) lymphocytes associated with SAR 24K. The residue may be involved in establishing and maintaining the reservoir. However, the molecular mechanisms by which the signature ultimately achieve such effect needs to be further investigated. It could be involved in mechanisms enforcing proviral silencing at the transcriptional level (post integration latency). In contrast, the residue is associated with low levels of CD4+ T cells. This observation suggests the SAR 24K is linked to processes that results in CD4+ depletion.

However, this signature residue was neither under positive selection nor negative selection using SLAC. Positive selection was inferred on 29R in the impaired group and on 29H using both SLAC. Regarding neurocognitive impairment, positive selection for Arginine at codon 29 supports a link between diversifying selection and neurocognitive impairment (Tilghman et al. 2014). Positive selection was also inferred at this position for Histidine 29H in the non-impaired group, indicating selective advantage of having this residue in participants without neurocognitive impairment. Positive selection on residues signifies genetic evolution. In addition, selective advantage for 29R residue has been shown to result in structural changes in the di-cysteine motif using secondary RNA prediction (Tilghman et al. 2014). Therefore, our finding is important in informing future functional studies on the effects of this mutation in neuropathogenesis, immune activation and immunosuppression.

We also observed differences in impairment status when examining Shannon entropy. We identified two conserved amino acids, Proline (P) at position 3 and Arginine at position 52 in the impaired group and Alanine at position 58 in the non-impaired. As observed in this study, amino acid information at each position in the alignment can be used to empirically predict biological phenotype (Pritišanac et al. 2019). In this study, we identified a tat exon 1 conservation pattern that was associated with neurocognitive impairment. Positional information on an alignment has also been used to predict protein structure, catalytic residues, protein binding and evolutionary relationships (Pritišanac et al. 2019; Ramazzotti et al. 2004) Therefore, the conserved amino acids at these positions may have a biological importance in the pathogenesis of HAND.

Conclusion

We conclude that HIV 1 subtype C Tat 24K signature amino acid residue is associated with neurocognitive impairment, markers of viral load and immune suppression. This implies that the mutation might be involved in pathogenesis of neurocognitive impairment in HIV infection and therefore, it can be targeted for diagnostic and therapeutic approaches to HAND.

Acknowledgements

This study was supported by the Poliomyelitis Research Foundation of South Africa (PRF), National Research Foundation (NRF), National Health Laboratory Services (NHLS) Research Trust and the South African Medical Research Council (SAMRC).

Authors' contributions

Study conception and design: VR, SE, RP. Acquisition of data: VR, RP, JJ, SS. Analysis, and interpretation of data: VR, SE, GJ. Drafting of manuscript and Critical revision: all authors

Competing interests

The authors declare no competing interests.

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are enriched for HIV-1 Tat variants with impaired transactivation activity. Virology 387:98-108. doi: 10.1016/j.virol.2009.01.013.

Figure legends

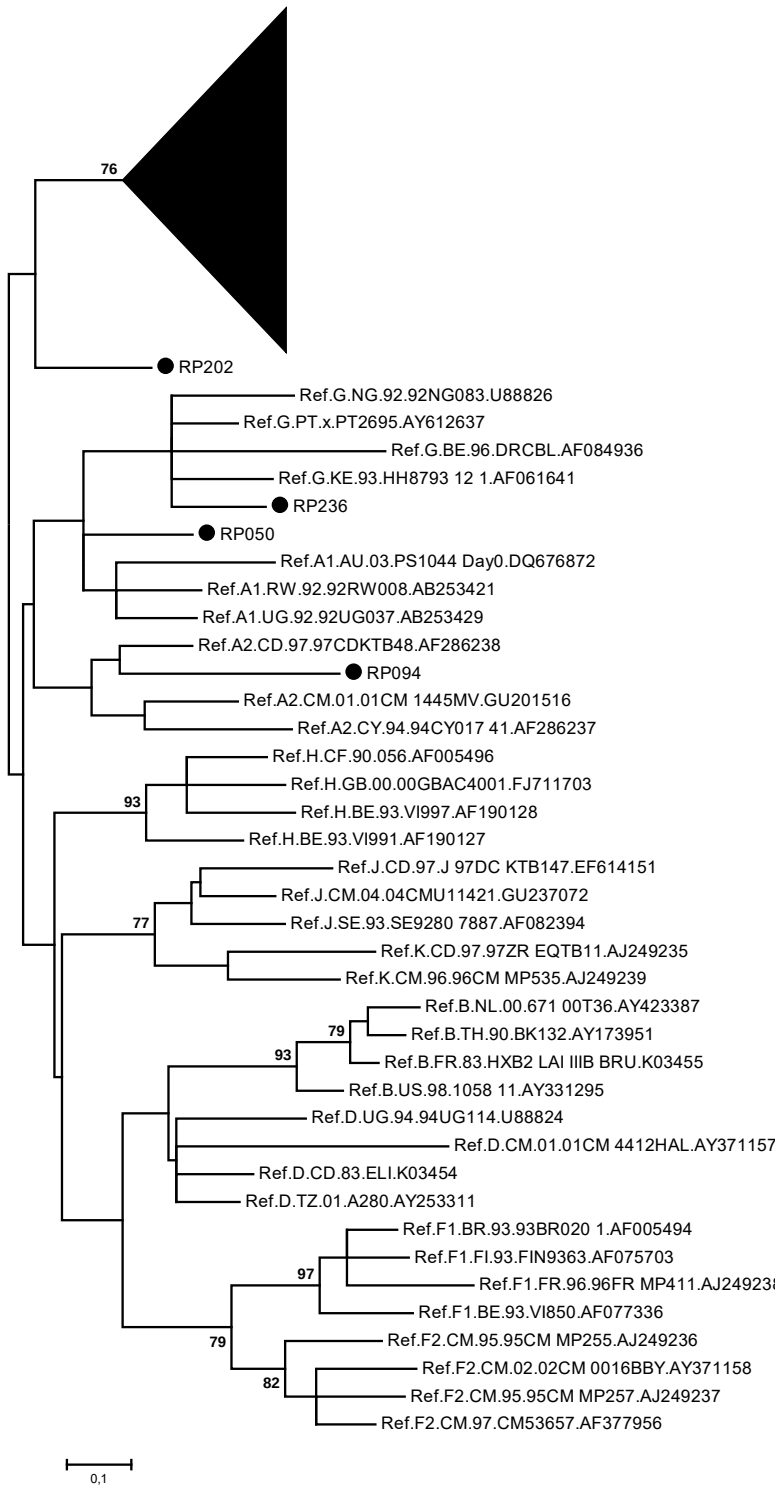

Figure 1. *Phylogenetic analysis of the tat gene. The evolutionary history was inferred by using the ML method based on the GTR+G+I model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There was a total of 216 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 with 1000 bootstraps. Triangle in the phylogenetic tree shows 143 (97.2%) tat sequences inferred as subtype C.*

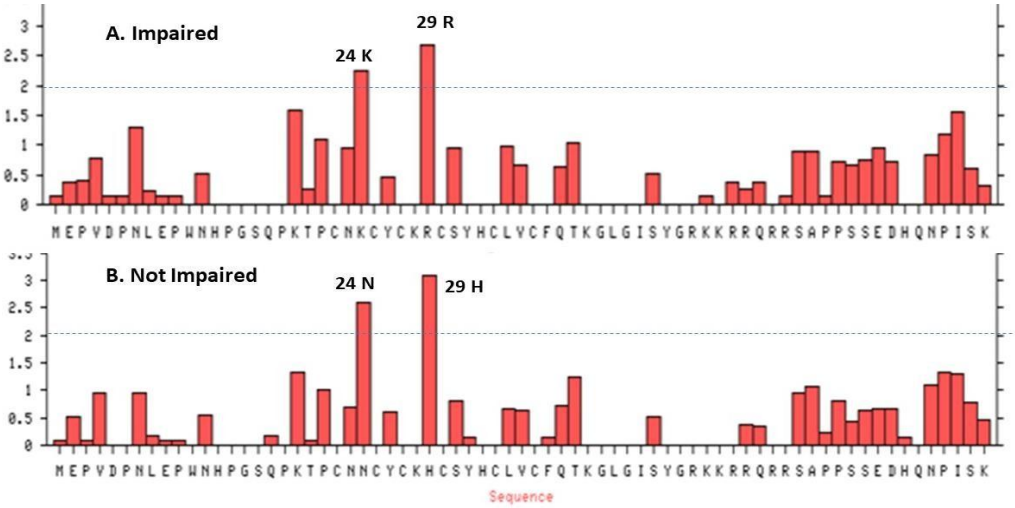

Figure 2. *Shannon variability plot in the consensus sequence of A.) impaired group and B.) non-impaired group. Positions were considered variable when the Shannon entropy (H) values were greater than 2. The signature amino acids in the impaired group, 24K and 29 R and signature residues in the non-impaired group, 24N and 29H are also indicated*

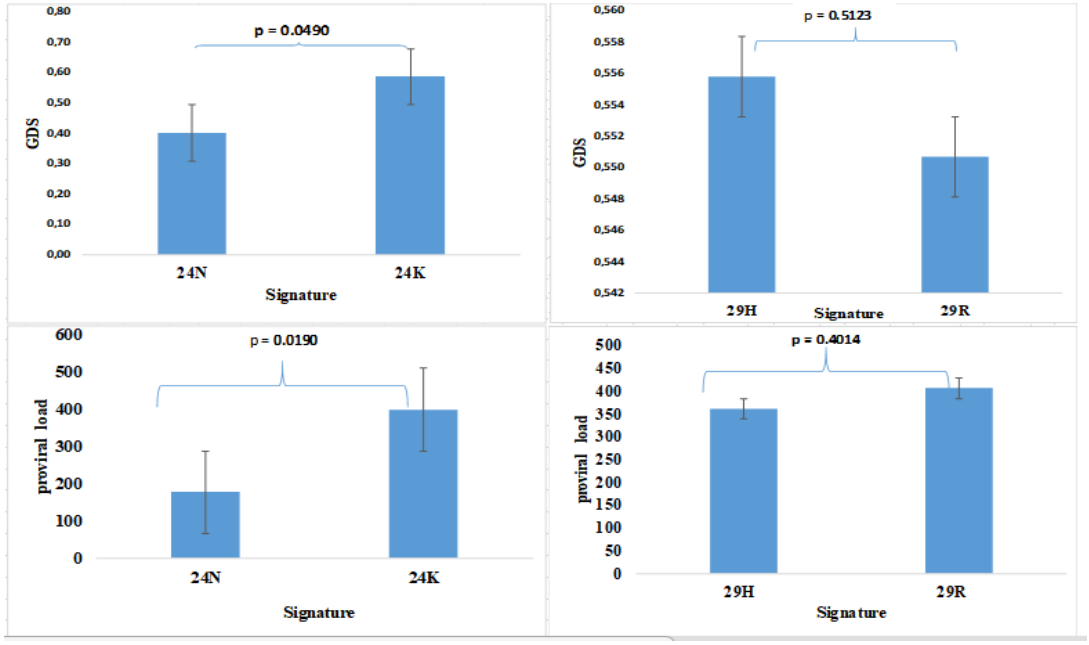

Figure 3: *Graphical comparison of the impact of N24K and H29R polymorphism on global deficit score (GDS) with 24 K and 29 H showing a higher impact on GDS than 24N and 29R. and comparison of the impact of the signatures on proviral load and cytokines. 24K showing significantly higher impact on proviral load (p = 0.0190) than 24N*

Paper 8: Ruhanya, V.; Jacobs, G.B.; Paul, R.; Joska, J.; Seedat, S.; Nyandoro, G.; Glashoff, R.H.; Engelbrecht, S. HIV-1 subtype C Vpr amino acid residue 45Y and specific conserved fragments are associated with neurocognitive impairment and markers of viral load. Journal of neurovirology 2021. NJIV-D-21-00204 (Completed - Currently under review)

HIV-1 subtype C Vpr amino acid residue 45Y and specific conserved fragments are associated with neurocognitive impairment and markers of viral load

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Summary

There is increasing evidence that HIV-1 viral protein R (Vpr) plays an important role in the pathogenesis of cognitive impairment. We investigated the impact of HIV-1 sub-type C Vpr sequence variation on HIV-associated neurocognitive impairment in treatment naive individuals using bioinformatic tools and statistical models. We identified a Tyrosine at position 45 (45Y) as signature amino acid residue (SAR) for neurocognitive impairment with a frequency of 58.9% and Histidine (45H) as a SAR in the non-impaired indviduals with a frequency of 49%. The presence of signature 45Y increased by 3.66 times the risk of neurocognitive impairment, resulted in 525 times higher plasma viral load, 15.84 times higher proviral load and 60% lower absolute CD4-T cell count compared those without the signature. Additionally, we identified 4 conserved Vpr fragment sequences, PEDQGPQREPYNEWTLE (5 to 21), LGQYIY(42 to 47), TYGDTW (49 to 54), PEDQGPQREPYNEW (5 to 18) that were associated with higher plasma viral load and proviral load. The implication of these findings is that variation of Vpr leads to neurocognitve impairment in HIV infection and worsens the progression of disease in general by promoting the production of provirus, promoting HIV replication and depletion of CD4+ T cells in the periphery.

***Key words**: HAND, Vpr, entropy, signature amino acid residue, Tyrosine, Histidine,*

1.0. Introduction

HIV-1 viral protein R (Vpr) is one of the 4 accessory proteins encoded by the HIV genome. It is a basic and highly conserved 96 amino acid protein which has been shown to be multifunctional but plays a central role in HIV pathogenesis (Cohen et al. 1990; Romani et al. 2009; Soares et al. 2016). Biological activities of Vpr include modulation of viral genome transcription (Sawaya et al. 2000), induction of T- cell apoptosis, nuclear transport of HIV-1 PIC (Vodicka et al. 1998), transactivation of HIV-1 LTR and protein transduction through cell membranes (Rogel et al. 1995; Henkein et al. 2000).

The cytotoxic effects of Vpr were postulated to be associated with clinical symptoms like dementia and neuropathy in HIV-1 infection (Ferucci et al. 2011; Mamik et al. 2017). The cell penetrating ability of Vpr could also lead to apoptosis of bystander cells (Sherman et al. 2002) in the brain. Elevated levels of Vpr have been found in the CSF of patients with HIV-1-related cognitive deficits and in the basal ganglia and frontal cortex of brains with HIV associated encephalitis (HIV-E) (Wheeler et al. 2006). The protein was also shown to induce apoptosis in the brain in both animal models and people with severe HIV associated dementia (HAD) (Rom et al. 2009). Soluble Vpr in neurons was shown to cause apoptosis involving p53 induction, release of cytochrome c and activation of caspase-9 (Soares et al., 2016). Extracellular Vpr causes astrocyte necrosis (Huang et al. 2000), stunted neuronal axon elongation (Kitayama et al. 2008), increased cytokine and chemokine release as well as increased reactive oxygen species (Ferrucci et al. 2013). This evidence points to possible mechanisms by which Vpr could be involved in the pathogenesis of HIV associated neurocognitive disorders (HAND)

Vpr like the rest of HIV-1 proviral genome is subject to variation due to mutation during reverse transcription (Lloyd et al. 2014). Variation in the Vpr gene may also arise due to continuous substitutions because of host selection pressure. Given the putative role of Vpr in HIV-1 associated neuropathogenesis, it is important to investigate how diversity/variations of Vpr impact clinical outcomes of HAND which occurs in more than 50% of HIV-1 infections (Heaton et al. 2010, Rao et al. 2014, Namanga et al. 2019). Previous studies in the USA have identified 4 Vpr amino acid, 41G, 41S, 41N, 55A and 37I that associate with cognitive function (Dampier et al. 2016). The results of the afore-mentioned study clearly demonstrate that there are clinical consequences of Vpr sequence variation. This study investigates the

possible links between the Vpr gene variations and clinical diagnosis of neurocognitive function in HIV subtype C.

2.0. Methods

A cross-sectional study was conducted on a cohort of HIV-1 positive individuals recruited from primary care HIV-1 clinics in Cape Town, South Africa. Participants were enrolled in a parent study that focused on neuropsychological and brain imaging signatures in HIV-1 subtype C as described earlier (Paul et al. 2014; Paul et al. 2017; Heaps-Woodruff et al. 2017; Ruhanya et al. 2020, 2021). Briefly, the tests were administered to assess learning, executive functions, and visuospatial and psychomotor speed. Learning was assessed. T-scores for individual cognitive tests were averaged to generate a global T-score and a global deficit score (GDS); GDS > 0.5 defined neurocognitive impairment as described elsewhere (Heaps-Woodruff et al. 2017; Jumare et al. 2017). This study was approved by the Health Research Ethics Committee (HREC) of Stellenbosch University, Ethics Reference #: S17/02/035. The study was conducted according to South African Guidelines for Good Clinical Practice and the Medical Research council, Ethical Guidelines for Research in line with the guidelines and principles of the international declaration of Helsinki.

2.1. Cell subset separation

PBMCs were separated from whole blood as described previously (Ruhanya et al., 2020). Thereafter, CD14+ monocytes were separated from PBMCs by magnetic cell sorting (MACS, Miltenyi Biotec GmbH, and Bergisch Gladbach, Germany) and monocyte depleted peripheral blood lymphocytes (CD14- fraction) were recovered. The total CD3+, CD4+ and CD45+ count and percentages were determined using standardized T cell subset protocols (BDMultiset) and routine flow cytometry analysis (BD FACS Calibur). Monocyte depleted (CD14-) lymphocytes were used to extract proviral HIV DNA.

2.2. Proviral DNA extraction from peripheral lymphocytes

HIV-1 DNA was extracted from lymphocytes using the QIAamp Blood Mini extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity and purity of extracted HIV-1 DNA was determined by NanoDrop® ND-1000, (Thermo Fisher Scientific, MA, USA) spectrophotometer.

2.3. Amplification and sequencing -Sanger sequencing of Vpr

Vpr was amplified from Lymphocyte derived proviral DNA using nested PCR using the Promega GoTaq® Flexi Kit, according to manufacturer's instructions (Promega Corporation, GmbH, Germany). To simplify the PCR process, we chose to amplify the two genes (vpr and tat exon 1) as a single fragment and then separated Vpr fragment from the Vpr-tat fragment after sequencing. Therefore, our primer sets targeted vpr (HXB2 5559-5850) and tat exon 1 (HXB2 5831-6045) as a single fragment. The vpr gene lies near, and slightly overlaps tat exon 1. A fragment of 1451 bp was amplified in the (pre-nested) first round of PCR using external primersVif-1 5'-GGGTTTATTACAGGGACAGCAGAG-3' (HXB2 Position: 4900 -4923) and CATH4R 5'-GTACCCCATAATAGACTGTGACC-3' (HXB2 Position: 6329-6351) as forward and reverse primers respectively. The PCR vials were incubated at 94 ^{o}C for 2min initial denaturation before the amplification. The amplification was performed for 40 cycles, each cycle consisting of melting at 94 ^{o}C for 30s, annealing at 60^{o}C for 30s, and extension at 68 ^{o}C for 1min, followed by final extension at 68^{o}C for 10 min. Two microliters of the first-round PCR products were transferred to the second-round PCR to amplify a fragment of 953 base pair fragment containing vpr-tat exon 1 using primers Vif-1F 5'-GGAATTTGGGTCATGGAGTCTCCATA-3' (HXB2 Position: 5276→5301) and Tat1_OR 5'-CTCATTGCCACTGTCTTCTGC-3' (HXB2 Position: 6209 – 6229) as forward and reverse primers respectively. The cycling conditions were identical to the first round of amplification. 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, CA, USA). The PCR products were performed with the Nucleospin PCR Clean-up kit, according to manufacturer instructions (Machery-Nagel, GmbH, Germany).

The sequencing of the purified PCR products was done using the internal primers Vif-1F and Tat1_OR on the Applied Biosystems BigDye® Terminator v3.1 Cycle Sequencing Kit, according to the user guide (ThermoFisher Scientific, MA, USA). and run on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, CA, USA). Sequences were read and assembled into contigs using Sequencher v 5.2.4 (Gene Codes Corporation, Ann Arbor, MI, USA). All sequences were checked for quality assurance using the HIV-1 Sequence Quality Analysis tool (http://www.hiv.lanl.gov/content/sequence/QC/index.html) before further analyses.

2.4. Sequence quality and phylogenetic analyses

All sequences were checked for quality assurance using the HIV-1 Sequence Quality Analysis tool (http://www.hiv.lanl.gov/content/sequence/QC/index.html) before further analyses. These tool identify similar database sequences and possible recombinant sequences using the recombinant identification program (RIP). Subtypes were also obtained using Context-based Modeling for Expeditious Typing (COMET) (Stuck et al., 2014) https://comet.lih.lu/

The latest HIV-1 subtype reference sequence dataset was obtained from the LANL database (http://www.hiv.lanl.gov) and aligned with Vpr sequences using MAFFT as implemented in Geneious Prime 2020.1.2 (http://geneious.com). Sequences were then codon-aligned and manually checked for insertions and deletions. The accession number for the sequences used in this study is MZ566636 - MZ566791. The Maximum likelihood (ML) method was used to infer phylogenetic trees with MEGA 6 (Tamura et al. 2013) using the best substitution model for the dataset with 500 bootstrap replicates.

2.5 Variability and signature pattern analyses of Vpr sequences

The SAR in subtype C Vpr was identified using the Viral Epidemiological Signature Pattern Analysis (VESPA) tool from the HIV sequence database (www.hiv.lanl.gov) which identifies positions in a sequence at which the most common amino acid residues differ between a query sequence alignment and a background sequence alignment. In our cohort, the query alignment was from participants with neurocognitive impairment and the background alignment was from participants without neurocognitive impairment. VESPA detects amino acid substitutions that may be unique to the query (impaired) group relative to the background (non-impaired) group. Briefly, VESPA 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 amino acids in the query group differs from the background group. The specific amino acid signature is obtained by looking for the set of amino acids that is conserved within each group but differs between the two groups (Korber & Myers 1992). Signature pattern analysis purposefully uses the most common amino acid at a position, rather than merely describing positions that exhibit differences in amino acid distributions (Huang et al. 2012).

Variability analysis of Vpr amino acid sequences

Information at each position in the alignment has been found to be empirically and theoretically predictive of biological function (Pristisanac et al. 2019). We used the entropy calculator software which offers a graphic interface that allows conservation inference. Since we were comparing two alignments, impaired and non-impaired, Entropy-two was used to compare variability in the two sets of aligned sequences, impaired and non-impaired sequences (https://www.hiv.lanl.gov/content/sequence/ ENTROPY/ entropy.html). We also used Protein variability sever (PVS)(http://imed.med.ucm.es/PVS/pvs-help.html). which calculated sequence variability within impaired alignment and non-impaired alignment separately using Shannon entropy as a variability metric. Briefly, the frequency of each amino acid residue is calculated for each position and the sum of the products of each frequency gives the entropy value for each position. The entropy (H) reaches its minimum value when the same amino acid residue or base is present at one position, while the maximum is reached when all the amino acids/nucleotide bases are present. H ranges from zero when only one residue is present at that position to 4.322, when all 20 amino acid residues are equally represented in that position (Liwtin and Jores 1992).

2.7. Evaluation of the selection pressure

Relative rates of non-synonymous (dN) and synonymous (dS) substitutions were calculated to measure the strength of positive selection on Vpr using Datamonkey a web-based suite of phylogenetic analyes tools for evolution (Weaver et al. 2018; Delport et al. 2010; Pond et al. 2005). Positive selection pressure analysis was performed at the Vpr gene site-by-site codon using two methods, single-likelihood ancestor counting (SLAC) and the Mixed Effects Model of Evolution (MEME). Briefly, SLAC uses a combination of maximum likelihood and counting methods to confer synonymous and nonsynonymous changes rates per site for a given coding and alignment. MEME combines fixed changes at site and random changes at a branch of a coding alignment.

Statistical analysis

We examined the association Vpr amino acid variation and neurocognitive as measured by GDS, and clinical parameters using generalized linear modelling (GLM) to determine the adjusted relative risk ratios. The dependent variables were neurocognitive impairment, measures of viral load, and CD4 cell count and the independent variables were amino acid

variations, clinical parameters, and demographic factors. The tests were performed at 5% significance level in Stata version 13.

3.0. Results

Subtyping and phylogenetic inference

One hundred and fifty-two sequences were obtained, 56 from individuals with cognitive impairment and 98 from individuals without neurocognitive impairment. RIP and COMET identified all sequences as subtype C, except for RP236 (subtype G) and RP050 and RP202 (subtype A1). RIP could not determine the subtype of RP094. COMET identified RP094 as A1 and suggested to check for CRF02_AG. The most similar sequence in the database was identified as sequence AJ251057|SN|02_AG|95.

The ML phylogenetic tree is shown in Figure 1. The general time reverse model with discrete gamma distribution (GTR+G+I) was the best model with the lowest Bayesian Information Criterion (BIC) score (21503.81469). From a total of 149 sequences, 143 (96.8%) were inferred as subtype C. Sequence RP050 was an outlier to subtype A1 and RP202 an outlier to subtype C. Both sequences were identified as A1 by the online tools. One sequence R236 clustered with subtype G, RP094 clustered within subtype A2 and RP127 clustered within subtype H. These five sequences were removed from the dataset before further analysis.

Signature pattern analysis of vpr sequences

A total of 33 (58.9%) out of 56 sequences from individuals with neurocognitive impairment had Tyrosine at residue 45. In the non-impaired group, 47(47.9%) out of 98 sequences had the same amino acid at position 45. Comparing the frequencies in the two groups VESPA identified Tyrosine at position 45 (**45Y**) as signature amino acid residue (SAR) for neurocognitive impairment in HIV-1 subtype C alignment. At the same position, 48 (49.0%) out of 98 sequences from individuals without neurocognitive impairment was Histidine. The frequency of Histidine in sequences with neurocognitive impairment was 23 (41.1%). Comparing the impaired and non-impaired group, VESPA identified Histidine at position 45 (**45H**) as SAR for non-impairment in HIV-1 subtype C alignment.

Vpr amino acid variability

There were 6 amino acid positions that were variable in the impaired amino acid alignment. The position 41 had highest variability/entropy of 1.884 and the consensus amino acid at this

position was Serine. Other positions which were variable include, 4, 37, 48, 55, and 88. The respective consensus amino acid at these positions are shown in **Fig 2**. Six conserved fragment sequences were also identified on the amino acid alignment of people with neurocognitive impairment. The longest fragment stretched from position 64 - 83 and the shortest conserved fragments were between 42 -27 and 49 - 54. Table 2 below shows the 6 conserved fragments and their amino acid position in consensus sequence.

Table1. Vpr conserved fragments of individuals with neurocognitive impairment

Fragments of 6 or more consecutive residues with V <= 1			
N	**Start**	**End**	**Sequence**
1	5	21	P E D Q G P Q R E P Y N E W T L E
2	23	36	L E E L K Q E A V R H F P R
3	42	47	L G Q Y I Y
4	49	54	T Y G D T W
5	64	83	L Q Q L L F I H F R I G C Q H S R I G I
6	86	93	Q R R A R N G A

The non-impaired group had a total of 12 variable positions/sites. Position 41 had the highest variability/entropy of 2.031 followed by position 84 with entropy of 1.655. The variability plot for the non-impaired group is shown in **Fig 3**. Generally, the impaired group had higher entropy than the non-impaired. PVS software analysis also identified 6 conserved fragment sequences of at least 6 consecutive bases. The longest fragment was located on 5 - 18 bases followed by 71 - 83. The shortest fragments were located from positions, 49 -54 and 64 - 69 as shown in Table 2 below.

Table 2. Vpr Conserved fragments of individuals without neurocognitive impairment

Fragments of 6 or more consecutive residues with V <= 1			
N	**Start**	**End**	**Sequence**
1	5	18	P E D Q G P Q R E P Y N E W
2	23	36	L E E L K Q E A V R H F P R
3	49	54	T Y G D T W
4	64	69	L Q Q L L F
5	71	83	H F R I G C Q H S R I G I
6	86	93	Q R R A R N G A

Entropy-two identified two positions with significant variability difference between impaired and non-impaired alignments. These were 42 (Hdiff = -0.33; p = 0.0 3) and 45 (Hdiff = 0.151; p = 0.05). Alanine at position 42 (42P) was relatively conserved in the impaired set while

Histidine at position 45 (45H) was conserved in the non-impaired set. **Fig 4** shows amino acid variability difference plot between impaired and non-impaired alignments.

Using generalized linear model (GLM) we evaluated the effect of vpr signature amino acids on GDS, controlling for age and sex. Tyrosine at residue (45Y) was associated with a higher GDS (IRR =3.667) but it was not statistically significant (p = 0.153). Signature amino acid 45H was again associated with higher GDS (IRR = 2.381; p = 0.039). However, a conserved sequence fragment TYGDTW stretching from residue 49 to residue 54 was significantly associated with risk of high GDS (IRR = 1.923; p = 0.042). The conserved fragment, QRRARNGA from residue 86 to residue 93 was associated with lower GDS (IRR = 0.7086; p = 0.039).

Using the same model, we observed that signature amino acid residue 45Y strongly associated with risk of high plasma viral load (IRR = 525.188; p < 0.000 1). Although lower, the same signature was associated with risk of higher proviral DNA IRR = 413.731; P < 0.0001). Signature 45H was also associated with both higher plasma viral load and proviral load as shown in Table 5. In addition to these signature amino acids, we identified four conserved vpr fragment sequences, PEDQGPQREPYNEWTLE (5 to 21), LGQYIY (42 to 47), TYGDTW (49 to 54), PEDQGPQREPYNEW (5 to 18) that were associated with higher plasma viral load. The same fragment sequences PEDQGPQREPYNEWTLE (5 to 21), PEDQGPQREPYNEW (5 to 18), TYGDTW (49 to 54), were also associated with higher proviral load. The details of impact of conserved sequences on measures of viral load are shown in **Tables 3 and 4**.

Table 3. Impact of HIV Subtype C Vpr signatures and conserved fragment on plasma viral load

Plasma viral load	IRR	Robust Std. Err.	95% Conf. Interval	P-value
Age	.9804298	.0261608	.9304734 - 1.033068	0.459
Sex/male	3.092177	1.120831	1.519591 - 6.292193	0.002
CD4+ T cell absolute count	.9966137	.0012724	.994123 - .9991107	0.008
Vpr 45Y	525.1881	389.1189	122.9273 - 2243.786	< 0.000 1
Vpr 45H	413.7311	301.1299	99.35277 - 1722.885	< 0.0001
PEDQGPQREPYNEWTLE_5to21	2.887108	.8538365	1.617066 - 5.15464	< 0.0001
LGQYIY_42to47	3.043523	1.226868	1.381181 - 6.706604	0.006
TYGDTW_49to54	4.526791	3.392572	1.04199 - 19.66606	0.044
PEDQGPQREPYNEW_5to18	2.391606	.9126965	1.132009 - 5.052768	0.022
Vpr 37 P	.2961796	.1490672	.1104448 - .7942642	0.016

Vpr 48 E	.3861305	.1220084	.2078614 - .7172892	0.003
Vpr 85L	7.195984	4.078164	2.369685 21.85193	< 0.0001
Vpr 94S	.1157661	.0531178	.0470998 - .2845406	< 0.0001

Table 4. Impact of HIV Subtype C Vpr signatures and conserved fragment on proviral load

Proviral Load	**IRR**	**Robust Std. Err.**	**95% Conf. Interval**	**p value**
Age	.99851	0.0198972	.9602631 - 1.038278	0.940
sex /male	1.94088	0.5744095	1.08663 - 3.466683	0.025
CD4+ T cell absolute count	0.99813	0.0007489	1.0000 - 1.000001	0.013
Vpr 45Y	15.8437	10.12012	4.530578- 55.40634	< 0.0001
Vpr 45H	8.1982	5.220475	2.353411 - 28.55934	0.001
PEDQGPQREPYNEWTLE_5to21	1.53523	0.38222	0.94243- 2.5009	0.085
PEDQGPQREPYNEW_5to18	1.9328	0.61674	1.0341 - 3.6124	0.039
TYGDTW_49to54	2.9438	1.2535	1.277759 - 6.7822	0.011
LQQLLF_64to69	2.5644	1.0126	1.182637 -5.560476	0.017
HFRIGCQHSRIGI_71to83	0.61970	0.16191	0.3713549 - 1.034127	0.067
Vpr 4P	0.34776	0.09758	0.2006448 - .6027515	< 0.0001
Vpr 41S	0.44677	0.10429	0.2827505 - .7059589	0.001
Vpr 55 E	0.68700	0.15583	0.4404426 - 1.071586	0.098
Vpr 60 I	0.58831	0.14218	0.3663456 - .9447427	0.028

Sites under positive/diversifying selection using SLAC and MEME

In total, 8 sites were under positive selection using single likelihood ancestor counting approach. Three positions, 3, 4 and 19 were in the acidic region, one site (48) was in the second α-helix and the last 4 sites, 84, 85, 90 and 95 were in the basic amino acid carboxyl-region. Using mixed effects model of evolution 13 sites were under episodic selection, 3 in the acidic region, 1 site 37 in the flexible loop joining the second helix and the third α-helices, 1 site, 48 in the second helix, 3 sites, 58 and 59 and 77, in the third helix, and the last 5 were located in the C-terminus.

Table 5: Sites under positive selection using SLAC

Site	ds	dN	P [dN/ds > 1]
4	2.00	20.0	0.00000975
95	0.00	13.7	0.000158
84	12.3	29.1	0.00170
85	11.7	24.5	0.0131
48	6.72	18.8	0.0162
3	0.00	6.68	0.0191
19	5.08	13.9	0.0193
90	3.01	8.50	0.0608

Table 6. Sites under episodic selection using MEME

Site	α	**β**	**p[ω(α/β) >1]**
3	0.00	0.51	0.02
4	0.12	3.42	0.00
19	0.33	0.96	0.06
37	2.76	15.27	0.02
48	0.56	3.84	0.01
58	1.18	11.10	0.04
59	1.68	4625.58	0.01
77	1.41	10.88	0.09
84	0.61	4.55	0.00
85	1.04	1.93	0.09
90	0.26	12.66	0.05
94	0.24	2.61	0.06
95	0.00	0.99	0.00

5. Discussion

Viral protein R exhibits molecular diversity which has been shown to be associated with clinical phenotypes including immunosuppression, viral replication, neuropathogenesis, neuroinvasion and non-progression (Na et al. 2011; Tcherepanova et al. 2009; Rajan et al. 2006; Mologni et al. 2006; Pomerantz et al. 2004) in functional studies and humans. We therefore evaluated the relationship between peripheral lymphocyte derived Vpr sequence

diversity, HIV associated neurocognitive impairment and clinical biomarkers of HIV infection. We discovered a mutation at position 45 on lymphocyte derived HIV-1 subtype C vpr sequences which distinguished HIV infected individuals with neurocognitive impairment (45Y) from those without neurocognitive impairment. The finding suggests that impact of Vpr on cognitive function can be altered by amino acid sequence changes that occur during the course of HIV infection (Dampier et al. 2018) as presence of this amino acid residue (45Y) was associated with a 3.67 times risk of having a higher GDS compared to those individuals without the residue.

We suggest that this residue may be involved in biological processes that contribute to neuropathogenesis of cognitive impairment in HIV-1 infection. This mutation located in the second α-helix which spans from positions 38-50, which is associated with virion packaging, reverse transcription, and oligomerization (Power et al. 2012). Oligomerization of Vpr is thought to be essential for virion incorporation property and may also have a role in the events associated with virus infection (Venkatachari et al. 2012) including virion incorporation and subcellular localization (Singh et al. 2000). Interestingly, this study has shown that the presence of this mutation is associated with 525 times higher plasma viral load and approximately 16 times proviral load than those without the mutation. Therefore, we suggest that the mutation may influence neurocognitive dysfunction through the establishment of peripheral HIV reservoir whose association with cognitive impairment has been demonstrated previously (Valcour et al. 2013, Jumare et al. 2017; Ruhanya et al. 2020). Plasma viral load could influence neurocognition in various ways, including possible crossing of the BBB by haematogenous spread and directly infecting brain cell like microglia and astrocytes or by releasing neurotoxic proteins. This mutation could therefore be associated with increased virus replication. This leads to increased antigenic load, which could promote a proinflammatory environment or dysregulated release of proinflammatory cytokines which are known to impair BBB integrity and results in uncontrolled influx of neurotoxins in the brain.

We have also identified a conserved fragment TYGDTW from position 49 to 54 which was significantly associated with a higher GDS (worse neurocognitive performance) compared to those without the conserved fragment. The same fragment was associated with 4.5 times risk of higher plasma viral load and 2.9 times risk of higher proviral load compared to those without. This is the first time that such an association between this Vpr fragment, GDS and viral load has been observed. What is known about this is that 51-52 act as connectors of α-

helix 2 and α-helix three allowing for both flexibility and steric stability of the protein's tertiary conformation (James et al., 2016). It has also been shown that this region of the vpr influences accuracy of reverse transcription due to the presence of Tryptophan (54W) (Wong-Staal et al. 1987; Mansky et al. 2000; Le Rouzic and Benichou 2005; Fabryova et al. 2019) and efficient replication of the virus macrophages (Chen et al. 2004). Therefore, we suggest future studies investigate the functional role of this conserved fragment on HIV neuropathogenesis.

In contrast, fragment QRRARNGA spanning from 86-93 was significantly related to lower GDS. This fragment is in the C-terminal. Although this fragment was associated with lower GDS compared to those without the fragment, it was not associated with neurocognitive impairment as defined by a GDS>.5. Previous investigations of polymorphisms of amino acid residues encompassing this fragment showed some polymorphisms without a dominant pattern and changes were not associated with any clinical outcome (Cavert et al. 2004). Although in vitro evidence suggests that critical determinants of apoptosis and cell-cycle arrest lie within the C terminus of Vpr, the presence of QRRARNGA in this region was not associated to decline or lower which are direct result of cell death or arrest in cell division.

When we evaluated the impact of vpr conserved fragments on clinical biomarkers of disease progression, four conserved fragments, PEDQGPQREPYNEW from position 5-18, PEDQGPQREPYNEWTLE from position 5- 21, and LGQYIY, from position 42-47, TYGDTW from position 49- 54 were significantly associated with higher plasma viral load and proviral load. The fragment is in the acidic region and the second spans from acidic region into the first 3 residues of the first α-helix. The acidic region and the α-helix1 are responsible for reverse transcription and virion packaging respectively (Power et al. 2012). Our observation suggests that these fragments or some residues contained in these fragments may play a role in promoting reverse transcription and virion packaging, thereby associating them with both proviral load and plasma viral load.

6. Conclusion

This study identified Tyrosine (Y) at position 45 (45Y) of Vpr HIV subtype C as signature amino acid residue for neurocognitive impairment in infected people. The signature was also linked to higher plasma viral load and proviral load implying that it could be involved in processes that lead to cognitive impairment involving viral load and the establishment of an

HIV reservoir. We propose that functional studies are needed to establish a mechanistic link between cognitive dysfunction and this residue.

Acknowledgements

This study was supported by the Poliomyelitis Research Foundation of South Africa (PRF), National Research Foundation (NRF), National Health Laboratory Services (NHLS) Research Trust and the South African Medical Research Council (SAMRC).

Authors' contributions

Study conception and design: VR, SE, RP. Acquisition of data: VR, RP, JJ, SS. Analysis, and interpretation of data: VR, SE, GJ. Drafting of manuscript and Critical revision: all authors

Competing interests

The authors declare no competing interests.

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Figure Legends

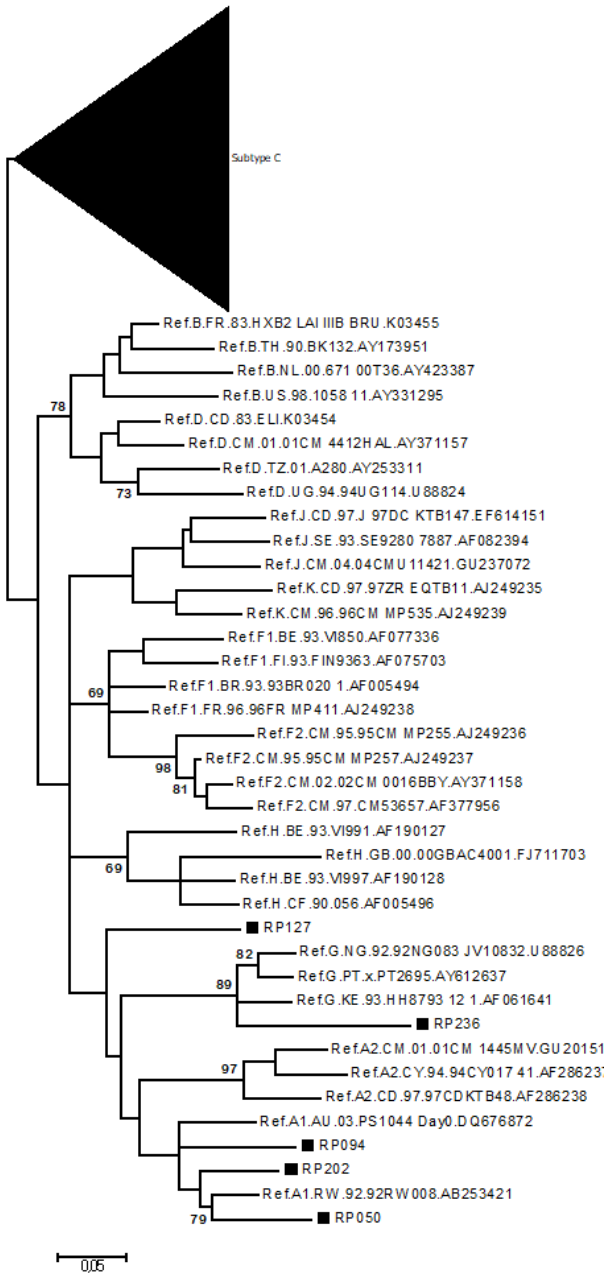

Figure 1. Phylogenetic analysis of the Vpr gene. *The evolutionary history was inferred by using the ML method based on the General Time Reversible model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There was a total of 291 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 with 500 bootstraps*

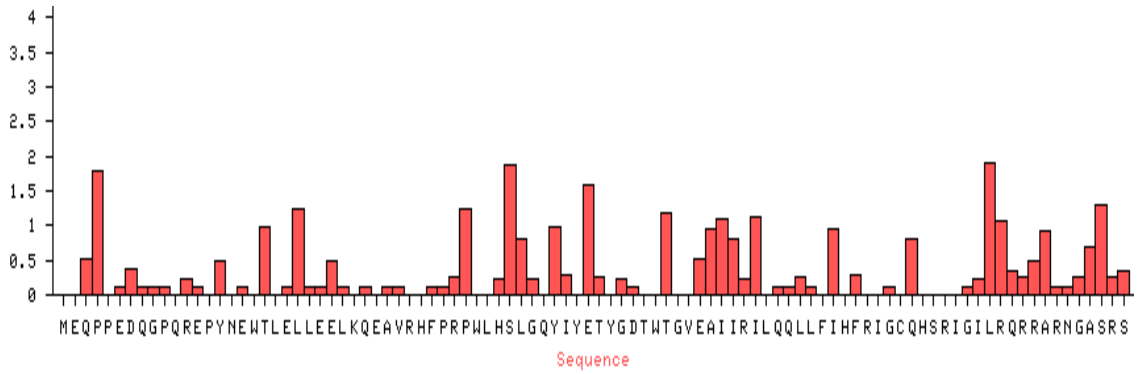

Fig 2. *VPR alignment variability plot of individuals with neurocognitive impairment, showing positions 4 (H = 1.799), 37) H = 1.254), 41 (H = 1.884), 48 (1.486), 55(1.196) and 85(1.067) having variability indices above 1. The plots also show the consensus amino acid at each position in the alignment***.**

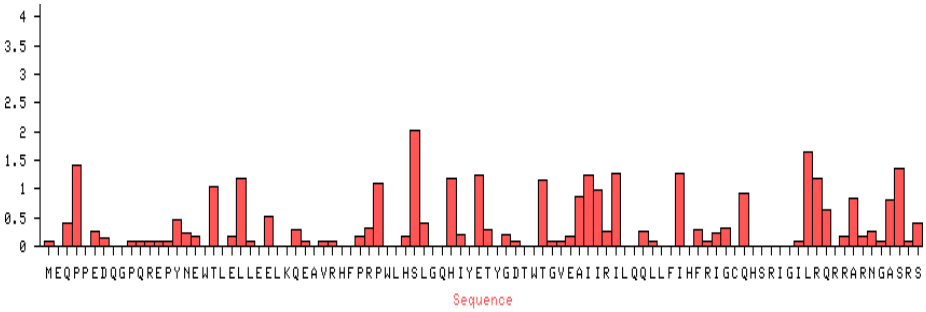

Fig3. *VPR alignment variability plot of individuals without neurocognitive impairment showing positions 4(H = 1.409),19(H = 1.035), 22(H = 1.172), 37(H = 1.086), 41(H = 2.031),45(H = 1.195), 48(H = 1.248), 55(H = 1.165), 60(H = 1.239), 84(1.655), 85(H = 1.174), 94(H = 1.367*)

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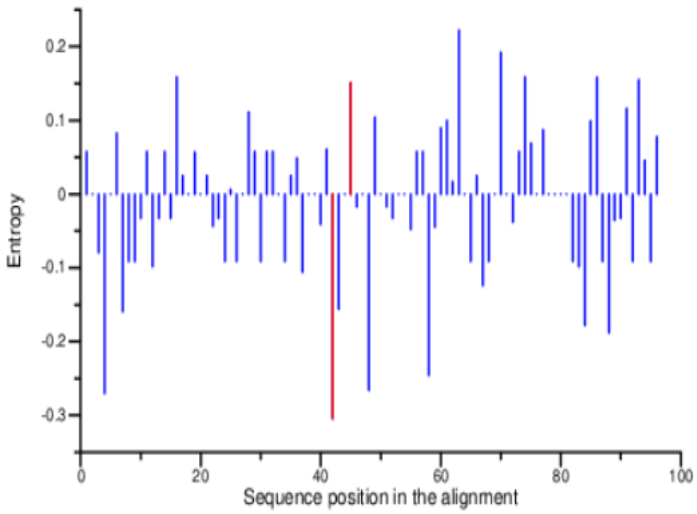

Fig 4. *Entropy differences between impaired and non-impaired amino acid alignments with significant sites p<=0.05 are shown in red in the difference plot*

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

The publications included in this dissertation summarized the findings of investigations characterising the relationship between NCI and peripheral proinflammatory cytokines, lymphocyte HIV proviral load and variation on lymphocyte-derived *tat* and *vpr* sequences. The aim was to identify potential peripheral biomarkers of neurocognitive impairment in HIV-1 subtype C infection using high through put immunotechnology, molecular methods, bioinformatic tools and statistical models in the context of South Africa. HAND screening in HIV is recommended to identify clinically important changes in cognitive function and allow early interventions [1], but this is not currently done in clinical practice in South Africa. This is due to inadequate staff with specialised skills and numerous PLWH competing for health care in a resource limited setting. The studies presented in this dissertation contribute a background for further investigations based on host immune response biomarkers and viral factors that reveal the underlying pathology of HAND to develop laboratory assays for prognosis and monitoring the condition. Biomarkers would become more useful in identifying patients with subtle neurocognitive deficits which contribute 88% of individuals with HAND [2], where psychometric performance testing is challenging [3].

In the second chapter, the relationship between lymphocyte-derived HIV-1 subtype C proviral DNA and HIV-associated neurocognitive impairment were investigated. The investigations were motivated by the fact that peripheral infection results in seeding of viral reservoirs which persist in different parts of the body including the central nervous system [4]. Lymphocytes, which include CD4+ T cells, are recognized as the major reservoir of HIV. This study demonstrated that the burden of HIV-1 peripheral blood lymphocyte proviral DNA corresponds to neurocognitive impairment among individuals infected with clade C disease. It also demonstrated that HIV-1 DNA in cognitively impaired PLWH was significantly higher than in cognitively normal individuals which was detailed in P**aper 2** (Chapter 1). The strength of this study is that further investigations were performed to determine lymphocyte-derived proviral HIV-1 DNA threshold value or cut-off value for neurocognitive impairment associated accuracy (detailed in **Paper 6)**. The sensitivity of 60% and specificity of 51% of lymphocyte proviral DNA threshold for neurocognitive impairment

fall in the range of diagnostic accuracies of international HIV dementia scale (IHDS) and other psychometric tests of HAND studies done in South Africa, whose specificities ranged from 37% to 81% and sensitivity from 45% to 100%. Therefore, this biomarker is promising as it compares well with standard screening tools for HAND. However, more studies are needed on diagnostic accuracy of proviral DNA cut-off values in both treated and treatment-naive individuals. As more data accumulates on lymphocyte proviral HIV DNA threshold values for neurocognitive impairment, therapeutic strategies to reduce the HIV-1 proviral DNA reservoir in lymphocytes may improve neurocognitive outcomes in PLWH.

Proviral DNA in PBMCs has been shown to be involved in neurocognitive impairment in HIV infection has generated important information that has a potential to translate into clinically beneficial outcomes. In our study, we explored the potential utility of proviral DNA for prognosis of HIV associated neurocognitive impairment. In the review paper (**Paper 2**), we conducted a detailed review on the clinical relevance HIV DNA in peripheral blood mononuclear cell compartments as a biomarker of HAND [5]. The advantage of PBMC compartments as samples for studying deterioration in neurological function in HIV is that it is a clinically more accessible as compared to brain biopsy and CSF [6]. Assays used to quantify HIV DNA by qPCR utilized both unfractionated PBMC and sorted cell subsets. However, choice must be made on whether to use unfractionated PBMC or cell subsets. The best clinical specimen should give the true size of peripheral HIV-1 DNA which enables diagnosis and monitoring disease progression [7] with its role in the pathology of the disease well characterised. An appropriate clinical specimen gives information which reflects on the condition of the disease by correlating with findings from clinical assessments. In this study we demonstrated that lyphocyte-derived proviral HIV DNA was positively correlated to global deficits in neurocognitive function. This PBMC fraction contains the CD57−CD4+ T memory cells which contain majority of HIV DNA. Our study is one of the first studies to utilize lymphocyte derived proviral HIV DNA to predict cognitive impairment in HIV-1 in Sub-saharan Africa [8]. Although the use of use of this sample incurs extra cost of sorting and separating PBMC fractions, it contains a primary target for HIV infection and represents HIV reservoir in the periphery.

The pattern of cytokines / chemokines released or persisting in individuals with a particular clinical disease, may offer important insights into the pathogenesis of that disease. HIV-associated neurocognitive impairment has been linked to increased peripheral cytokine

profiles [9] and chronic systemic inflammation [10]. This points to the potential utility peripheral cytokine markers for understanding underlying neuropathological mechanisms in cognitive dysfunction as well as prognostic tools for the condition [11, 12]. We identified novel associations between plasma IP-10/CXCL10, RANTES cognitive impairment, plasma viral load, cell-associated viral load and absolute CD4+ T cell count [13]. The fact that IP-10 and RANTES were strong predictors of GDS, strongly associated with measures of viral load and formed a separate cluster indicates that we have identified a signature plasma chemokine biomarker linked to neurocognitive impairment in PLWH (**Paper 3**).

Further investigation using domain-specific cognitive function showed that there was differential impact of different cytokines on different cognitive domains. IL-2 was linked to impaired recall, recognition, mental control, and executive function (**Paper 4**). In addition, neuroimaging investigations showed that higher levels of inflammatory chemokines including RANTES, and MCP-1 were related to lower white and grey matter volumes (**Paper 5**). The results support earlier findings linking peripheral cytokines to neurodegerative processes resulting in inflammation-associated brain atrophy in HIV infection [12, 14]. Virus-induced systemic immune activation and inflammatory processes have been linked to brain dysfunction in HIV infection [15] and bidirectional neuro-immune pathways mediating inflammatory signals from the periphery to CNS have been identified in HIV infection [16].

Neuroimaging has also revealed that brain structures and complex neuro-circuits in the CNS are modulated by cytokine signalling from the periphery [17]. Cytokines also alter neurometabolism of neurotransmitters, serotonin, dopamine which in turn leads to decreased growth factors necessary for neurogenesis. Our study has shown the effect of cytokines on the cognitive function at a clinical level demonstrated by their association with global and domain-specific cognitive impairment and a ‘pathological’ level by association with lower gray matter and white matter volumes. Therefore, we suggested that cytokines could be used as peripheral indices of HIV-associated neurocognitive impairment in concert with behavioural assessments. As potential biomarkers for HIV-associated brain dysfunction, we determined cytokine threshold / cut-off values and their associated diagnostic accuracies as a first step for assessing possibilities of using them in clinical practice (**Paper 6**). The pooled diagnostics accuracy for the cytokine biomarkers assessed in this study ranged from sensitivity of 46% to 67% and specificity of 39% to 72%. Considering psychometric tests of HAND studies done in South Africa whose specificities ranged from 37% to 81% and

sensitivity from 45% to 100%, the diagnostic parameters obtained in this study are comparable. We suggest that more studies which combine the biomarkers may improve the possible diagnostic accuracy of cytokine biomarkers.

Genetic diversity, which is the hallmark of HIV-1, affects different aspects of the infection including pathogenesis, diagnosis and antiretroviral therapy. In this study we hypothesized that naturally occurring polymorphisms on two HIV-1 subtype C neurotoxic genes, *tat* and *vpr* were related HIV associated neurocognitive disorders. In support of this hypothesis, we identified two amino acid residues on Tat, Lysine at codon 24, (24K) and Arginine at codon 29 (29R) which could distinguish individuals with HIV-associated neurocognitive impairment. The findings are detailed in **paper 7**. These two signature amino acids for HIV associated neurocognitive impairment were related to classical markers for monitoring HIV infection like plasma viral load, proviral load and CD4+ T cell count, implying that that they are involved in processes in neuropathogenesis. These signatures should be further investigated as potential virological markers for the condition.

We also identified Tyrosine (Y) at position 45 (45Y) of Vpr HIV subtype C as signature amino acid residue for neurocognitive impairment in infected people. Additionally, we identified 4 conserved Vpr fragment sequences, PEDQGPQREPYNEWTLE (5 to 21), LGQYIY (42 to 47), TYGDTW (49 to 54), PEDQGPQREPYNEW (5 to 18) that were higher in plasma viral load and proviral load. The implication of these findings is that variation on Vpr leads to neurocognitive impairment in HIV infection and worsen the progression of disease in general by promoting establishment of provirus, HIV replication and depletion of CD4+ T cells in the periphery.

Overall strength of the Study

The study demonstrated that there are multiple peripheral blood factors that are related to neurocognitive dysfunction in a cohort which was rich in clinical, demographic, and neuroimaging profiles of individuals recruited. We applied high throughput immunoassays and powerful statistical models to determine novel associations between inflammatory cytokine biomarkers and HIV-associated neurocognitive impairment. This study also determined virological and plasma cytokine cut-off values for neurocognitive impairment in HIV infection and their associated diagnostic accuracies. This is an important milestone in search for biomarkers for HAND. The biomarker cut-offs can be used as treatment endpoints

or screening tools particularly in SSA where the prevalent of the disease is high (72%) but limited specialised training for screening and monitoring.

The two Tat signatures for HIV-associated neurocognitive impairment, 24K and 29R have not been identified before in HIV subtype C. The strength of this finding is important because it correlates with clinical markers that are routinely used to screen and monitor progression of HIV infection like viral load and absolute CD4+ T cell count. Of note, 24K was also related to two important cytokines, IP-10 and RANTES which were identified as plasma cytokine biosignature for neurocognitive from a panel of 27 plasma cytokines. This result indicate that it might be involved in processes that amplify peripheral inflammatory environment in HIV infection which in turn negatively impacts on cognitive function.

A third signature for neurocognitive impairment, Tyrosine at position 45 (45Y) was on Vpr which was also strongly related to the clinical markers of HIV infection. This signature has not been described before. Using protein variability sever (PVS), we also described conserved fragments on Vpr which were related to clinical markers of HIV-1 infection, particularly plasma viral load and lymphocyte. This observation supports the importance of Vpr in virus replication in dividing and non-dividing cells and to the pathogenesis of HIV-1 infection [18]. The bioinformatic and statistical tools have revealed the multifaceted effect of Vpr on HIV-1 infection.

Overall limitations of the work

The study is limited in several ways. It was a cross-sectional study based on cohort sampled mostly in primary care HIV clinics in Cape Town. It would be desirable to have a wider geographical representation of sampling. The spectrum of neurocognitive impairment ranged from mildly to moderately impaired individuals. The mild and the moderate impairment categories which are the most prevalent forms of HAND globally were predominant in our cohort. This showed that our cohort represented the current clinical picture of the condition. However, it was desirable to have severe forms HAND to have a clear picture of how the detected biomarkers and viral genetic signatures relate to the mild / asymptomatic impairment and the severe form, HAD and to stratify cytokines in immune suppressed (< 200 CD4+ T cell counts/ul) and immunocompetent (> CD4+ T cell counts/ul). In addition, it would be important to have functional data to support our findings which were based on clinical data. Other variables including host factors which did not investigate may have

affected our findings. Therefore *in vitro* studies which are well-controlled should be done to provide a mechanistic link between the novel plasma biomarkers and viral genetic signatures for HAND identified in this study. It would be also be insightful to compare variation and possibly genetic signatures in monocytes (CD14+) and lymphocytes (CD14-) derived HIV proviral DNA sequences

Conclusions

Proviral load and plasma inflammatory biomarkers are a practical and feasible tool for screening and monitoring neurocognitive impairment in HIV infection. However, the clinical validity of these biomarkers could be improved with use of algorithms that combine virological and inflammatory biomarkers as suggested in the thesis publications. In addition, the three identified genetic signatures for cognitive impairment in HIV-1 infection need to be supported by functional data. Investigations should also investigate the impact of identified genetic signatures and variation on cytokine biomarkers and MRI measures of brain and in future studies to compare cytokine levels in different subtypes for example subtypes B vs. C).

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Appendix

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Approval Notice
New Application

16-Mar-2017
Ruhanya, Vurayai V

Ethics Reference #: **S17/02/035**

Title: **In depth analysis of HIV-1 diversity and inflammatory markers in different cell compartments in HIV associatedneurocognitive disorders (HAND)**

Dear Mr Vurayai, Ruhanya,

The **New Application** received on **10-Feb-2017**, was reviewed by Health Research Ethics Committee 2 via Committee Review procedures on **15-Mar-2017** and has been approved.
Please note the following information about your approved research protocol:

Protocol Approval Period: **15-Mar-2017 -14-Mar-**

2018Present Committee Members:
Hendricks, Johann
JAMorris, Linzette
LD Holgate,
Sandilee S
Du Plessis,
Lisanne LM
Masiye, Francis
FD Bloem, Josua
JJF Naidoo, Vikesh
VT
Van der Merwe,
Anita ASBlouws,
Firnley F Makiwane,
Memela MM
Langenegger, Eduard
EJ Mann, Theresa
TNC Sewram,
Vikash V Kruger,
Wilma WM
Swanepoel, Carmen
CC Edwards, C E

Please remember to use your **protocol number** (**S17/02/035**) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:

Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.
Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number:
IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel:
+27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.
For standard HREC forms and documents please visit: www.sun.ac.za/rds

If you have any questions or need further assistance, please contact the HREC office at .

Included Documents:
HREC_Application_Ruhanya_PhD_2017.d
oc Paul_2016_JNVi_tat.pdf
06_CV_Vurayai_Ruhanya.pdf
N08-01-011 Ethics Approval Letter for
Continuation.pdf 06_CV_Supervisor_Engelbrecht.pdf
General_Checklist_Ruhanya_PhD.doc
Registration_PhD_Ruhanya_Checklist.docx
Letter_PhD_Ruhanya_2017.docx
06_CV_Co-supervisor_Jacobs.pdf
Investigator_Declaration_Engelbrecht_signed.pdf
05_Synopsis_PhD_Ruhanya_Stellenbosch_updated.do
cx Paul_2014_JNVi_tat.pdf
06_CV_Co-supervisor_Glashoff.pdf
Investigator_Declaration_Glashoff.pdf
S. AfricaAimsThruRefs.pdf
Letter_Ethics_Ruhanya_2017.docx
05_Full_protocol_Ruhanya_PhD-
aplication_updated.docx
Investigator_Declaration_Jacobs.pdf
Investigator_ declaration_Ruhanya.pdf

Sincerely,

Francis Masiye

HREC Coordinator
Health Research Ethics Committee

Investigator Responsibilities

Protection of Human Research Participants

Some of the responsibilities investigators have when conducting research involving human participants are listed below:

1. Conducting the Research. You are responsible for making sure that the research is conducted according to the HREC approved research protocol. You are also responsible for the actions of all your co-investigators and research staff involved with this research.

2. Participant Enrolment. You may not recruit or enrol participants prior to the HREC approval date or after the expiration date of HREC approval. All recruitment materials for any form of media must be approved by the HREC prior to their use. If you need to recruit more participants than was noted in your HREC approval letter, you must submit an amendment requesting an increase in the number of participants.

3. Informed Consent. You are responsible for obtaining and documenting effective informed consent using **only** the HREC-approved consent documents, and for ensuring that no human participants are involved in research prior to obtaining their informed consent. Please give all participants copies of the signed informed consent documents. Keep the originals in your secured research files for at least fifteen (15) years.

4. Continuing Review. The HREC must review and approve all HREC-approved research protocols at intervals appropriate to the degree of risk but not less than once per year. There is **no grace period.** Prior to the date on which the HREC approval of the research expires, **it is your responsibility to submit the continuing review report in a timely fashion to ensure a lapse in HREC approval does not occur.** If HREC approval of your research lapses, you must stop new participant enrolment, and contact the HREC office immediately.

5. Amendments and Changes. If you wish to amend or change any aspect of your research (such as research design, interventions or procedures, number of participants, participant population, informed consent document, instruments, surveys or recruiting material), you must submit the amendment to the HREC for review using the current Amendment Form. You **may not initiate** any amendments or changes to your research without first obtaining written HREC review and approval. The **only exception** is when it is necessary to eliminate apparent immediate hazards to participants and the HREC should be immediately informed of this necessity.

6. Adverse or Unanticipated Events. Any serious adverse events, participant complaints, and all unanticipated problems that involve risks to participants or others, as well as any research-related injuries, occurring at this institution or at other performance sites must be reported to the HREC within **five (5) days** of discovery of the incident. You must also report any instances of serious or continuing problems, or non-compliance with the HRECs requirements for protecting human research participants. The only exception to this policy is that the death of a research participant must be reported in accordance with the Stellenbosch Universtiy Health Research Ethics Committee Standard Operating Procedures www.sun025.sun.ac.za/portal /page/portal/Health_Sciences/English/Centres%20and%20Institutions/Research_Development_Support/Ethics/Application_package All reportable events should be submitted to the HREC using the Serious Adverse Event Report Form.

7. Research Record Keeping. You must keep the following research-related records, at a minimum, in a secure location for a minimum of fifteen years: theHREC approved research protocol and all amendments; all informed consent documents; recruiting materials; continuing review reports; adverse or unanticipated events; and all correspondence from the HREC

8. Reports to the MCC and Sponsor. When you submit the required annual report to the MCC or you submit required reports to your sponsor, youmust provide a copy of that report to the HREC. You may submit the report at the time of continuing HREC review.

9. Provision of Emergency Medical Care. When a physician provides emergency medical care to a participant without prior HREC review and approval, to the extent permitted by law, such activities will not be recognised as research nor will the data obtained by any such activities should it be used in support of research.

10. Final reports. When you have completed (no further participant enrolment, interactions, interventions or data analysis) or stopped work on your research, you must submit a Final Report to the HREC.

11. On-Site Evaluations, MCC Inspections, or Audits. If you are notified that your research will be reviewed or audited by the MCC, the sponsor, any other external agency or any internal group, you must inform the HREC immediately of the impending audit/evaluation.