AN INVESTIGATION OF A NEURO-INFLAMMATORY PROFILE OF HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS

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WLLMON009

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Professor John Joska

Professor Dan Stein

“Peace amidst our earthly fears,

strength through trials and through tears;

joy which sorrow cannot dim,

hope which draws me close to Him.

God gave me the very best,

He my heart has richly blest

Grace and mercy are my share,

boundless wealth beyond compare;

gifts abundant came to me,

well can I contented be.

God gave me the very best,

He my heart has richly blest!”

A dedication to my parents, Marlene and Edward Williams

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DECLARATION

I, Monray Edward Williams, do hereby declare that this thesis is based on five journal manuscripts: Four which have been published (Chapters 2, 3, 4 and 5) and one which is in preparation for submission (Chapter 6). These manuscripts have been formatted uniformly for the purposes of this thesis, with regards to referencing style and use of terms. The content of each manuscript remains unchanged from that which has been either published or submitted for publication, but the introduction and conclusion of each have been edited in a manner to underscore the coherence of the entire thesis i.e. how each chapter links to the next and the others. I confirm that I have been granted permission by the University of Cape Town's Doctoral Degrees Board to include the following publication(s) in my PhD thesis, and where co-authorships are involved, my co-authors have agreed that I may include the publication(s). The manuscripts included are listed below, with a description of my contribution to each.

Chapter 2

Williams, M.E., Ipser, J.C., Stein, D.J., Joska, J.A., Naudé, P.J.W., 2020. ***Peripheral immune dysregulation in the ART era of HIV-associated neurocognitive impairments: A systematic review.*** **Psychoneuroendocrinology 118, 104689. https://doi.org/10.1016/j.psyneuen.2020.104689**

This systematic review summarized clinical research of the association of peripheral immune markers with HIV-associated neurocognitive impairments in ART-experienced participants. This manuscript identified the inconsistencies in the current literature and highlighted the need for investigation of new immune markers (Chapter 3). For this manuscript, together with Dr. Pieter Naudé, I have generated the concept for the manuscript. With guidance from Dr. Jonathan Ipser, I have generated the search strategy. Both Dr. Pieter Naudé and I reviewed all papers for potential inclusion and established our level of agreement. I then analysed and summarized all data myself and generated the first full draft of the manuscript. All co-authors reviewed the draft, made conceptual and intellectual contributions, and edited the final draft. I managed all revisions.

Chapter 3

Monray E. Williams, Jonathan C. Ipser, Dan J. Stein, John A. Joska, Petrus J.W. Naudé. *The association of immune markers with cognitive performance in South African HIV-positive patients. Journal of neuroimmune pharmacology.* 2019. 14, 679-687, https://doi.org/10.1007/s11481-019-09870-1

This is the first manuscript (Chapter) of the thesis which presented empirical data. Here, I investigated several blood immune markers for their association with HIV-associated neurocognitive impairment. The immune markers were upregulated in people living with HIV (PLWH), with markers having an association with domain-based neurocognitive impairment. This study was performed in samples that were obtained from cohorts of Prof. John Joska and Dr. Jonathan Ipser. Together with Dr.

Pieter Naudé, I generated the concept of the manuscript. I did the literature mining and searches. I performed the laboratory ELISA myself. Dr. Pieter Naudé assisted with the statistical analysis. I wrote the first full draft of the manuscript. All authors provided conceptual and intellectual contributions to the final drafts. I managed all revisions.

Chapter 4

Williams, M. E., Joska, J.A., Amod, A.R., Paul, R.H., Stein, D.J., Ipser, J.C., Naudé, P.J.W., 2020. *The association of peripheral immune markers with brain cortical thickness and surface area in South African people living with HIV*. J. Neurovirol. https://doi.org/10.1007/s13365-020-00873-w

This manuscript served as the second chapter presenting empirical data and was a follow-up of chapter 3. Several immune markers were associated with HIV-associated neurocognitive impairment (Chapter 3). The current chapter determined the association of blood immune markers with cortical thickness and surface area measured by MRI. I conducted the laboratory ELISA experiments myself. This study was performed in samples that were obtained from cohorts of Prof. John Joska and Dr. Jonathan Ipser. Together with Ms. Alyssa Amod and Dr. Jonathan Ipser, I conducted the quality control of all MRI data and I generated the output data myself. Dr. Pieter Naudé assisted with the statistical analysis. I wrote the first draft of the manuscript myself. All authors provided conceptual and intellectual contributions to the final drafts. I managed all revisions.

Chapter 5

Monray E. Williams, Simo S. Zulu, Dan J. Stein, John A. Joska, Petrus J.W. Naudé. ***Signatures of HIV-1 subtype B and C Tat proteins and their effects in the neuropathogenesis of HIV-associated neurocognitive impairments*** **(2020)** ***Neurobiology of disease.*** **https://doi.org/10.1016/j.nbd.2019.104701**

This manuscript reviewed and summarized the current literature between HIV-1B and HIV-1C, in particular, Tat-B and Tat-C and its differential effects on the underlying mechanisms of HIV-associated neurocognitive impairment. Together with Dr. Pieter Naudé and Prof. John Joska, I generated the concept for the manuscript. Dr. Simo Zulu contributed to the animal model section. I did the literature mining and searches and wrote the first draft myself. Dr. Pieter Naudé assisted in reviewing the relevant drafts. All authors provided conceptual and intellectual contributions to the final drafts. I managed all revisions.

Chapter 6

Monray E. Williams, Ruhanya Vurayai, Susan Engelbrecht, Dan J. Stein, John A. Joska, Petrus J.W. Naudé. ***Impact of the HIV Tat $C_{31}S$ and $R_{57}S$ mutations on peripheral immune marker levels in subtype-C infection: An exploratory study. In submission: Journal of AIDS Research and Human Retroviruses***

The last manuscript was as an exploratory/pilot study with secondary data analysis. This manuscript investigated the effect of Tat protein sequence variation on

peripheral immune marker levels. Prof. John Joska, Dr. Pieter Naudé and I have generated the concept for the manuscript. I did the laboratory ELISA experiments myself. HIV-1 genotyping was done by Mr. Ruhanya Vurayai and Prof. Susan Engelbrecht from Stellenbosch University, Division of Medical Virology. This study was performed in samples that were obtained from cohorts of Prof. John Joska. Dr. Pieter Naudé assisted with the statistical analysis. I wrote the first full draft of the manuscript. All authors provided conceptual and intellectual contributions to the final drafts.

I confirm that no part of this thesis has been submitted in the past, or is being, or is to be submitted for a degree in this or any other university. I hereby grant the University of Cape Town free license to reproduce this thesis in whole or part for the purpose of research or teaching.

This thesis is presented for examination in fulfilment of the requirements for the degree of Doctor in Philosophy.

Name: Monray Edward Williams

Student number: WLLMON009

Signature: Signed by candidate

Date: 10 February 2020

ABSTRACT

Title: An Investigation of a Neuro-Inflammatory Profile of HIV-Associated Neurocognitive Disorders

Background

HIV-associated neurocognitive disorder (HAND) is the consequence of the effects of HIV-1 within the central nervous system (CNS). HIV-associated neurocognitive impairment differs in severity with milder forms presenting in 50% of people living with HIV (PLWH), regardless of treatment status. Chronic immune dysregulation has been associated with HAND; in particular, it has been noted that inflammation persists despite the successful treatment with antiretroviral therapy (ART). However, the nature to which (neuro)inflammation influences cognitive performance and brain integrity remain unclear. Further, it is not clear how sequence variation in neurotoxic viral proteins, including Tat, affects inflammation in PLWH. This study aimed to 1) perform a systematic review of the existing literature to identify changes in peripheral immune markers that are associated with HAND in ART-experienced PLWH, 2) determine the association of blood peripheral immune markers with domain-based neurocognitive performance and structural brain changes in South African PLWH, and 3) lastly, to evaluate the possible influence of Tat sequence variation on a dysregulated immune profile in HIV-1C infection (i.e. Tat-C).

Methods

A systematic review of the published literature was performed to identify the most common markers associated with HAND in the ART-era. A panel of markers was

measured in a treatment naïve South African cohort by enzyme-linked immunosorbent assays (ELISA). Cognitive performance was established using a battery of tests sensitive to HIV-associated neurocognitive impairment, with domain-based scores utilized in analysis. Thickness and surface area of all cortical regions were derived using automated parcellation of T1-weighted images acquired at 3T. Markers were correlated with neurocognitive performance and cortical thickness and surface area. Further, a prospective review of the literature was performed to determine the association between Tat sequence variation and underlying mechanisms (and inflammation) of HAND. The HIV-1 was genotyped and the influence of Tat sequence variation on immune marker levels was evaluated in a subset of South African participants.

Results

A systematic review of the existing literature suggested that peripheral immune markers of monocyte activation (sCD14 and sCD163) and inflammation (IL-18 and IP-10) were associated with HAND in the majority of studies. Evaluation of blood immune markers in a treatment naïve South African cohort showed that thymidine phosphorylase (TYMP) and neutrophil gelatinase-associated lipocalin (NGAL) levels were significantly higher, while matrix metalloproteinase (MMP)9 levels were significantly lower in PLWH. The results further showed that in PLWH, worse psychomotor processing speed was associated with higher TYMP and NGAL levels and worse motor function was associated with higher NGAL levels. Further, in imaging analysis, it was reported that higher NGAL levels were associated with the reduced thickness of the bilateral orbitofrontal cortex. The association of NGAL with

worse motor function was mediated by the cortical thickness of the bilateral orbitofrontal cortex. The associations between higher NGAL and TYMP levels with cortical thickness were largely found in the regions of the frontal cortex. A review of the literature suggests that key protein signatures ($C_{31}S$ and $R_{57}S$) present in the Tat protein from HIV-1 subtype C (Tat-C) infection may contribute to the lowered inflammation. Supporting this hypothesis, the results from this thesis showed that HIV-1C participants with the $R_{57}S$ mutation had lower peripheral TYMP levels.

Conclusions

Current literature supports the premise that chronic inflammation may be an important contributor to the development of the milder forms of HAND. For patients on ART, other strategies are required to address the ongoing peripheral inflammation, in addition to simply suppressing the viral load. In a South African context, TYMP and NGAL may be promising markers for their involvement in HAND. Patients were largely treatment-naïve; therefore, these markers may represent HIV-related effects without the potential confounding effects of ART. Therefore, these findings may represent long-standing effects which might persist in treatment-experienced participants. In HIV-1C infection, the level of certain inflammatory markers may be influenced by the $R_{57}S$ Tat protein signature. To our best knowledge, this is the first thesis to report the association of these markers with HAND. These immune markers need to be investigated for their potential role in the underlying mechanisms of HAND.

ACKNOWLEDGEMENTS

Thank you to God, for providing me undeserved grace and mercy throughout this challenging journey.

Support for the research presented in this thesis was provided by the South African National Research Foundation and the Poliomyelitis Research Foundation.

I would like to acknowledge my supervisors, Prof. John Joska and Prof. Dan Stein, for your invaluable contributions, guidance and mentorship throughout this project. I am thankful to have had an opportunity to learn from you. To my supervisor, Dr. Pieter Naude' (including Nynke and Stella), I would not have made it through this journey without your constant support, motivation and guidance. You kept the fire burning. I was extremely fortunate to have worked with what may be the greatest minds around.

I would like to thank those who contributed to the research presented in this thesis, including; Dr. Jonathan Ipser, Dr. Simo Zulu, Prof. Rob Paul, Ms. Alyssa Amod and Mr. Ruhanya Vurayai. This thesis would've been incomplete without your contribution. Thank you to Prof. Fleur Howells and her group for including in me in many hours of journal club, which I hope is reflected in this thesis.

More importantly, this thesis would not have been possible if I did not have the support of my family. Firstly, to my parents, supportive is an understatement. I hope

I have made you proud. God truly gave me the best. To my siblings, Grant and Tarrenqiue, I thank you for always believing in me. To my beautiful wife, Tarryn, who has seen every side of this thesis, thank you for sticking around. I could not have asked for a better partner and support system to what you have provided me.

Finally, to each family member, friend and minister that has remembered me and prayed for me, I cannot thank you enough.

This thesis is a shared gift for all those mentioned above, and I simply cannot claim it for myself.

God gave me the best, he my heart has truly blest!

ABBREVIATIONS AND ACRONYMS

Acquired immunodeficiency syndrome (AIDS)

American Academy of Neurology (AAN)

Amyloid beta 1-42 ($A\beta_{1-42}$)

Amyloid precursor protein (APP)

Amyloid-beta (Aβ)

Analyses of covariance (ANCOVA)

Antiretroviral therapy (ART)

Antiretroviral (ARV)

Arginine (R)

Asymptomatic neurocognitive impairment (ANI)

Blood–brain barrier (BBB)

C-C chemokine receptor type (CCR)

C-reactive protein (CRP)

Camp response element-binding protein (CREB)

Catalase (CAT)

Central nervous system (CNS)

Chemokine (C-C motif) ligand (CCL)

Chemokine (C-X-C motif) ligand (CXCL)

Chemokine receptor (CCR)

Cluster of differentiation (CD)

Combination antiretroviral therapy (cART)

Cysteine (C)

Endoplasmic Reticulum (ER)

Enzyme-linked Immunosorbent assay (ELISA)

Erythropoietin (EPO)

FMS-related tyrosine kinase 4 (FTK-4)

Glutamic acid (E)

Glutamine (Q)

Glutathione (GSH)

Glutathione peroxidase 1 (gpx1)

Glutathione synthetase (GSS)

Glycoprotein 120 (gp120)

Hepatitis C (HCV)

Highly active antiretroviral therapy (HAART)

HIV-associated neurocognitive disorders (HAND)

HIV-associated neurocognitive impairment (HANI)

HIV neurobehavioral research center (HNRC)

HIV-1 clade B (HIV-1B)

HIV-1 clade C (HIV-1C)

HIV-associated dementia (HAD)

Human astrocytes (HA)

Human brain microvascular endothelial cells (hbmvecs)

Immature dendritic cells (IDC)

Indoleamine-2,3-dioxygenase (IDO)

Interferon (IFN)

Interferon gamma-induced protein (IP-10)

Interleukin (IL)

Interquartile rang (IQR)

Junctional adhesion molecule (JAM)

Kynurenine (KYN),

Limulus amebocyte lysate (LAL)

Long terminal repeat (LTR)

Low-density lipoprotein receptor-related protein (LRP)

Macrophage colony stimulating factor (M-CSF)

Macrophage Inflammatory Protein (MIP)

Magnetization-prepared rapid acquisition gradient echo (MPRAGE)

Matrix metalloproteinase (MMP)

Memorial Sloan Kettering (MSK)

Mild neurocognitive disorder (MND)

Mitogen-activated protein kinase (MEK) ½,

Monocyte (MC)

Monocyte-derived macrophages (MDM)

Multi-drug resistance protein (MRP)

N-methyl-D-aspartate (NMDA)

N-methyl-D-aspartate receptor (NMDAR)

NADPH oxidase (NOX)

Neuroblastoma cells (SK-N-MC)

Neurocognitive impairment (NCI)

Neutrophil gelatinase-associated lipocalin (NGAL)

Nitric oxide synthase (NOS)

Normal cognition (NC)

Not Available (N/A)

Nucleotide-binding oligomerization domain containing 2 (NOD2)

People living with HIV (PLWH)

Phosphoinositide 3-kinases (PI-3K)

Polymerase chain reaction (PCR)

Progranulin (PGRN)

Quinolinic acid (QUIN)

Reactive oxygen species (ROS)

Redox-sensitive kinase (PYK2)

Regions of interest (ROI)

Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES)

Soluble cluster of differentiation (sCD)

Soluble Tumour Necrosis Factor (TNF) receptor (sTNFR)

Solute carrier family 11 (SLC11A1)

Stromal cell-derived factor (SDF)

Superoxide dismutase 1 (SOD1)

Kynurenine pathway (KP)

The L-type voltage-gated calcium channels (L-channels)

Thymidine phosphorylase (TYMP)

Tissue inhibitors of metalloproteinases (TIMP)

TNF-related apoptosis-inducing ligand (TRAIL)

Transactivation response element (TAR)

Transactivator of transcription (Tat)

Transforming growth factor (TGF)

Tumor Necrosis Factor (TNF)

Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor receptor-2 (VEGFR-2)

Viral protein r (Vpr)

Zona occuldens (ZO)

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Note: The numbers of the tables and figures are presented as in the chapters in which they appear.

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Chapter 1

Introduction

The (neuro)inflammatory profile of HIV-associated neurocognitive impairment in the South African context

1.1. Context

HIV-associated neurocognitive disorder (HAND) is the consequence of the effects of HIV-1 within the central nervous system (CNS) (González-Scarano and Martín-García, 2005). Approximately 50% of people living with HIV (PLWH) may develop milder forms of HAND despite viral suppression (Heaton et al., 2010). The onset and severity of HAND are affected by HIV subtype differences (Tyor et al., 2013), with HIV-1 subtype-B (HIV-1B) and HIV-1 subtype-C (HIV-1C) having different effects (Santerre et al., 2019). HIV-1B is present in America, Western Europe, and Australasia and represents about 12% of all HIV infections (Taylor et al., 2008). In contrast, the more prevalent HIV-1C is present in countries of Southern Africa and India and represents about 50% of the world's HIV infected population (Geretti, 2006).

The neuropathogenesis of HIV-1 is multifactorial and not clearly understood. Our current understanding of the HIV-1 neuropathogenesis is largely derived from studies of HIV-1B infection (Taylor et al., 2008). There is a need for further investigation of the underlying neuropathology of HIV-1C infection. This is important as HIV-1C is responsible for the highest number of HIV-1 infections globally (Geretti, 2006).

With the neuro-invasion of HIV-1 into the CNS, a sequel of neuropathology influences the current phenotype of HAND (González-Scarano and Martín-García, 2005). Amongst several underlying mechanisms of HAND, there has been a great

interest in understanding the role of (neuro)inflammation and its dysregulation in HIV-1 infection. Several studies have reported associations of peripheral immune markers with HAND (Cohen et al., 2011; Correia et al., 2013; Falasca et al., 2017) and brain volume alterations (Gongvatana et al., 2014; Ragin et al., 2010). Multiple immune markers have been studied; however, none were shown to consistently and robustly correlate with HIV neuropathology and clinical neurocognitive impairment. Furthermore, there are inconsistencies in the direction of the reported associations between immune markers and HAND (Cohen et al., 2011; Falasca et al., 2017). The associations of peripheral immune markers with cortical thickness and surface area were not previously assessed in HIV-1 infection. This is important as cortical structures may be indirectly affected via several (inflammatory) mechanisms and observed as changes in cortical thickness, surface area and cognitive status. Further, there are currently no established/proven adjunctive treatments for HAND. Therefore, there is a need to identify new markers that are associated with HAND and brain structural changes. These markers may improve our understanding of the mechanisms involved in the neuropathophysiology of HAND. The identified markers may be surrogate endpoints during trials of HAND treatments. Further investigation would be required to determine their potential as prognostic, diagnostic and therapeutic targets.

Inflammation may be influenced by the activity of the HIV-1 viral proteins. HIV-1 viral proteins; including glycoprotein 120, transactivator of transcription (Tat) protein and Viral protein r (Vpr) (Jones et al., 2007; Rao et al., 2014) are important in the neuropathogenesis of HIV-1, however, there is a strong research interest in Tat

because it remains present despite the introduction of ART (Johnson et al., 2013; Mediouni et al., 2012). Sequence polymorphisms in the Tat protein influences the neurovirulence of HIV-1. Pre-clinical studies have identified that the $C_{30}S$ and $R_{57}S$ mutations of Tat are major contributors to the reduced immune activity and neurovirulence of HIV-1C infection (Campbell et al., 2007; Rao et al., 2013; Ruiz et al., 2019; Wong et al., 2010). However, it is not clear which Tat protein signatures influence differential inflammation in PLWH.

Therefore, this thesis will attempt to address the gaps in the research of HAND within South African participants, which are primarily HIV-1C infected. These gaps will be addressed by (1) investigating the association of peripheral immune markers with neurocognitive performance and structural brain integrity in a South African cohort and (2) evaluating the influence of Tat sequence variation on immune marker levels. Findings from this thesis may help with the understanding of the underlying mechanisms of HAND and provide targets for the development of improved therapies.

1.2. Research aims

The overall aim of this thesis is to determine the association of peripheral immune markers with HIV-associated neurocognitive impairments, based on their putative neurobiological function. In particular, we aimed to 1) perform a systematic review of the existing literature to identify changes in peripheral immune markers that are associated with HAND in ART-experienced PLWH (Chapter 2), 2) determine the

association of blood peripheral immune markers with domain-based neurocognitive performance (Chapter 3) and structural brain changes (Chapter 4) in South African PLWH, and 3) lastly, to evaluate the possible influence of Tat sequence variation on a dysregulated immune profile in HIV-1C infection (i.e. Tat from HIV-1C) (Chapter 5 and Chapter 6) (Figure 1).

1.3. Coherence of the thesis

This thesis has a common underlying theme, which includes the investigation of a dysregulated immune profile of HIV-associated neurocognitive impairments in the South African (HIV-1C) context. This thesis resulted in a series of manuscripts, of which four have been published to date.

For this thesis, I have included two review papers (chapters 2 and chapters 5). The first, which has been published, is a systematic review which summarized findings from clinical studies and identified 1) peripheral immune markers that are altered in ART experienced PLWH compared to HIV-negative controls and 2) peripheral immune markers that are associated with HAND. Findings from this systematic review strengthen the evidence that a pro-inflammatory environment is associated with HAND. However, inconsistencies in the associations of peripheral immune markers with HIV-associated neurocognitive impairment suggest the need for further investigations to identify new markers for HAND. In addition to the markers related to inflammation/monocyte activation reported in this chapter, I selected

immune markers based on their putative neurobiological relation to pathways of HAND for investigation in Chapter 3.

The second review, which has been published, explored studies presenting data on the differential effects of Tat sequence variation on the underlying mechanisms (inflammation) of HAND. This paper highlighted the key protein signatures in Tat from HIV-1C (predominant in South Africa) which may influence lower immune responses. The identified protein signatures as reviewed (Chapter 5) were used to investigate the current South African cohort (Chapter 6).

This thesis also includes three original empirical papers (Chapters 3, 4, and 6). The first of which has been published (Chapter 3), shows the associations of several immune markers with domain-based neurocognitive impairment in a South African cohort. Several new immune markers were identified in this chapter. Chapter 4, which has been published, reported the associations of immune markers with cortical thickness and surface area in South African PLWH. In the final empirical paper, chapter 6, I investigated the influence of Tat sequence variation on immune marker levels in a subset of South African HIV-1C participants. The empirical papers (Chapters 3, 4, and 6) investigated PLWH that were largely treatment naïve. Firstly, this population represents participants with a higher risk of developing dementia and therefore this investigation may provide an opportunity to investigate the immune response in these individuals. Secondly, this allowed me to investigate the nature of inflammation without the possible confounding effects of ART-related neurotoxicity. Thirdly, recent statistics for South Africa indicated that by 2018, only

62% of the HIV population were on antiretroviral (ARV) treatment and 54% of the HIV population were virally suppressed (UNAIDS, 2019). Considering these statistics, the need to understand the underlying mechanisms of treatment-naïve HAND are emphasized. Lastly, inflammation may persist regardless of treatment status, therefore, findings presented in this thesis may be relevant for future investigations of treatment-experienced participants.

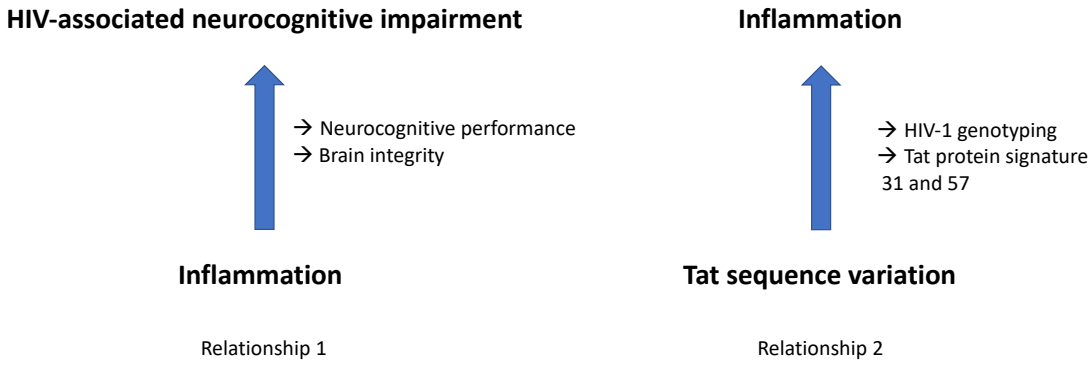

Figure 1: Description of thesis aims. The overall aim is to determine the relationship between inflammation and HIV-associated neurocognitive impairment (relationship 1) and the relationship between Tat sequence variation and inflammation (relationship 2). Relationship 1 investigated the association of peripheral immune markers with neurocognitive performance and brain integrity. Relationship 2 investigated the influence of Tat protein signatures (at position 31 and 57) on peripheral immune marker levels.

NOTE: Minor adjustments to the format and structure of the original manuscripts have been made in order to maintain consistency, connectedness and clarity throughout this body of work.

1.4. References

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Chapter 2

Peripheral immune dysregulation in the ART era of HIV-associated neurocognitive impairments: A systematic review

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Abstract

Human immunodeficiency virus-associated neurocognitive disorder (HAND) remains problematic despite the effective use of antiretroviral therapy (ART) and viral suppression. A dysregulated immune response contributes to the development of HAND but findings on the association between peripheral blood immune markers and HAND have been inconsistent. We therefore conducted a systematic review of studies of the association of peripheral blood immune markers with neurocognitive performance in ART experienced people living with HIV (PLWH). Thirty-seven studies were eligible, including 12 longitudinal studies and 25 cross-sectional studies. Findings consistently show that PLWH have altered immune marker levels, including elevated markers of monocyte activation (neopterin, sCD14, sCD163) and inflammation (CCL2, IL-8, IL-18, IP-10, IFN-α, sTNFR-II and TNF-α). These elevated levels persist in PLWH despite ART. The majority of studies found associations of HIV-associated neurocognitive impairment with immune markers, including markers linked to monocyte activation (sCD14 and sCD163) and inflammation (IL-18 and IP-10). Despite the heterogeneity of studies reviewed, due to the presence of raised peripheral markers, our narrative review provides evidence of chronic inflammation despite ART. The raised levels of these markers may suggest certain mechanisms are active, potentially those involved in the neuropathophysiology of HAND.

2.1. Introduction

The human immunodeficiency virus (HIV) has complex interactions among the brain and the immune system and is responsible for the development of a spectrum of HIV-associated neurocognitive disorders (HAND) (Hong and Banks, 2015). HIV-associated neurocognitive impairment differs in impairment severity and remains problematic for people living with HIV (PLWH) regardless of treatment status. Approximately 50% of PLWH may develop milder forms of HAND (Heaton et al., 2010). The neuroinvasion of HIV-1 into the central nervous system (CNS) is explained by the "trojan horse" hypothesis which states that HIV finds passage into the CNS through infected monocytes (González-Scarano and Martín-García, 2005). Here, infected monocytes that subsequently differentiate into macrophages are able to elicit an immune response. Immune activation or more specifically, monocyte/macrophage activation, is an important step in the normal immune response to pathogens and other relevant stimuli (Ivanova and Orekhov, 2016). Depending on the stimulus, macrophages acquire either a pro- or anti-inflammatory phenotype which are characterized by the secretion of cytokines and signaling molecules (Ivanova and Orekhov, 2016). As with HIV, the disturbance of these processes by immunopathologies results in abnormal monocyte activation and immune regulation.

The persistence of HAND in the ART era is hypothesized to be due to the continued immune activation and low-grade inflammation experienced by PLWH (Harezlak et al., 2011). Immune activation is characterized by monocyte activation (Imp et al.,

2017; Ivanova and Orekhov, 2016) and inflammation (Krebs et al., 2016; Rubin et al., 2018). PLWH experience a dysregulated immune response within both the CNS (Yuan et al., 2013) and the periphery (Lyons et al., 2011). An aberrant regulation of immune markers within the periphery demonstrates an association with neurocognitive impairment as shown in several empirical studies (Burdo et al., 2013; Lyons et al., 2011; Xing et al., 2017; Yuan et al., 2015). This immune dysregulation is also present in the human brain as indicated by increased monocyte/macrophage activation (Tavazzi et al., 2014) and increased levels of pro-inflammatory markers (Shapshak et al., 2004) in post mortem brain tissue of cognitively impaired PLWH. Thus, immune-related processes as shown in both the CNS and periphery may contribute to neuronal dysfunction and consequent neurocognitive impairment in PLWH (Harezlak et al., 2011).

Even though CSF markers may be a more accurate representation of neuroinflammation and pathways that are associated with or lead to HAND, the ease and limited invasiveness of obtaining peripheral blood create an attractive opportunity to investigate peripheral immune markers that are associated with HAND. To a certain extent, peripheral inflammation may present the state of inflammation within the CNS (Capuron and Miller, 2011). Therefore, the identification of peripheral markers with the potential for predicting or stratifying the risk of HAND may contribute to strategies in the early identification of HAND and assessing the efficacy of therapeutic interventions. Further, this line of research could advance our understanding of the underlying mechanisms involved in HAND.

Several studies have investigated the associations between immune peripheral blood markers (monocyte activation and inflammation) and cognitive impairments in HIV. Based on significant associations with HAND, evidence suggests that the most important and promising peripheral immune markers are those related to monocyte activation (e.g. soluble cluster of differentiation (sCD)14, sCD163) (Imp et al., 2017; Lyons et al., 2011; Xing et al., 2017) and inflammation (e.g. tumor necrosis factor (TNF)-α and interleukins) (Ancuta et al., 2008; Cohen et al., 2011; Falasca et al., 2017). However, there are inconsistencies in the association between peripheral immune markers and HAND.

These inconsistencies are seen with both pro- and anti-inflammatory immune markers. Some studies have found that tumor necrosis factor (TNF)-α (Falasca et al., 2017) and interleukin (IL)-10 (Correia et al., 2013) are higher in PLWH and have an association with HAND. However, an inverse association has also been found, with lower TNF-α and IL-10 in PLWH and the lower levels were associated with HAND (Cohen et al., 2011). The differences in these findings may be due to the measures of cognitive functioning in PLWH that vary between studies and disparate study designs which include the number of study participants, type of measurement assays (Breen et al., 2011), ART regimen and CNS penetration (Nightingale et al., 2016; Valcour et al., 2015), presence of comorbidities (Keating et al., 2017), age (De Oliveira et al., 2015), sex (Krebs et al., 2016; Mathad et al., 2016) and viral clade (Rao et al., 2013). Therefore, a clearer understanding of the association of peripheral monocyte activation and inflammatory markers with HAND is important as these findings may

suggest an involvement of peripheral immune markers in the neuropathophysiology of HAND.

Therefore, this systematic review aimed to summarize findings from clinical studies to identify 1) immune markers that may be altered in ART experienced PLWH compared to HIV-negative controls and 2) immune markers that may be associated with HAND. These findings may delineate a set of immune markers for future studies in this research area and provide insights into the neuropathophysiology of HAND.

2.2. Methodology

2.2.1. Study Design

This is a descriptive and narrative systematic review aimed at summarizing the extant literature of the association between the peripheral blood immune markers and neurocognitive performance in ART experienced PLWH.

2.2.2. Eligibility criteria

Eligible studies were those which included 1) HIV seropositive clinical samples from adults with relevant neuropsychological and medical assessments, 2) a relevant control group to the respective study (e.g. HIV-negative, normal cognition or baseline ART status in longitudinal studies), 3) cohorts on a regimen of ART. All medication types were included, and no cut-off was applied for treatment duration,

as this was not declared in all studies and 4) marker measurement done from plasma/serum using enzyme-linked immunosorbent assay (ELISA) or Multiplex/Milliplex analysis for cytokines, chemokines and monocyte-associated immune markers.

Pre-clinical (animal and cell culture models) studies and reviews were excluded. Also, studies investigating only cerebrospinal fluid (CSF) markers were excluded, as these were considered outside the scope of this study.

2.2.3. Data Sources

We electronically searched for publications in PubMed, Scopus and Web of Science databases based on all studies published until 11/03/2020. Eligible studies included published studies in English only. The search strategy was executed without publication date limitations. The full search criteria for each database is included in the supplementary file. The following search terms were applied to PubMed: (HIV [mh] OR HIV [tw] OR Acquired Immunodeficiency Syndrome [mh] OR "Acquired Immunodeficiency Syndrome" [tw] OR AIDS [tw]) AND (HIV associated neurocognitive disorders [mh] OR HAND [tw] OR neurocognitive [tw] OR cogniti* [tw] OR Executive Function [mh] OR executive [tw] OR Memory [mh] OR memory [tw] OR Attention [mh] OR attention [tw] OR Neuropsychological Tests [mh]) AND (Cytokines [mh] OR cytokin* [tw] OR Chemokines [mh] OR chemokine [tw] OR Inflammation [mh] OR inflammation [tw] OR Neurogenic Inflammation [mh] OR neuroinflammation [tw] OR TNF [tw] OR Interleukins [mh] OR interleukins [tw]) AND

(Microglia [mh] OR microglia [tw] OR Monocytes [mh] OR monocyte* [tw] OR sCD163 [tw] OR sCD14 [tw] OR sCD40 [tw]) AND (Anti-HIV Agents [mh] OR Antiretroviral Therapy, Highly Active [mh] OR combination antiretroviral therapy [tw] OR cART [tw] OR HAART [tw] OR ART [tw]).

In addition, we also 1) reviewed reference sections of eligible articles and manually searched for relevant publications, 2) consulted with the contact authors of the included studies and 3), contacted experts in the field for any further papers. This search strategy and the retrieved articles are shown in Figure 1.

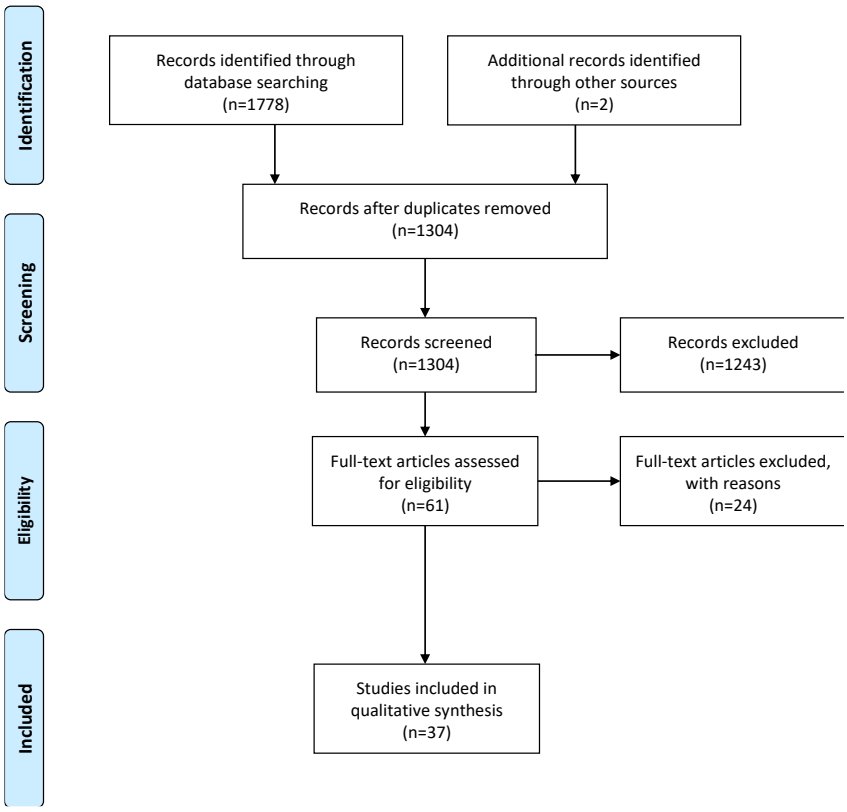

Figure 1: Preferred Reporting Items for *Systematic Reviews* and Meta-Analyses (PRISMA) flow diagram for results of search strategy

2.2.4. Data selection

All articles were retrieved and loaded onto a single database using the reference manager computer program. Two authors, MEW and PJWN independently identified studies meeting the inclusion criteria. Where there was a discrepancy in article inclusion/exclusion, this was discussed amongst all authors, and a decision was made regarding its suitability. Using this criterion, 1304 abstracts and titles were screened. Full-text articles assessed for eligibility were done for 61 studies which further identified 37 research papers for data extraction and analysis (Figure 1).

The quality of the included studies was assessed using a validated and published Likert scale (Likert 1932) for the following areas: (1) Did the study report on potential confounders such as substance misuse, comorbid conditions (e.g. Hepatitis C (HCV)), neurological conditions and psychiatric disorders and were these controlled for upon statistical analysis? (2) Did the study utilize a test battery of at least 5 domains of function commonly affected by HIV? (3) Did the study report on the current regimen of ART and the duration of treatment before the assays were done for cross-sectional studies? For longitudinal studies, did the study report on the current regimen of ART and the duration of treatment between each time point? Each question was rated for 0=no, 1=partly and 2=yes. Studies that addressed all of the quality questions and had a total rating of 6 were classified as high quality. Studies with a rating between 3 and 5 were considered as intermediate quality and less than 3 as low quality.

2.3. Results

2.3.1. Study characteristics

The search strategy yielded 1780 research studies as described in Figure 1. Duplicates were removed resulting in 1304 research studies. Thereafter, abstracts and titles were screened. A total of 1243 studies were excluded. Majority of the excluded studies were review articles (n = 434) and pre-clinical investigations (n = 516). Studies which have not investigated immune markers in general (n = 114), and studies which have only investigated CSF markers (n = 26) were excluded. Studies which have not done any neuropsychological evaluation of participants were excluded (n = 15). Immune marker measurements not done by ELISA/multiplex (n = 3) were excluded. Treatment naïve participants (n = 14) and studies including participants under the age of 18 were excluded (n = 10). Studies investigating neuroimaging data only (n = 47) were excluded. Studies not reporting statistical analysis for immune marker levels and neurocognitive performance were excluded (n = 8). Lastly, studies not published in English were excluded (n = 21).

The full-text analysis was done for 61 studies, and 24 studies were excluded based on the inclusion criteria. A total of 37 studies were eligible for inclusion. A total sample size of n = 3852 participants (HIV-positive and HIV-negative) were included in the eligible studies. This included 3105 PLWH with an age ranging between 18-71 years. Approximately two-thirds of the eligible studies employed a cross-section design (25; 68%), with the remainder employing longitudinal study designs (12; 32%). Investigations were conducted using plasma, serum and CSF. For this study, we

only included results obtained from that analyses of plasma and serum, as CSF immune markers were outside the scope of this review. For all cross-sectional studies and the majority of longitudinal studies, participants were on a regimen of ART (exact regimen not stated in all studies). Several longitudinal studies recruited participants that were treatment naïve and initiated ART as part of the study (long term treatment studies). For longitudinal studies that recruited treatment naïve patients at baseline, only follow-up results were considered as participants had to be ART experienced to be included. The minimum ART duration across all studies were 12 weeks. However, no restriction was applied for the minimum required duration of ART exposure. Only 14/37 studies reported on treatment duration (range of 3-274 months) before analysis. For the measurements of immune markers, ELISA, multiplex, or a combination of the two, were utilized.

Several peripheral blood immune markers were investigated across all studies. To provide clinical practicality, these markers were clustered into (1) monocyte activation and (2) inflammation. The formal classification of these markers remains controversial as many have an overlapping function (i.e. both inflammation and monocyte activation) (Table 1). A description of all investigated markers is described in Supplementary Table 1.

Table 1: Immune markers investigated across all reviewed studies

Monocyte activation	Inflammation	
Neopterin	Chemokine ligand (CCL)2/MCP-1	IL-17
sCD14	CCL5/RANTES	IL-18
sCD163	Chemokine (C-X-C motif) ligand (CXCL)9/MIG	IL-1β
sCD40 ligand	C-reactive protein (CRP)	Macrophage colony-stimulating factor (M-CSF)
	Cystatin-C	Macrophage inflammatory protein (MIP)-1α
	Eotaxin	MIP-1β
	Erythropoietin (EPO)	Matrix metalloproteinases (MMP)-1
	Interferon (IFN)-α2	MMP-2
	IFN-γ	MMP-7
	IFN- gamma-induced protein (IP)-10	MMP-9
	IL-2	MMP-10
	IL-4	Osteopontin
	IL-5	Progranulin (PGRN)
	IL-6	Soluble tumour necrosis factor (TNF) receptor (sTNFR)-I
	IL-7	sTNFR-II
	IL-8	Stromal cell-derived factor (SDF)-1α
	IL-10	Transforming growth factor (TGF)-β1
	IL-12	TGF-β2
	IL-13	TNF-related apoptosis-inducing ligand (TRAIL)
	IL-16	TNF-α

2.3.2. Neuropsychological evaluation

Global neurocognitive impairment was evaluated in the included studies using a range of criteria, as set out by the Memorial Sloan Kettering (MSK) (n = 4), American Academy of Neurology (AAN) (n = 6) and Frascati (n = 7). Furthermore, n = 18 studies utilized test batteries which measured seven domains commonly affected by HIV. Two studies investigated <7 domains (2). The term “HAND” was used when the neuropsychological evaluation diagnosed HAND according to a relevant criterion (e.g. Frascati, AAN or MSK). The term “HIV-associated neurocognitive impairment” was used when the neuropsychological evaluation did not formally diagnose HAND

according to a relevant criterion and instead, utilized domain-based scores in analysis. This has been applied throughout the thesis. Across all studies, domain measurements included action and verbal (i.e., animal) fluency, attention, working memory, psychomotor speed, visuospatial reasoning, executive function, learning and memory, and motor function. In the case of HAND, significant deficits were observed in memory, psychomotor speed, motor skills, executive function, processing speed, attention and learning. This is aligned with previous findings for domains commonly affected by HIV (Woods et al., 2009).

2.3.3. Quality assessment of the included studies

A majority of the included studies were rated as intermediate quality (n=28), with six considered as high quality and three as low quality. Even though according to this criterion a large number of studies are of intermediate quality, not all of these studies met the quality criteria. Specifically, only n = 6 studies received a rating of 2 for each of the quality questions and a total score of 6 for the overall quality criteria. As an inclusion criterion, all study participants had to be treated with ART (or initiate treatment in longitudinal studies), however, only n = 17 studies reported on the duration of therapy before assays. In addition, only n = 15 studies reported the exact ART regimen used (Supplementary Table 2). Lastly, only n = 13 studies reported on both the duration of treatment before the relevant assays as well as the exact treatment regimen (Supplementary Table 2).

The reporting and adjustment of potential confounders upon statistical analysis were done in 78% (n=29) of all studies included. The majority of the studies included in this review evaluated multiple immune markers, and 37% (n=14) of all studies controlled for multiple comparisons by utilizing either Benjamini-Hochberg, Bonferroni corrections or Dunn's multiple comparisons

2.3.4. Immune marker levels and HAND

Irrespective of neurocognitive status, findings show that PLWH had altered levels of several blood immune markers compared to seronegative controls (Table 2). Studies show that PLWH had higher levels of cystatin-c, IL-6, IL-8, IL-10, IL-18, IP-10, IFN-α, CCL2, MIP-1β, MMP-2, MMP-9, neopterin, sCD14, sCD163, sTNFR-I, sTNFR-II, TIMP-1, TIMP-2 and TNF-α. Several studies consistently reported higher blood marker levels of IL-8, IL-18, IP-10, sCD14, sCD163, sTNFR-II (all $p < 0.05$) in PLWH (Table 2).

PLWH may also have lower blood levels of IL-1β, IFN-γ and SDF-1α (all $p < .025$) (Table 2). Contradictory findings for several markers were also reported, with PLWH also having lower levels of IL-6, CCL2, MMP-2 and TNF-α (Table 2).

Findings from longitudinal treatment studies showed that PLWH had a dysregulated immune response over various time points despite treatment status. Studies indicate that immune markers remain elevated at subsequent times compared to HIV-negative participants (Table 2). These included the markers IL-8, IL-18, IP-10, IFN-α,

CCL2, neopterin, sCD14, sCD163, sTNFR-II and TNF-α. Across all studies, a consistent direction in findings were reported for the elevated blood levels of IP-10, CCL2, neopterin, sCD14, sTNFR-II, TNF-α (all $p < 0.05$) at subsequent time points. Longitudinal studies did not report any marker to be significantly lower in PLWH. Two studies similarly reported that IP-10 and neopterin were decreased over time, however, levels remained consistently and significantly higher than controls (Krebs et al., 2016; Valcour et al., 2015). Similarly, in chronic HIV infection, sCD163 decreased over time but remained significantly higher than controls ($p < .0001$) (D'Antoni et al., 2018a). One study also showed that plasma sTNFR-II and TNF-α increased significantly between time points (Marcotte et al., 2013). Several markers were shown to be increased in PLWH and have an association with HAND.

Table 2: Immune marker levels in PLWH

Markers higher in PLWH	***P*-value (Effect size)**	**Reference**
Cystatin-c	$p < .001$ (d = .79)	(Sakoda et al., 2017)
IL-6	$p < .001$ (N/A)	(Ancuta et al., 2008)
	$p < .0001$	(Kamat et al., 2012)
IL-8	$p = .003$ (N/A)	(Correia et al., 2013)
	$p < .001$ (N/A)	*(Krebs et al., 2016)
IL-10	$p = .0046$ (N/A)	*(Krebs et al., 2016)
IL-18	$p = .002$ (N/A)	(Correia et al., 2013)
	$p = .05$ (N/A)	*(Rubin et al., 2018)
IP-10	$p = .001$ (Partial η2 = .162)	(Cohen et al., 2011)
	$p < .001$ (N/A)	(Correia et al., 2013)
	$p < .001$ (N/A)	*(Krebs et al., 2016)
	$p = .05$ (N/A)	*(Rubin et al., 2018)
	$p = .007$ (N/A)	*(Valcour et al., 2015)
IFN-α	$p = .0439$ (N/A)	*(Krebs et al., 2016)
CCL2	$p < .001$ (N/A)	(Ancuta et al., 2008)
	$p = .01$ (N/A)	(Kamat et al., 2012)
	$p = .007$ (N/A)	*(Krebs et al., 2016)
	$P < .001$ (r = .37)	(Montoya et al., 2019)
	$p < .05$ (N/A)	*(Rubin et al., 2018)

	p = .02 (d = .51)	(Yu et al., 2017)
MIP-1β	*p* = .034 (Partial η2 = .073)	(Cohen et al., 2011)
MMP-2	*p* < .01 (N/A)	(Xing et al., 2017)
MMP-9	*p* < .01 (N/A)	(Xing et al., 2017)
Neopterin	*p* < .001 (N/A)	*(Krebs et al., 2016)
	p < .001 (N/A)	*(Valcour et al., 2015)
sCD14	*p* < .001 (N/A)	(Ancuta et al., 2008)
	p = .03 (N/A)	*(Burdo et al., 2013)
	p < .0001 (N/A)	(Kamat et al., 2012)
	p = .002 (N/A)	*(Krebs et al., 2016)
	p = .007 (r = .2)	(Montoya et al., 2019)
	p = .001 (N/A)	(Muñoz-Nevárez et al., 2019)
	p < .01 (N/A)	(Ryan et al., 2001)
	p < .001 (N/A)	(Sakoda et al., 2017)
	p = .007 (N/A)	(Sun et al., 2010)
	p = .01 (N/A)	(Xing et al., 2017)
sCD163	*p* = .01 (N/A)	*(Burdo et al., 2013)
	p < .01 (N/A)	(Xing et al., 2017)
sTNFR-I	*p* < .01 (N/A)	(Ryan et al., 2001)
sTNFR-II	*p* < .001 (N/A)	*(Krebs et al., 2016)
	p = .03 (N/A)	*(Marcotte et al., 2013)
	p < .05 (N/A)	*(Rubin et al., 2018)
	p < .01 (N/A)	(Ryan et al., 2001)
TIMP-1	*p* < .001 (N/A)	(Xing et al., 2017)
TIMP-2	*p* < .01 (N/A)	(Xing et al., 2017)
TNF-α	*p* < .001 (N/A)	*(Krebs et al., 2016)
	p = .017 (N/A)	*(Marcotte et al., 2013)
	p = .003 (r = .22)	(Montoya et al., 2019)
	p < .05 (N/A)	*(Rubin et al., 2018)
	p < .01 (N/A)	(Ryan et al., 2001)
Markers lower in PLWH		
IL-1β	*p* = .004 (Partial η2 = .129)	(Cohen et al., 2011)
IL-6	*p* = .003 (Partial η2 = .134)	(Cohen et al., 2011)
	P < .05 (N/A)	(Rubin et al., 2018)
IFN-γ	*p* = .002 (Partial η2 = .143)	(Cohen et al., 2011)
CCL2	*p* = .04 (Partial η2 = .066)	(Cohen et al., 2011)
MMP-2	*p* < .001 (N/A)	(Li et al., 2013)
SDF-1α	*p* = .025 (N/A)	(Correia et al., 2013)
TNF-α	*p* = .003 (Partial η2 = .136)	(Cohen et al., 2011)

**Longitudinal studies, N/A: Not Available.*

Dysregulated levels (higher and lower levels) of blood immune markers were associated with both global and domain-based HIV-associated neurocognitive

impairment (Table 3). Across all studies and all markers investigated (Table 1), several markers including CRP, IL-6, IL-10, IL-16, IL-18, IL-1β, IP-10, IFN-γ, MMP-1, MMP-2, MMP-7, MMP-9, MMP-10, CCL2, MIP-1β, neopterin, sCD40L, sCD14, sCD163, sTNFR-II, TIMP-1, TIMP-2, TNF-α and TRAIL were associated with HAND (Table 3). From these markers, several cross-sectional and longitudinal studies reported consistent associations of increased IL-18, IP-10, sCD14 and sCD163 with HIV-associated neurocognitive impairment.

Mixed findings were reported for the blood levels of CCL2 and TNF-α and its association with HAND. Compared to the relevant controls, both higher (Ancuta et al., 2008; Woods et al., 2006) and lower CCL2 blood levels (Cohen et al., 2011) were respectively associated with HAND. Similar findings were reported for TNF-α where both higher (Falasca et al., 2017; Sevigny et al., 2004) and lower (Cohen et al., 2011) levels were respectively associated with HAND when compared to controls. The contradictory findings for both CCL2 and TNF-α were found by a study which included HIV patients co-infected with HCV (Cohen et al., 2011).

Long term treatment studies reported associations of immune markers with HAND (Table 3). Compared to the relevant controls, increased levels of neopterin (Krebs et al., 2016), sTNFR-II (Krebs et al., 2016), sCD14 (Burdo et al., 2013; Monnig et al., 2017) and sCD163 (Burdo et al., 2013) were associated with global HIV-associated neurocognitive impairment at follow-up analysis (all $p < .0082$) (Table 2). Furthermore, in a cohort of seventy-two PLWH (36 with HAND) the change of CRP levels at subsequent visits was negatively associated with global HAND (Rubin et al.,

2018). The change of CRP levels at subsequent time points may be associated with worse executive function, attention/working memory, and psychomotor speed in HIV-positive women (Rubin et al., 2018). Higher levels of sCD14 may be associated with worse processing speed in HIV-positive men (Monnig et al., 2017) and higher sCD163 may be associated with impaired learning and executive function when compared to PLWH with normal cognition (NC) (Burdo et al., 2013) (Table 5). Therefore, based on their consistent findings across several studies, monocyte activation and inflammatory markers are promising targets for future investigations. These will be described in the latter part of the review

Table 3: Immune markers associated HIV-associated neurocognitive impairment in PLWH

Marker associated with HIV-associated neurocognitive impairment	***P*-value, (Effect size)**	**Impaired domain**	**Reference**
CRP	$p = .04$ (N/A)	Global	*(Rubin et al., 2018)
	$p = .01$ (Rs = -.30)	Executive function	*(Rubin et al., 2018)
	$p = .004$ (Rs = -.34)	Psychomotor speed	*(Rubin et al., 2018)
	$p = .01$ (Rs = -.24)	Attention/working memory	*(Rubin et al., 2018)
IL-6	$p = .027$ (N/A)	Global	(Ancuta et al., 2008)
	$p = .023$ (β = -.348)	Attention/working memory	(Falasca et al., 2017)
	$p = .003$ (rho = .18)	Global	(Sattler et al., 2015)
IL-10	$p = .04$ (β = .26)	Processing speed/flexibility	(Cohen et al., 2011)
	$p = .001$ (β = .42)	Processing speed	(Cohen et al., 2011)
	$p = .003$ (β = .38)	Attention/working memory performance	(Cohen et al., 2011)
	$p = .036$ (β = -1.194)	Memory performance	(Correia et al., 2013)
IL-16	$p = .0007$ (β = -.06)	Processing speed/flexibility	(Cohen et al., 2011)
	$p < .0001$ (β = -.14)	Processing speed	(Cohen et al., 2011)
	$p = .01$ (β = -.08)	Predictor of Motor skills	(Cohen et al., 2011)
IL-18	$p = .008$ (β = .07)	Processing speed/flexibility	(Cohen et al., 2011)
	$p < .001$ (β = .09)	Attention/executive function	(Cohen et al., 2011)
	$p = .050$ (β = -3.314)	Memory	(Correia et al., 2013)

	p = .015 (β = .283)	Psychomotor processing speed	(Falasca et al., 2017)
	p = .049 (β = -.298)	Attention/working memory	(Falasca et al., 2017)
IL-1β	p = .04 (β = 2.34)	Processing speed/flexibility	(Cohen et al., 2011)
1P-10	p = .02 (β = -.003)	Processing speed/flexibility	(Cohen et al., 2011)
	p = .003 (β = -.006)	Processing speed	(Cohen et al., 2011)
	p = .02 (β = -.004)	Predictor of processing speed	(Cohen et al., 2011)
	p = .0377 (N/A)	Global	(Yuan et al., 2015)
IFN-γ	p = .02 (β = -1.82)	Processing speed/flexibility	(Cohen et al., 2011)
MMP-1	p = .019 (R = -.283)	Verbal memory	(Li et al., 2013)
	p = .003 (R = .350)	Frontal executive performance	(Li et al., 2013)
MMP-2	p < .001 (N/A)	Global	(Xing et al., 2017)
MMP-7	p = .03 (R = .359)	Motor speed	(Li et al., 2013)
MMP-9	p < .01 (N/A)	Global	(Xing et al., 2017)
MMP-10	p = .035 (R = .254)	Frontal executive performance	(Li et al., 2013)
CCL2	p < .01 (N/A)	Global	(Ancuta et al., 2008)
	p = .007 (β = .02)	Attention/executive function	(Cohen et al., 2011)
	p = .01 (R = -.49)	Prospective memory	(Woods et al., 2006)
MIP-1β	p = .044 (β = -.03)	Attention/executive function	(Cohen et al., 2011)
Neopterin	p = .0455 (N/A)	Global	*(Krebs et al., 2016)
sCD40L	p < .01 (N/A)	Global	(Sui et al., 2007)
sCD14	p = .03 (N/A)	Global	(Ancuta et al., 2008)
	p = .029 (N/A)	Executive function	*(Burdo et al., 2013)
	p < .05 (β = -.20)	Executive function	(Imp et al., 2017)
	p = .02 (N/A)	Global	(Kamat et al., 2012)
	p = .03 (N/A)	Learning	(Lyons et al., 2011)
	p = .015 (N/A)	Attention	(Lyons et al., 2011)
	p = .007 (N/A)	Global	(Lyons et al., 2011)
	p < .008 (β = -.003)	Processing speed	*(Monnig et al., 2017)
	p = .037 (r = -.14)	Global	(Muñoz-Nevárez et al., 2019)
	p < .01 (N/A)	Global	(Ryan et al., 2001)
	p < .001 (N/A)	Global	(Xing et al., 2017)
sCD163	p = .028 (N/A)	Global	*(Burdo et al., 2013)
	p = .005 (N/A)	Learning	*(Burdo et al., 2013)
	p = .029 (N/A)	Executive function	*(Burdo et al., 2013)
	p < .001 (β = -.30)	Global	(Imp et al., 2017)
	p < .05 (β = -.33)	Verbal memory	(Imp et al., 2017)
	p < .05 (β = -.24)	Psychomotor speed	(Imp et al., 2017)
	p < .05 (β = -.21)	Fine motor skills	(Imp et al., 2017)
	p < .01 (N/A)	Global	(Xing et al., 2017)
sTNFR-II,	p = .0082 (N/A)	Global	*(Krebs et al., 2016)
TIMP-1	p < .001 (N/A)	Global	(Xing et al., 2017),

TIMP-2	$p < .001$ (N/A)	Global	(Xing et al., 2017),
TNF-α	$p = .003$ (β = 2.02)	Attention/executive function	(Cohen et al., 2011)
	$p = .024$ (β = -.282)	Worse executive function	(Falasca et al., 2017)
	$p = .03$ (N/A)	Global	*(Sevigny et al., 2004)
TRAIL	$p = .05$ (β = .23)	Predictor of Processing speed	(Cohen et al., 2011)
	$p = .02$ (β = .23)	Predictor of Attention/working memory	(Cohen et al., 2011)

Longitudinal studies, N/A: Not Available.

Not all included studies have reported on treatment durations before analysis as this may have affected immune reconstitution and potentially the peripheral immune marker levels. To account for immune status ($CD4^+$ count) and its potential effect on immune marker levels, studies were stratified according to their $CD4^+$ count. We subdivided studies into two categories, specifically having a current (or follow-up for longitudinal studies) mean/median $CD4^+$ count of <200 cells/µl or >200 cells/µl. These included 8 studies with <200cells/µl and 24 studies with >200 cells/µl (Supplementary Table 3). Five studies did not provide sufficient information to stratify according to $CD4^+$ count (i.e. did not include follow up CD4 counts (D'Antoni et al., 2018a; Monnig et al., 2017; Robertson et al., 2019; Sevigny et al., 2004) or did not report statistical associations (Valcour et al., 2015). From the 8 studies (<200cells/µl), the majority investigated immune markers that had an association with HIV-associated neurocognitive impairment. However, in studies with cohorts having >200cells/µl, half of the studies investigated markers with an association with HIV-associated neurocognitive impairment (Supplementary Table 3). When stratified by CD4 count, study cohorts with >200 cells/µl consistently report associations of immune markers with HAND. Therefore, despite cross-sectional and longitudinal studies investigating patients with varying CD4 counts (both higher and lower than

200cells/µl), peripheral blood immune markers continue having a reported association with HIV-associated neurocognitive impairment. A similar trend is noted in cognitively impaired PLWH regardless of viral suppression.

For this review, participants were considered as being virally suppressed with <1000 copies/ml. Studies were stratified according to mean/median viral suppression (<1000 copies/ml) and non-viral suppression (>1000 copies/ml) (Supplementary Table 4). Across all studies, viral load was reported as a mean (SD), median (range) or percentage distribution. Thirty-three studies provided sufficient information to be stratified according to viral load. From the 33 studies, 20 studies investigated patients that were largely virally suppressed, and 50% of these studies reported an association with immune markers. The remaining 13/33 studies investigated non-virally suppressed participants, and 69% of these studies reported markers with an association with HAND. This suggests that despite plasma viral suppression, a dysregulated peripheral immune response present in PLWH has an association with HIV-associated neurocognitive impairment. This potentially represents unchecked monocyte activation and inflammation, and this might be a trigger for ongoing neuroinflammation and cognitive impairment

2.3.5. Monocyte activation and neuroinflammation in HAND

Studies included in this review indicate that monocyte activation markers sCD14 (Ancuta et al., 2008; Burdo et al., 2013; Kamat et al., 2012; Krebs et al., 2016; Montoya et al., 2019; Muñoz-Nevárez et al., 2019; Ryan et al., 2001; Sakoda et al.,

2017; Sun et al., 2010; Xing et al., 2017) and sCD163 (Burdo et al., 2013; Xing et al., 2017) are consistently elevated in PLWH compared to controls. The higher blood levels of monocyte activation markers have a reported association with HIV-associated neurocognitive impairment.

Studies collectively show that the increased sCD14 blood levels have an association with both global and domain-based HIV-associated neurocognitive impairments. sCD14 has been implicated in global HAND (Ancuta et al., 2008; Kamat et al., 2012; Lyons et al., 2011; Muñoz-Nevárez et al., 2019; Ryan et al., 2001; Xing et al., 2017) as well as impairment in domains of executive function (Burdo et al., 2013; Imp et al., 2017), attention and learning (Lyons et al., 2011) and processing speed (Monnig et al., 2017). sCD163 has been associated with global HAND (Burdo et al., 2013; Imp et al., 2017; Xing et al., 2017) and impairment in verbal memory, psychomotor speed, fine motor skills (Imp et al., 2017), learning and executive function (Burdo et al., 2013) (Table 2). Similarly, in long term treatment investigations, sCD14 (Burdo et al., 2013; Monnig et al., 2017) and sCD163 (Burdo et al., 2013) were associated with HAND at multiple time points.

Various inflammatory cytokines and chemokines were investigated across studies. However, only some markers were consistently higher in PLWH. These included a combination between pro- and anti-inflammatory blood markers; IL-8, IL-18, IP-10 and sTNFR-II. From these markers, several studies reported that the consistently increased blood levels of IL-18 (Cohen et al., 2011; Correia et al., 2013; Falasca et al., 2017) and IP-10 (Cohen et al., 2011; Yuan et al., 2015) were associated with HAND.

Inconsistent findings were found for the inflammatory cytokines/chemokines IL-6, CCL2 and TNF-α. Studies reported higher IL-6 levels in PLWH (Ancuta et al., 2008; Kamat et al., 2012). However, IL-6 has also been shown to be lower in PLWH (Cohen et al., 2011; Rubin et al., 2018). The higher IL-6 levels were associated with global HAND (Ancuta et al., 2008) and impairment in attention/working memory (Falasca et al., 2017).

Several studies reported higher levels of the monocyte recruitment chemokine, CCL2 in PLWH (Ancuta et al., 2008; Krebs et al., 2016; Montoya et al., 2019; Rubin et al., 2018; Yu et al., 2017). Another study, on the contrary, showed lower levels of CCL2 in PLWH (Cohen et al., 2011). Two studies showed that higher CCL2 levels in PLWH were associated with HAND (Ancuta et al., 2008; Woods et al., 2006), whereas an inverse direction of this association was also reported (Cohen et al., 2011). Higher CCL2 was found to be associated with global HAND (Ancuta et al., 2008), worse attention/executive function (Cohen et al., 2011) and prospective memory performance (Woods et al., 2006) (Table 4 and Table 5).

Conflicting results were also reported for TNF-α levels, with studies showing higher levels (Krebs et al., 2016; Marcotte et al., 2013; Montoya et al., 2019; Rubin et al., 2018; Ryan et al., 2001) and opposing lower levels (Cohen et al., 2011) in PLWH when compared to controls. Higher (Falasca et al., 2017; Sevigny et al., 2004) and lower (Cohen et al., 2011) levels of TNF-α were found to be associated with HAND. Despite the varying associations of TNF-α levels with HAND, the dysregulated TNF-α

levels were shown to have an association with attention and executive function (Cohen et al., 2011; Falasca et al., 2017). A full description of all studies investigated in this review is described in Table 4 and Table 5.

Table 4: Cross-sectional studies reporting the association of peripheral blood immune markers with cognitive performance in HIV-ART cohorts

References	Type of Study	Treatment	Sampling technique	Cohort	Neuro test	Covariates	Markers	Major findings
(Ancuta et al., 2008)	Cross-sectional	HAART	ELISA and LAL	HIV+: n= 119 HIV+ NCI: n=73 NCI (other causes): n= 20, HIV+ NC: n=32 and HIV-: n=25 Female: 20% (n=30) Age: 45 (±8) CD4 count: 110 cells/µl (±197) Viral load: 150600 copies/ml (±33100)	AAN and HNRC criteria	N/A	sCD14, IL-6 and CCL2	1. CCL2 ($p < .001$), IL-6 ($p < .001$) and sCD14 ($p < .001$) were significantly higher in AIDS participants compared to negative controls. 2. NCI participants had higher plasma levels of CCL2 ($p = .01$), IL-6 ($p = .027$) and sCD14 ($p = .03$) compared to NC patients.
(Brown et al., 2011)	Cross-sectional	HAART	ELISA	HIV+ NCI: n=81 HIV+ NC: n=21, inflammatory: n=30 non-inflammatory: n=27 Female: (N/A) Age: 42.2 (±6.12) CD4 count: 127.2 cells/ml (±21.8)	MSK	N/A	Osteopontin	1. No significant differences in plasma Osteopontin levels between NCI HIV+ and NC HIV+ participants.

				Viral load: range between 39811-199526 copies/ml				
(Cohen et al., 2011)*	Cross-sectional	HAART	Multiplex	HIV+: n=30 HIV-: n=34 Female: 42% (n=27) Age: 44.8 (±9.06) Current CD4 count: 463.9 cells/µl (± 284.56) Nadir CD4 count: 168.6 cells/µl (±130.76) Viral load: 75% <75 copies/ml	Neurocognitive test battery (7 domains)	Age, education, HIV status, HCV status, lifetime substance dependence history, alcohol, opiates and cocaine	IL-1β, IL-6, IL-8, IL-10, IL-16, IL-18, IFN-γ, IP-10, MIP-1β, CCL2, SDF-1 α, TRAIL and TNF- α	1. Plasma IP-10 (p = .001 partial η2=0.162) and MIP-1β (p = .034, partial η2=0.073) were higher in HIV+ participants whereas IL-1β (p = .004, partial η2=0.129), IL-6 (p = .003, partial η2=0.134), IFN-γ (p = .002, partial η2=0.143), CCL2 (p = .04, partial η2=0.066), and TNF-α (p = .003, partial η2 = 0.066) were lower in HIV+ participants. 2. Higher plasma IL-16 (p = .0007, β = -0.06), IP-10 (p = .02, β = -.003), IFN-γ (p = .02, β = -1.82) and Lower IL-1β (p = .04, β = 2.34), IL-10 (p = .04, β = 0.26), and IL-18 (p = .008, β = 0.07) was associated with worse processing speed/flexibility performance. 3. Higher plasma IL-16 (p < .0001, β = -.14) and IP-10

								(p = .003, β = -.006) and lower IL-10 (p = .001, β = .42) was associated with worse processing speed performance. 4. Higher plasma MIP-1β (p = .044, β = -.03) and lower IL-18 (p < .001, β = .09), CCL2 (p = .007, β = .02), and TNF-α (p = .003, (β = 2.02) was associated with worse attention/executive function performance. 5. Higher plasma IP-10 (p = .02, β = -.004) and lower TRAIL (p = .05, β = .23) were significant predictors of processing speed. 6. Lower plasma IL-10 (p = .003, β = .38) and TRAIL (p = .02, β = .23) were significant predictors of attention/working memory performance. 7. Higher plasma IL-16 (p = .01, β = -.08) was a significant predictor of motor skills.

(Correia et al., 2013)*	Cross-sectional	cART	Multiplex	HIV+: n=74 HIV-: n=50 Female: 37% (n=46) Age: 46.22 (±11.65) Current CD4 count: 528.83 cells/µl (±254.69) Nadir CD4 count: 187.15 cells/µl (±166.88) Viral load: 68% had <75 copies/ml	Hopkins Verbal Learning Test-Revised	HCV status	IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-16, IL-18, IP-10, CCL2, MIP-1β, SDF-1α, TNF-α and TRAIL	1. HIV+ participants had higher levels of IL-8 (p = .003), IL-18 (p = .002) and IP-10 (p < .001) compared to HIV- controls. 2. HIV+ participants had significantly lower SDF-1α (p = .025) compared to HIV- controls. 3. Higher plasma levels of IL-8 (p = .027), and IFN-γ (p = .005) were associated with better performance on memory measures. In contrast, higher IL-10 (p = .036, β = -10.194) and IL-18 levels (p = .050, β = -3.314) were related to poorer memory performance.
(De Oliveira et al., 2015)	Cross-sectional	ART	ELISA and electrochemiluminescence multiplex assay	HIV + (Younger group 20-46): n=18 HIV+ (Older :50-71): n=26 HIV-: n=86 Female: 16% (n=21) Age: 32 (22-40) and 57(50-71)	Frascati	HIV DNA levels, age group, estimated duration of infection and the interaction between	sCD163, CCL2, IL-8, IL-6 and TNF-α	1. Older HIV+ participants demonstrated higher levels of IL-6 (p = .03) and CCL2 (p = .03) compared to younger individuals. 2. None of the inflammatory markers were associated with NCI.

				Current CD4 count: 726 cells/µl (603-1051) Nadir CD4 count: 374 cells/µl (287-505) Viral load: 100% <50 copies/ml		HIV DNA level and age group		
(Falasca et al., 2017)*	Cross-sectional	ART	ELISA	HIV+: n=40 HIV+ NCI: 40 Female: 12.5% (n=5) Age: 47.8 (N/A) CD4 count: 646 cells/µl (523-964) Viral load: 100% <40copies/ml	Neurocognitive test battery (7 domains)	Hepatic fibrosis, treatment type, age, gender and education	IL-6, IL-8, IL-18 and TNF-α	1. Overall serum cytokine levels were significantly associated with performances on many neurocognitive measures. 2. Lower IL-8 were associated with better executive function (p = .008). 3. Higher IL-6 was associated with better executive function (p = .032). 4. Higher levels of TNF-α were associated with worse executive function (p = .024, β = -.282). 5. IL-18 serum levels were associated with worse psychomotor processing (p = .015, β = .283) and worse attention/working memory (p = .049, β = -.298).

(Imp et al., 2017)*	Cross-sectional	cART	ELISA	HIV+: n=186 HIV-: n=67 Female: 0% (n=0) Age: HIV+ 48.29 (±8.63) HIV- 44.15 (±10.27) Nadir CD4 count: 261 cells/µl (±210) >500cells/µl : n=101 200-500cells/µl : n=63 <200 cells/µl : n=22 Viral load: 50% <500 copies/ml 37% <10000 copies/ml 13% >10000 copies/ml	Neurocognitive test battery (7 domains)	Site, HIV and HCV status, anti-depressants, depressive symptoms, hypertension, income, and number of previous cognitive test exposures	sCD163, sCD14, CRP and IL-6	1. Marker levels between HIV+ and HIV- participants were not reported. 2. Serum IL-6 levels were not associated with overall cognitive performance or with any specific cognitive domain. 3. Higher serum CRP levels were associated with better verbal memory in aviremic women (p = .04). 4. Higher sCD163 was associated with overall performance (β = -.30), verbal memory (β = -.33), psychomotor speed (β = -.24), and fine motor skills (β = -.21) (all p < .05) in the overall samples. 5. Higher sCD14 remained associated with worse executive function (p < .05, β = -.20). 6. In aviremic participants, sCD163 was significantly associated with global NCI (β = −.24, p = .04), verbal memory (β = −.36, p

								= .003), psychomotor speed (β = −.28, *p* = .01), and fine motor skills (β = −.34, *p* = .004). 7. In aviremic participants, sCD14 was associated with executive function (β = −.25, *p* = .02), and a trend for significance was noted for psychomotor speed (β = −.19, *p* = .05).
(Kamat et al., 2012)	Cross-sectional	cART	ELISA and multiplex	HIV+ NCI: n=45, HIV+ NC: n=19, HIV+ Unknown: n=3 Female: 17% (n=23) Age: 46 (32–69) Nadir CD4 count: 75 cells/µl (±93) CD4 count: 155 (±162) Viral load: 31% <400 copies/ml 67% >400 copies/ml Mean: 127975 (±236077)	Neurocognitive test battery (7 domains)	Age and cART	sCD14, IL-6, CCL2, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, IFN-α, IFN-γ, TNF, MIP-1α, MIP-1β, IP-10, MIG, eotaxin and RANTES	1. sCD14 (*p* < .0001), CCL2 (*p* = .01) and IL-6 (*p* < .0001) were significantly higher in HIV+ participants. 2. Plasma sCD14 (*p* = .02), was higher in HIV+ participants with NCI compared to NC HIV+ participants.

(Gianella et al., 2019)	Cross-sectional	ART	ELISA	HIV+ NCI: n=31 HIV+ NC: n=30 Female: 0% (n=0) Age: 48 (27–63) CD4 count: 589 (407-776) Viral load: 95% undetectable HIV RNA	Frascati	Age	sCD14	1. sCD14 plasma levels were not associated with NCI.
(Gougeon et al., 2017)	Cross-sectional	ART	Multiplex and LAL	HIV+ NCI: n= 30 HIV+ NC: n= 73 HIV-: n=10 Female: N/A Age: 43 (38-50) Nadir CD4 count: 227 (76-350) cells/µl CD4 count: 495 (358-747) cells/µl Viral load: 65% undetectable viral load Median: 40 copies/ml	AAN revised	Age, gender and years of education	IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-13, IL-15, IL- 17, Eotaxin, IFN-γ, IP-10, CCL2, MIP-1α, MIP-1ß, RANTES, TNF-α	1. No significant differences in immune marker levels between HIV+ NCI and NC groups.
(Li et al., 2013)*	Cross-sectional	ART	MilllIplex	HIV+: n=52 HIV-: n=21	Neurocognitive test	N/A	MMP-1, MMP-2, MMP	1. HIV+ participants had lower plasma MMP-2

				Female: 15% (n=11) Age: 33.2 (±9.9) CD4 count: 548 cells/µl (±252) Viral load: 1291 (±16.5) Aviremic: 22.4%	battery (7 domains)		7, MMP-9, and MMP-10	levels compared to the HIV- control group (p < .001). 2. MMP-9 levels were significantly higher in the ARV-initiated than ARV-naïve subgroup (p = .016). 3. MMP-1 was correlated with verbal memory (p = .019, R = -.283) and frontal executive performance (p = .003, R = .350). 4. MMP-7 was correlated with motor speed (p = .003, R = .359). 5. MMP-10 was correlated with frontal executive performance (p = .035, R = .254).
(Lyons et al., 2011)	Cross-sectional	cART	ELISA	HIV+ NCI: n=47 HIV+ NC: n=20 HIV+ Unknown: n=5 HIV+ NCI (other causes): n= 25 Female: 23% (n=23) Age: 47.1 (± 8.0)	AAN	N/A	sCD14 and CCL2	1. CCL2 was not associated with a significant difference in global T-scores. 2. Higher sCD14 was associated with global NCI (p = .007) and impaired attention (p = .015) and learning (p = .03).

				Nadir CD4 count: 63 (±65) CD4 count: 138 (±139) Viral load: 36% <400 copies/mL 61% >400 copies/mL Mean : 83868 (±162893)				
(Montoya et al., 2019)	Cross-sectional	cART	Multiplex and ELISA	HIV+: n=90 HIV-: n=94 Female: 48% (n=90) Age: 36-45 Nadir CD4 count: 160 (74-407) CD4 count: 627 (398-884) Viral load: 100% <50 copies/ml	Neurocognitive test battery (7 domains)	Hypertension, hyperlipidemia, diabetes mellitus, lifetime cannabis use disorder, lifetime methamphetamine use disorder and demographic variables	d-dimer, IL-6, CCL2, sCD14, TNF- α .	1. CCL2 (r = .37), sCD14 (r = .2), and TNF- α (r = .22) were higher in the HIV+ group (all $p < .05$), 2. Higher inflammation composite score was associated with cognitive impairment; however, no individual marker was associated with NCI.

(Muñoz-Nevárez et al., 2019)	Cross-sectional	cART	ELISA	HIV+: n=290 HIV-: n=104 Female: 55% (n=220) Age: HIV+: 41 (19-68) HIV-: 39 (19-69) Nadir CD4 count: 247 (258) CD4 count: 402 (232) Viral load: 69% <500 copies/ml	Neurocognitive test battery (6 domains)	$CD4^{+}$count, plasma HIV RNA and location	sCD163, sCD14 and neopterin	1. Plasma sCD14 was significantly higher in HIV+ compared HIV- participants (p = .001). 2. Higher plasma sCD14 levels were associated with lower global scores in both HIV+ and HIV- participants (r = .0001, p = .037). 3. Plasma sCD163 (p =.938) and neopterin were not associated with lower global scores in the overall sample or any sub-group (p = .963, p = .835).
(Ryan et al., 2001)	Cross-sectional	HAART	ELISA	HIV+ NCI: n=20 HIV+ NC: n=8 HIV-: n=6 Female: 10% (n=3) Age: 28 (29-61) CD4 count: 237 cells/µl (±41) Viral load: 77091 copies/ml (±195372)	Neurocognitive test battery (7 domains)	N/A	sCD14, TNF-α, sTNFR-I and sTNFR-II.	1. TNF-α, sTNFR-I and sTNFR-II were significantly higher in HIV+ participants (p < .01). 2. There was no significant association for TNF-α, sTNFR-I and sTNFR-II with NCI in HIV+ participants. 3. Higher sCD14 was associated with NCI (p < .01) in HIV+ participants.

(Sakoda et al., 2017)	Cross-sectional	ART	ELISA	HIV+: n=77 HIV-: n= 47 Female: 25% (n=31) Age: 58.26 (6.06) Nadir CD4 count: 185 cells/µl (±192) CD4 count: 646 cells/µl (±341) Viral load: 100% <50 copies/ml	Neurocognitive test battery (7 domains)	Demographic factors (age, sex, ethnicity/race), HIV disease characteristics (estimated duration of HIV infection, current and nadir CD4+ T-cell count, AIDS diagnosis), substance use disorder diagnosis (current and lifetime), and major depressive disorder (MDD) diagnosis	Cystatin-c and sCD14	1. Plasma cystatin-c (p < .001, d = 0.79) and sCD14 (p < .001) levels were significantly higher in HIV+ than in HIV− participants. 2. Cystatin-c levels tended to be higher in those with global NCI compared to those with NC (p = .055).

						(current and lifetime)		
(Sattler et al., 2015)	Cross-sectional	ART	ELISA and multiplex	HIV+: 152 Female: 15% (n=23) Age: 49 (22-69) Current CD4 count: 549 cells/µl (65-3199) Viral load: 50 copies/ml (40-1000)	Neurocognitive test battery (12 domains)	Age, education and race	High sensitivity IL-6, sCD163, and sCD164	1. Only higher IL-6 was associated with lower GDS (rho = .18, *p* = .003), however, this was limited to participants with a waist circumference of > 99cm.
(Suh et al., 2014)	Cross-sectional	cART	ELISA and multiplex	HIV+ NCI: n=58 HIV+ NC: n=49 Female: 13% (n=14) Age: 43,7 (±8.7) CD4 count: 396 cells/µl (190-582) Viral load: 49.5% undetectable	Neurocognitive test battery (7 domains)	Demographics, HIV Viral load, and inflammatory mediators.	IFN- γ, IL-1β, IL-4, IL-6, IL-10, IL-13, IL-17, TNF-α, CCL2, IP-10, PGRN	1. Plasma PGRN levels (*p* = .003) were significantly higher in viremic compared to aviremic participants. 2. Plasma PGRN was not associated with NCI.
(Sui et al., 2007)*	Cross-sectional	HAART	ELISA	HIV+ NCI: n=16 HIV+ NC: n=9 Female: 24% (n=6) Age: 41 (±11)	MSK	N/A	CD40L and TNF-α	1. Plasma sCD40L was significantly higher in participants with NCI,

				CD4 count NCI: 266 cells/µl (±182) CD4 count NC: 257 cells/µl (±69) Viral load: NCI: 1287 copies/ml (±33,28) NC: 3234 copies/ml (±16,93)				when compared with NC HIV+ controls ($p < .01$).
(Sun et al., 2010)	Cross-sectional	HAART	ELISA	HIV+ NCI: n=17 HIV+ NC: n=38 HIV-: n=11 Female: 0% (n=0) Age: 50.9 (± 7.4) CD4 count: 493.3 cells/µl (±410) Viral load: mean 4014 copies/ml (±32)	Neurocognitive test battery (7 domains)	Educational level, gender and age	sCD14	1. sCD14 was higher in HIV+ participants ($p = .007$). 2. Higher sCD14 levels were associated with better information-processing speed (p = .047).
(Woods et al., 2006)*	Cross-sectional	HAART	ELISA	HIV+: n=35 Normal for ProM: n=28 Mild ProM impairment: n=2 Moderate ProM impairment: n=3	Memory for Intentions Screening Test, Hopkins	ART status and CD4 lymphocyte count	CCL2 and sTNFR-II	1. Higher plasma CCL2 was associated with prospective memory (ProM) ($p < .05$, R = -.49).

				Severe ProM impairment: n=2 Female: 22% (n=8) Age: 43.1 (±7.8) CD4 count: 510.5 cells/µl (288-622.2) Viral load: Median: 50 copies/ml (0.-31623)	Verbal Learning Test-Revised and the Brief Visuospatial Memory Test-Revised			
(Woods et al., 2010)	Cross-sectional	ART	ELISA	HIV+ NCI: n=44 HIV+ NC: n=30 Female: 40% (n=30) Age: 45.1 (±9.0) Nadir CD4 count: 217.5 cells/µl (46.5-360.3) CD4 count: 533 cells/µl (361-826.3) Viral load: Undetectable in 60.8%	AAN	Current cART, current CD4 lymphocyte count, and global cognitive functioning	CCL2, EPO and IP-10	1. There was no correlation between any of the plasma markers and action/noun fluency in HIV+ participants.
(Xing et al., 2017)	Cross-sectional	cART	ELISA and multiplex	HIV+ NCI: n=19 HIV+ NC: n=9 HIV-: n=9	Frascati	Age, sex and race	MMP-2, MMP-9, TIMP-1, TIMP-2,	1. MMP-2 ($p < .01$), MMP-9 ($p < .01$), TIMP-1 ($p < .001$), TIMP-2 ($p < .01$), sCD14 ($p = .01$) and

				Female: 17% (n=5) Age: HIV+ CI: 45 (±2) HIV+ NC: 48 (±2) CD4 count: HIV+ NCI: 150 cells/µl (±34) HIV+ NC: 301 cells/µl (±91) Viral load: NCI: 10000 copies/ml (±2) NC: 2512 copies/ml (±3)			sCD14 and sCD163	sCD163 ($p < .01$) were higher in HIV+ participants compared to controls. 1. HIV+ NCI participants had higher MMP-2 ($p < .001$), MMP-9 ($p < .01$), TIMP-1 ($p < .001$) and TIMP-2 ($p < .001$), sCD14 ($p < .001$) and sCD163 ($p < .01$) compared to HIV- controls.
(Yuan et al., 2015)	Cross sectional	HAART	Milliplex	HIV+ NCI: n=52, HIV+ NC: n=33, Female: 30% (n=26) Age: 38 (31–76) CD4 count: HIV+ NCI: 41 cells/µl (21-120) HIV+ NC: 69 cells/µl (21-120) Viral load: NCI: 796347 copies/ml (2541-12500000)	MSK	N/A	IL-8, eotaxin, IFN-α2, IFN-γ, IP-10 and MCP- 1	1. HIV + participants with NCI had higher levels of plasma IP-10 ($p = .0377$) compared to HIV+ NC controls.

				NC: 7943 copies/ml (501-158489)				
(Yu et al., 2017)	Cross-sectional	ART	ELISA	HIV+: 41 HIV-: 42 (Total sample NCI: 25 and NC: 58) Female: 15% (n=15) Age: 42.6 (±8.9) CD4 count: 422.6 cells/µl (±192.5) Viral load: 87% <50 copies/ml	Neuro-cognitive test battery (7 domains)	AIDS status, nadir or current CD4+ T-cell count, addictive drug use variables, age, sex, or ethnicity	CCL2	1. Plasma CCL2 levels were increased in HIV+ participants compared to HIV− controls (*p* = .02) but were not associated with NCI.

Neurocognitive test of 7 domains: attention; working memory; psychomotor speed; visuospatial reasoning; executive function; learning and memory, and gross motor. Age: mean (Range/Standard deviation), CD4 count: mean (IQR/standard deviation) and viral load: mean (SD), median (range) or percentage distribution. Inflammatory: participants with inflammatory conditions (multiple sclerosis (MS), relapsing-remitting MS, secondary progressive MS, meningitis, and brachial neuritis). Non-inflammatory: participants with non-inflammatory conditions (Intracranial hypertension, pseudotumor cerebri, hydrocephalus, headaches, and migraine).

[*] = *Studies reporting a mean/median CD4 count* >200cells/µl and an association with HIV-associated neurocognitive impairment

Abbreviations*: AAN: American Academy of Neurology, cART: combination antiretroviral therapy, CCL2: Chemokine (C-C motif) ligand 2, ELISA: Enzyme-linked immunosorbent assay, GDS: Global deficit score, HAART: Highly active antiretroviral therapy, HCV: Hepatitis C, HNRC: HIV neurobehavioral research center, IFN: Interferon, IL: Interleukin, IP-10: interferon gamma-induced protein, LAL: Limulus amebocyte lysate, CCL2: Monocyte chemotactic protein 1, MIP: Macrophage Inflammatory Protein, MMP: Matrix metalloproteinases, MSK: Memorial Sloan Kettering, N/A: Not Available, NC: Normal cognition, NCI: Neurocognitive impairment, PGRN: Progranulin, RANTES, CD: cluster of differentiation, SDF: stromal cell-derived factor, (sTNFR)-I: soluble TNF receptor, TIMP: tissue inhibitors of metalloproteinases, TNF: tumour necrosis factor and TRAIL: TNF-related apoptosis-inducing ligand*

Table 5: Longitudinal studies reporting the association of peripheral blood immune markers with cognitive performance in HIV-ART cohorts

References	Type of Study	Treatment	Sampling technique	Cohort	Neuro test	Covariates	Markers Investigated	Major findings
(Airoldi et al., 2012)	Longitudinal (12 weeks)	ART	ELISA	HIV+ NCI: n=6 HIV+NC: n=6 HIV-: n=0 Female: 16% (n=2) Age: 40 (25-59) Baseline CD4 count: HIV+ NCI: 42 cells/µl (42-74) HIV+ NC: 88 cells/µl (44-98) Follow-up CD4 count: HIV+ NCI: 188 cell/µl (178–205) HIV+ NC: 175 cell/µl (123–394) Viral load: Baseline: 31833 copies/ml (15849–316228) Follow up: 200 copies/ml	Frascati	N/A	IL-6, IL- 10, INF-γ, TNF-α, TGF-β1, TGF-β2, MIP-1α, MIP-1β and CCL2	1. No significant differences for marker levels between NCI and NC HIV+ participants after 12 weeks of ART.

				(63–251)				
(Burdo et al., 2013)*	Longitudinal (128 weeks)	ART	ELISA	HIV+ NCI: n=15 HIV+ NC: n=19 HIV-: n=34 Female: 4% (n=3) Age: HIV+ NCI: 42.8 (8.8) HIV+ NC: 44.0 (±12.2) Baseline CD4 count: HIV+NCI: 240 cells/µl (197–356) HIV+ NC: 215 cells/µl (180–300) Follow up: HIV+NCI: 658 cells/µl (442–876) HIV+ NC: 628 cells/µl (462–796) Viral load: 100% <50 copies/ml	Frascati and GDS	Age, education, sex and ethnicity.	sCD163 and sCD14	1. Higher plasma sCD163 (p = .01) and sCD14 (p = .03) in HIV+ participants compared to HIV- participants at visit A. 2. sCD163 decreased significantly at visit B (p = .01) whereas sCD14 had no significant difference at visit B. 3. sCD163 was higher in NCI HIV+ participants (p = .028). 4. Higher sCD163 was associated with impaired learning (p = .005) and executive function (p = .029).
(D'Antoni et al., 2018a)	Longitudinal (48 weeks)	cART	ELISA	HIV+: 51 HIV-: 18	Neurocog nitive test	N/A	sCD163 and neopterin	1. Fiebig I/II: After cART initiation, plasma

				Female: HIV+ Fiebig I/II: 5% (n=1) HIV+ Fiebig III: 9% (n=3) HIV-: 50% (n=9) Age: HIV+ Fiebig I/II: 31 (26-39) HIV+ Fiebig III: 28 (23-29) HIV-: 33 (28-39) Baseline CD4 count: HIV+ Fiebig I/II: 447 (307-567) HIV+ Fiebig III: 370 (293-470) Follow up CD4 count: N/A Viral load: Baseline HIV+ Fiebig I/II: 1000000 (19953-316228) HIV+ Fiebig III: 794328 (19953-7943282) Follow-up N/A	battery (7 domains			sCD163 levels increased by week 24 (p = .034), and by week 48 decreased matching pre-cART levels (p < .0001). 2. Fiebig III: After cART initiation, at week 48, plasma sCD163 levels decreased matching uninfected controls (p < .0001). 3. Chronic infection: After cART, plasma sCD163 decreased but remained higher than controls (p < .0001). 4. Before cART, higher plasma sCD163 were associated with better psychomotor performance (p = .044), however, no associations were found after cART initiation.

(D'Antoni et al., 2018b)	Longitudinal (24 weeks)	ART	ELISA	HIV+: n=17 Female: 5% (n=1) Age: 55 (47–58) Baseline Nadir CD4 count: 200 cells/µl (90-280) CD4 count: 545 cells/µl (404-731) Follow-up analysis: 732 cells/µl (397-900) Viral load: 100% <50 copies/ml	Neurocognitive test battery (7 domains) and global cognitive function were defined.	Age, sex and education	sCD163, sCD14 and neopterin	1. Plasma neopterin (p < .01), sCD163 (p = .001) and sCD14 (p = .003) levels decreased significantly at subsequent visits (24 weeks). 2. Neopterin and sCD163 were not associated with cognitive performance at any time point. 3. At baseline, higher sCD14 was associated with worse psychomotor speed (p = .064). 4. With the reduction in all markers after 2 weeks, no association with NCI was found at subsequent visits.
(Krebs et al., 2016)*	Longitudinal (96 weeks)	cART	ELISA	HIV+: n=60 HIV-: n=18 Female: 44% (n=35) Age: 35 (22-47) Baseline CD4 count:	Frascati	HIV-1 viral load, CD4 counts, and/or severity of neurocognitive disease as covariates. In	IFN-γ, TNFα, TNF-RII, IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-15, CCL2, t-Tau,	1. In HIV+ females, plasma neopterin (p < .001), IP-10 (p < .001), TNF-α (p < .001), TNF-RII (p < .001), IFN-α (p

				Males: 239 (29-532) Female: 230 (23-553) Follow up CD4 count: Increased significantly for both sexes (p = .002) Viral load: Baseline Female: 40503 copies/ml (1585-639659) Male: 100000 copies/ml (1279-639659) Follow-up Viral load decreased significantly p < .0001. Majority of participants were aviremic.		addition, sex was used as an independent variable	IP-10, neopterin, IFNα and sCD14	= .0439), CCL2 (*p* = .007), IL-8 (*p* < .001),IL-10 (*p* = .0046) and sCD14 (*p* = .002) levels were decreased after 48 weeks but remained elevated compared to uninfected controls. 2. HIV+ females with NCI reported higher neopterin levels (*p* = .0114) at baseline as well as after 48 weeks of treatment (*p* = .0445) compared to NC HIV+ females. 3. HIV+ females with NCI reported significantly higher sTNFR-II (*p* = .0082) compared to HIV+ females with NC after 48 weeks.
(Lentz et al., 2010)	Longitudinal (40 weeks)	cART	ELISA	HIV+ NCI: n=30 HIV+ NC: n=24 Female: 31% (n=17) Age: HIV+ NCI: 41.4 (±7.6) HIV+ NC: 40.2 (±5.9)	MSK	N/A	M-CSF	1. Plasma M-CSF were significantly decreased after 10 months of therapy *(p* = .008).

				CD4 count: Baseline: 184 cells/µl (±164) Follow up: 256 cells/µl (± 270) (40 weeks) Viral load Baseline 50545 copies/ml (±4) Follow-up: 4025 copies/ml (±19.8)				2. Plasma M-CSF had no significant relationship to cognitive measures at any time point or be able to predict the cognitive outcome.
(Marcotte et al., 2013)	Longitudinal (48 weeks)	ARV	Multiplex bead array and ELISA	HIV+: n=98 Cognition stably Normal (SN): n=25 Worsened (Wo): n=25 Stably Impaired (SI): n=25 Cognition Improved (Im): n=23 Female: 34% (n=34) Age: 44.65 (±1.7) CD4 count: Baseline:	Neurocognitive test battery (7 domains) using the global deficit score.	Age, sex, education and ethnicity	CCL2, IL-6, TNF-α, IP-10, SDF-1α, sTNFRII and sCD14	1. Plasma sTNFRII (*p* = .03) and TNF-α (*p* = .017) increased significantly between visits. 2. No significant associations between plasma markers and neurocognitive performance.

				SN: 462 cells/µl (321-667), Wo: 335 cells/µl (194-481), SI: 463 cells/µl (304-642) and Im: 260 cells/µl (102-540) Follow up: Groups who remained neurocognitively stable had higher CD4 counts at the first visit ($p < .01$) Viral load: Baseline 54% <50 copies/ml Follow-up: N/A				
(Monnig et al., 2017)	Longitudinal (12 weeks)	ART	ELISA	HIV+: n=21 Female: 0% Age: 46.7 (26-63) CD4 count: Baseline 643 cells/µl (±245) Follow-up: NA Viral load:	Neurocognitive test battery (7 domains)	Age, race, education	sCD14	1. Higher sCD14 was associated with worse processing speed *($p < .008$, $\beta = -.003$).*

				100% <75 copies/ml				
(Robertson et al., 2019)	Longitudinal (48 weeks)	Maraviroc and Tenofovir containing ART	ELISA	HIV+: n=230 Female: 9% Age: 33 CD4 count: Baseline 389 cells/µl (293-508) Follow-up: NA Viral load: Baseline 31,623 (10000-100000) copies/ml Follow-up: N/A	Frascati	Age, education, gender and race	sCD14, sCD163, IP-10, sTNFRII and high sensitivity IL-6	1. No associations were found between NCI and immune markers at week 48.
(Rubin et al., 2018) *	Longitudinal (288 weeks)	HAART	Multiplex, milliport and ELISA	HIV+ NCI: n=36 HIV+ NC: n=36 HIV− NCI: n=29 HIV− NC: n=29 Female: 100% (n=130) Age: 43.9 (25 -70) CD4 count: Baseline:	Neurocognitive test battery (7 domains)	Viral load, current and nadir CD4 count, and CD4/CD8 ratio	IL-10, IL-1β, IL-6, IP-10, CCL2, MIP-1β, TNF-α, IL-16, TRAIL, sTNFR-I, sTNFR-II, MMP-9, CRP and IL-18	1. HIV+ women reported higher serum levels of IL-18, sTNFR-II, CCL2, IP-10, TNF-α, and lower levels of IL-6 (*p* values < .05) compared to controls. 2. Greater CRP variability predicted decreased executive function (Rs = -.30),

				HIV+ NCI: 641 cells/µl (379-799) HIV+ NC: 620 cells/µl (396-788) Follow-up: N/A Viral load: NCI: 86% <80 copies/ml NC: 72% <80 copies/ml				attention/working memory (Rs = -.24), and psychomotor speed (Rs = -.34) in HIV+ women (all *p* < .05).
(Sevigny et al., 2004)	Longitudinal (84 weeks)	ART	ELISA	HIV+ NCI: n=74 HIV+ NC: n=129 Female: 26% (n=54) Age: 42 (±7.2) CD4 count: Baseline: 127.5 cells/µl (69-194) Follow-up: N/A Viral load Baseline 11500 (270-66,000) Follow-up: NA	AAN	Age, sex, years of education, duration of HIV infection, type of antiretroviral use, premorbid IQ score, and presence of minor cognitive motor disorder	CCL2, TNF- α, M-CSF and MMP-2	1. Plasma TNF-α (*p* = .03) tended to be associated with the development of HIV-associated dementia over time.

(Valcour et al., 2015)	Longitudinal (24 weeks)	cART	ELISA	HIV+ cART: n=30, HIV+ cART+ integrase inhibitor + CCR5 antagonist: n=32 HIV-: n=29 Female: 18% (n=17) Age: 27 (18-47) CD4 count: Baseline HIV+ cART: 369 cells/µl (±277) HIV+ cART+: 391 cells/µl (±218) Follow-up: No significant difference in CD4 counts Viral load Baseline cART 316228 (13.1) cART+ 398107(20.4) Follow-up cART: 53 copies/ml (50–102) cART+: 234 copies/ml (50–5596)	AAN	Age, gender and mean educational level.	IL-6, IP-10, CCL2 and neopterin	1. Plasma neopterin (p < .001) and IP-10 (p = .007) levels were decreased in HIV+ participants however, it remained higher compared to controls. 2. The associations between immune markers and cognitive performance were not reported.

Neurocognitive test of 7 domains: attention; working memory; psychomotor speed; visuospatial reasoning; executive function; learning and memory, and gross motor. Age: mean (Range/Standard deviation), CD4 count: mean (IQR/standard deviation) and viral load: mean (SD), median (range) or percentage distribution.

*= *Studies reporting a mean/median CD4 count* >200cells/µl and an association with HIV-associated neurocognitive impairment

***Abbreviations**: AAN: American Academy of Neurology, cART: combination antiretroviral therapy, CRP: C-reactive protein, CCL2: Chemokine (C-C motif) ligand 2,* CCR: C-C chemokine receptor type, *ELISA: Enzyme-linked immunosorbent assay, HAART: Highly active antiretroviral therapy, HCV: Hepatitis C, HNRC: HIV neurobehavioral research center, IFN: Interferon, IL: Interleukin, IP-10: interferon gamma-induced protein, LAL: Limulus amebocyte lysate,* M-CSF: Macrophage colony-stimulating factor *MIP: Macrophage inflammatory Protein, MMP: Matrix metalloproteinases, MSK: Memorial Sloan Kettering, N/A: Not Available, NC: Normal cognition, NCI: Neurocognitive impairment, PGRN: Progranulin, RANTES, CD: cluster of differentiation, SDF: stromal cell-derived factor, sTNFRI: soluble TNF receptor, TIMP: tissue inhibitors of metalloproteinases, TNF: tumour necrosis factor and TRAIL: TNF-related apoptosis-inducing ligand, TGF: Transforming growth factor*

2.4. Discussion

The main findings from studies reviewed here show that 1) immune markers were altered in ART experienced PLWH compared to HIV-negative controls, and 2) an alteration of immune markers linked to key pathways of monocyte activation (sCD14 and sCD163) and inflammation (IL-18 and IP-10) were was associated with HIV-associated neurocognitive impairment. With consistent evidence across several studies reviewed here, immune markers linked to key pathways of monocyte activation (sCD14 and sCD163) and inflammation (IL-18 and IP-10) may be important markers in the neuropathophysiology of HAND. Even with ART and studies including patients with varying $CD4^+$ counts/reconstituted immune systems and viral suppression, there is a continued association of monocyte activation and inflammation immune markers with HAND.

Our findings suggest that certain monocyte activation markers may be promising markers in the neuropathophysiology of HAND, due to their consistent findings across the majority of studies. sCD14 is a marker of monocyte activation and represents a state of activated immune cells. Due to the higher levels of sCD14 in PLWH and its association with HAND, sCD14 may have a functional role in the neuropathology of HIV. sCD14 is significantly increased in PLWH with cerebral atrophy compared to PLWH without cerebral atrophy (Ryan et al., 2001). sCD163 is a complex scavenger receptor which is cleaved and shed from activated monocytes/macrophages. Similar to sCD14, sCD163 represents an activated immune

system, which is a crucial step in the neuropathogenesis of HAND (Burdo et al., 2013).

In addition to chronic immune activation, a dysregulated inflammatory profile in HIV-associated neurocognitive impairment is reported in this review (Table 2). Promising inflammatory markers included the pro-inflammatory cytokines, IL-18 (Cohen et al., 2011; Correia et al., 2013; Falasca et al., 2017) and anti-inflammatory cytokine, IP-10 (Cohen et al., 2011; Yuan et al., 2015). IL-18 is a key inflammatory cytokine in HIV infection and may be an important cytokine in activating microglia during stress (Sugama et al., 2007). IP-10, also known as C-X-C motif chemokine 10 (CXCL10), is an anti-inflammatory cytokine that is increased in both the periphery (Cohen et al., 2011) and the CNS (Yuan et al., 2013) of PLWH. IP-10 can exacerbate the neurotoxic effects of HIV-1 and mediates pro-inflammatory cytokine production (Williams et al., 2009). Due to the higher levels of IP-10 and IL-18, this may suggest active mechanisms, potentially those involved in the neuropathophysiology of HIV-associated neurocognitive impairment. Higher plasma IL-18 and IP-10 levels were associated with lower volumes of the putamen, pallidum, thalamus, hippocampus, amygdala, gray and white matter, as well as higher ventricular volumes in PLWH (Gongvatana et al., 2014). IL-18 and IP-10 may, therefore, be involved in mechanisms involved in HIV-associated CNS abnormalities.

As shown in this review, the direction of the associations of CCL2 and TNF-α with HAND are inconsistent. This is an interesting finding, as this may suggest that the dysregulation of these markers beyond homeostatic immunological levels (higher or

lower), may contribute to the neuropathophysiology of HIV-associated neurocognitive disorders. However, this warrants further investigation as these studies differed in study design, cohort size, treatment duration and regimen and relevant statistical analysis. All of which may have affected these findings. The contradictory findings presented in this review were largely reported by a study by (Cohen et al., 2011), which included participants co-infected with HCV. It may be that the presence of HCV may affect inflammatory markers in treated HIV patients (Taye and Lakew, 2013), and this may be a plausible reason for the inconsistent findings presented by these studies (Cohen et al., 2011; Rubin et al., 2018).

This systematic review supports the hypothesis of continuous low-grade (neuro)inflammation despite treatment status. Long term treatment studies showed that inflammatory levels remained elevated or unchanged at subsequent visits on various treatment regimens. This continuous inflammatory profile may be responsible for the development of milder forms of HAND despite the introduction of ART (Gannon et al., 2011)

Furthermore, two longitudinal studies recruited treatment-naive patients. These studies suggest that $CD4^+$ counts are significantly increased at subsequent time points. Inflammatory marker levels were decreased at follow up analysis, however marker levels remained significantly higher than levels found in HIV-negative controls. This further supports previous findings that in the era of ART, immune marker levels may decrease but not to levels matching HIV- controls.

Even though $CD4^+$ counts may increase with ART, using $CD4^+$ count as predictors of cognitive impairment need further consideration (Ellis et al., 2011; Sacktor et al., 2001). To extend on this area, studies were stratified according to their $CD4^+$ count and viral suppression. Regardless of $CD4^+$ count/reconstituted immune system and viral suppression, peripheral cytokines still had an association with HAND, further supporting the argument that other mechanisms may contribute and trigger chronic immune activation and inflammation. Not all studies accounted for the effect of $CD4^+$ count in their analyses, which may have affected findings.

Several studies have reported an association between viral load and HAND (Marcotte et al., 2003; Mccombe et al., 2013). With the effective roll-out of ART, current viral load is no longer strongly associated with HAND and the dominant hypothesis for ongoing brain dysfunction has been linked to persistent inflammation (Clifford and Ances, 2013). ART may significantly reduce the viral load and systemic inflammation, but not with resolution to normal levels (Clifford and Ances, 2013). As reviewed here, studies including participants with higher viral load, more commonly reported associations between peripheral immune markers and HIV-associated neurocognitive impairment (69%) compared to studies including virally suppressed participants (50%). Therefore, with the effective use of ART, there may be a decrease in the level of inflammation and the development of HAND, but not to levels matching HIV-negative controls. Further, in a recent 24-month longitudinal treatment study, volume decline of the basal ganglia was reported in young adults despite the initiation of ART within the first 4 weeks of infection (Kallianpur et al., 2019). This further indicates that ART alone may not completely eradicate HIV from the CNS, resulting in the continued expression of HIV viral DNA and this, in turn, may

trigger ongoing chronic inflammation which may be partly responsible for the development of milder forms of HAND.

It is well reported that demographics including sex (Krebs et al., 2016; Mathad et al., 2016) and age (De Oliveira et al., 2015) may affect inflammatory marker levels, and this may have affected the interpretation of findings from the studies included in this review. Only one study stratified according to age (De Oliveira et al., 2015) and one according to sex (Krebs et al., 2016). The majority of studies included sex and age (60%) as covariates in the statistical analysis, however, studies which have reported findings for the associations between age/sex and immune marker levels have found inconsistent results (n = 5). Three studies reported significant associations between immune marker levels and sex. Plasma IFN-y (p = .017, R = .447) was associated with sex in HIV-positive participants (Cohen et al., 2011). Plasma neopterin (p = .0445) and TNFRII (p = .0082) were higher in HIV-positive cognitively impaired females when compared to normal cognition, whereas males did not display elevated levels between groups (Krebs et al., 2016). Further, a higher composite inflammation burden score (number of elevated immune markers in a panel) was associated with female sex in HIV-positive participants (p < .05, hedge's g = .45) (Montoya et al., 2019). The remaining two studies reported no significant associations between immune marker levels and sex (Sevigny et al., 2004; Suh et al., 2014)

For the associations between immune marker levels and age (n = 6), one study reported significant associations between the peripheral immune markers CCL2 (p = .01) and IL-6 (p = .03) with increased age (De Oliveira et al., 2015). Increased plasma

sCD163 was associated with increased age after controlling for estimated duration of infection (p = .03) (De Oliveira et al., 2015). The remaining five studies found no associations with age (Brown et al., 2011; Correia et al., 2013; Falasca et al., 2017; Montoya et al., 2019; Suh et al., 2014). Studies reporting findings for associations between immune marker levels and sex/age were limited, therefore no significant conclusions could be made.

Despite the heterogeneity of studies included here, the present study does suggest markers that may be commonly associated with HAND. Furthermore, the consistent evidence found for several markers may suggest a functional role in the neuropathogenesis of HAND. Therefore, these markers may also potentially be investigated for the development of improved diagnostics and therapeutics.

2.5. Limitations

The literature included in this review has several limitations and deserve emphasis. First, the heterogeneity and shortcomings of the included studies should be noted. Many studies in this review had a low number of participants and therefore lacked statistical power. Further, studies did not declare socioeconomic status and mode of HIV transmission of the included HIV populations. The majority of studies where from the United States of America, with two studies from Italy and one study each from France, Thailand and China. Therefore, the majority of studies reported were from high-income countries and these may have influenced the interpretation of the

findings reported in this review. Despite the inclusion criteria of this review, heterogeneity was evident in study design and characteristics of included study participants. This made the interpretation of findings reported from the studies much more challenging. Furthermore, <1000 copies/ml was considered as "viral suppression" as this has been benchmarked by several other studies (Chouraya et al., 2019; Jordan et al., 2009; Lokpo et al., 2020; McMahon et al., 2013). We do acknowledge that studies also consider undetectable viral loads as viral suppression, however, this may have been punitive with respect to the threshold for classifying studies within the review. A meta-analysis was not conducted due to the heterogeneity of the studies pooled for review. Heterogeneity was evident in study design. Varying cognitive measures and inflammatory markers were analysed in the studies pooled for investigation. For studies that reported an association of immune markers with HAND, more than half failed in reporting of effect sizes.

Second, several confounders may have been present, including ART-related inflammation and toxicity, ART regimen and CNS penetration, presence of comorbidities, age, sex and viral subtype. A large percentage (58%) of studies included in this review did not report on treatment durations before the relevant testing assays. The extended use of ART as recorded in long term investigations may indicate lower levels of plasma viral load, which may affect peripheral immune markers (Hattab et al., 2015). Further, it may also be related to the CNS/plasma discordance, whereby HIV-1 can be detected at higher levels in the CSF than in blood. CNS/plasma discordance coincides with the effective penetration of ART into the CNS (Nightingale et al., 2016; Valcour et al., 2015). The exact ART regimens have

not been declared for every study and were therefore not included in this systematic review. The brain may act as a HIV-1 reservoir which enables HIV an escape from the full effect of ART within the CNS (Schrager and D'Souza, 1998) and different ART regimens report different scores of CNS penetration (Decloedt et al., 2015; Eisfeld et al., 2013). This may also affect the levels of peripheral markers and subsequently the observed associations with cognitive performance (Nightingale et al., 2016; Valcour et al., 2015). This may also suggest that viral products within the CNS may more sensitively represent occurrences within the brain. A systematic comparison and investigation of the association of CSF peripheral markers with cognitive performance in HIV may be required to address the above.

Third, the included studies also utilized various criteria for measures of cognitive function. Cognitive diagnostic criteria differ in stringency when categorizing impairment severities. It is estimated that 20% of cognitively normal PLWH is classified as suffering from HAND (De Francesco et al., 2016; Gisslén et al., 2011). This may also have affected the reported findings of this review.

Fourth, critical factors influencing marker levels may include age and sex. Even though majority of studies included age and sex as covariates in the statistical analysis, majority of studies has not stratified participants according to age/sex. From the limited studies reporting on these associations, the findings were mixed, and no conclusion could be drawn. Therefore, future studies should include analysis for the effect of sex and age on immune markers.

Fifth, HIV viral subtype has not been taken into consideration for this systematic review. The majority of the included studies have not declared subtype status. It is assumed that HIV-1 subtype B is the universal strain responsible for the development of HAND. This may be because the majority of our understanding of the neuropathogenesis of HAND is derived from studies of HIV-1 subtype B (Taylor et al., 2008) and this may explain the lack in reporting of HIV-1 subtypes. Even though HIV viral subtype reportedly affects the prevalence of HAND (Rao et al., 2013), limited studies exist for its effect on inflammatory levels. One study conducted by de Almeida and colleagues showed that HIV-1 positive patients had increased inflammatory markers but no significant differences between Subtype-B and Subtype-C (de Almeida et al., 2016). However, this study had a small number of participants and therefore limited statistical power. Thus, the effect of HIV subtypes on the association of inflammatory markers with cognitive impairments cannot be neglected and needs further investigation.

Sixth, seven studies did not report and statistically adjust for confounders. Several covariates including; sex (Krebs et al., 2016; Mathad et al., 2016), age (De Oliveira et al., 2015), comorbidities (Keating et al., 2017) and substance abuse (Cadet and Krasnova, 2007; Liu et al., 2012) may similarly affect marker levels. In the studies that did not report on the above, a trend is noted that these studies attempted to create stricter inclusion criteria. Even though a stricter inclusion criterion may be used, the lack of adjustment and reporting of confounders may still affect findings presented in these studies and ultimately the interpretation thereof. Only 40% (n = 14) of studies controlled for multiple comparisons by utilizing either Benjamini-

Hochberg, Bonferroni corrections or Dunn's multiple comparisons. Controlling for multiple comparisons provide support for the findings presented in each study, and the lack to control for this may affect the interpretation of the findings.

Several markers were investigated more often than others across studies. Here we reviewed the most common markers found. Other exploratory studies may have identified immune marker candidates which were not highlighted here. Thus, the need for further investigation into the identification of candidate immune markers for HAND is required.

Lastly, there are also significant variations across assays used to measure blood immune markers, and these variations may lead to inconsistent associations observed in the reported studies. It was shown that in a multisite investigation of HIV positive serum/plasma samples, current multiplex assays vary significantly in their ability to measure serum and/or plasma concentrations of cytokines. Therefore, findings may not be reproducible for repeated determinations over a long-term study or in multiple laboratories. However, it may be useful for longitudinal studies in which relative, rather than absolute, changes in cytokines are important (Breen et al., 2011). This further emphasizes the need for uniformity of studies investigating immune markers in HIV-positive participants.

2.6. Recommendations

Monocyte activation and inflammatory markers, in particular, peripheral sCD14, sCD163, IL-18 and IP-10 are promising immune markers, given their consistent findings for their correlation with HAND. These markers, therefore, may potentially be investigated as crucial markers in the neuropathophysiology of HAND. Studies pooled for investigation differed in ART regimen, treatment duration (CNS penetration), viral subtype and statistical protocols, and this may have affected the interpretation of the findings. Greater uniformity in all studies investigating general markers and cognitive performance will allow for improved comparability. Specifically, investigations of cognitive severities and patterns in PLWH will allow the teasing out of which markers may be related to HAND and not to the presence of HIV-1 in general. Finally, future studies should investigate the association of a combination of immune markers with HAND. This approach may be more beneficial than investigating the association of individual markers with HAND. It was recently shown that using a composite score for a combination of inflammation markers were associated with neurocognitive impairment in PLWH, whereas, no association was found when investigating individual immune markers (Montoya et al., 2019).

2.7. Conclusions

Despite the heterogeneity of study conditions and patient populations, this study reports that PLWH with HAND experiences a chronic inflammatory regulation that

may persist regardless of ART. Results show that chronic inflammation is associated with HIV-associated neurocognitive impairment, particularly monocyte activation markers; sCD14 and sCD163 and inflammatory markers; IL-18 and IP-10. Based on these associations there is a possibility that these markers may be involved in the neurobiological mechanisms that manifest into HAND. Future studies in cell cultures and animal models are needed to determine specific neurobiological and molecular signaling pathways that can contribute to the neuropathophysiology of neurocognitive impairment in PLWH.

The findings presented in this review may contribute to this line of research. Firstly, this review addressed the inconsistency for the association between peripheral immune markers and HIV-associated neurocognitive impairment and suggests a panel of markers which were most commonly associated with HIV-associated neurocognitive impairment. Secondly, based on the consistent findings, the suggested peripheral immune markers may be important contributors to the underlying neuropathophysiology of HIV-associated neurocognitive impairment and may be fundamental for future research. We believe that this review presents researchers with a panel of markers which may be the basis for future research, without the need to scour the extensive literature of the field. Lastly, monocyte activation and inflammation may persist despite successful ART. Therefore, in addition to simply suppressing the viral load, other strategies are required to address the ongoing peripheral inflammation in PLWH.

The majority of studies investigating the association of peripheral immune markers with HAND were done in HIV-1B infection. Limited investigations exist in HIV-1C infected participants. Associations between immune markers of monocyte activation and inflammation with HAND were the most consistent outcome between the majority of the studies. However, I highlighted the inconsistency in the direction of the association of these peripheral immune markers with HAND. Therefore, in the next chapter, I investigated a new combination of markers for their potential association with HIV-associated neurocognitive impairments. This was in the aim of identifying additional markers that are potentially associated with HIV-associated neurocognitive impairments. It is also of interest to investigate the nature of inflammation without the possible confounding effects of ART-related neurotoxicity. Therefore, in Chapter 3 and Chapter 4, I investigated treatment-naïve populations. These populations represent participants with a higher risk of developing dementia and therefore these investigations may provide an opportunity to investigate the immune response in these individuals without the possible confounding effects of ART.

I expect immune marker levels to be higher in treatment naïve PLWH. However, as reported in the current review, although ART may reduce immune marker levels, it does not return to levels matching HIV-negative controls. Further, despite elevated CD4 counts and viral suppression, immune marker levels are associated with HIV-associated neurocognitive impairment. This is suggestive of ongoing inflammation in the modern ART-era. I therefore hypothesize that the findings of treatment-naïve

PLWH (Chapter 3 and Chapter 4) may be relevant to studies investigating treatment-experienced PLWH.

2.8. References

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2.9. Supplementary: Chapter 2

Search terms

PubMed

(HIV [mh] OR HIV [tw] OR Acquired Immunodeficiency Syndrome [mh] OR "Acquired Immunodeficiency Syndrome" [tw] OR AIDS [tw]) AND (HIV associated neurocognitive disorders [mh] OR HAND [tw] OR neurocognitive [tw] OR cogniti* [tw] OR Executive Function [mh] OR executive [tw] OR Memory [mh] OR memory [tw] OR Attention [mh] OR attention [tw] OR Neuropsychological Tests [mh]) AND (Cytokines [mh] OR cytokin* [tw] OR Chemokines [mh] OR chemokine [tw] OR Inflammation [mh] OR inflammation [tw] OR Neurogenic Inflammation [mh] OR neuroinflammation [tw] OR TNF [tw] OR Interleukins [mh] OR interleukins [tw]) AND (Microglia [mh] OR microglia [tw] OR Monocytes [mh] OR monocyte* [tw] OR sCD163 [tw] OR sCD14 [tw] OR sCD40 [tw]) AND (Anti-HIV Agents [mh] OR Antiretroviral Therapy, Highly Active [mh] OR combination antiretroviral therapy [tw] OR cART [tw] OR HAART [tw] OR ART [tw]).

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(HIV OR acquired immunodeficiency syndrome OR “acquired immunodeficiency syndrome” OR aids) AND (hiv associated neurocognitive disorders OR hand OR neurocognitive OR cogniti* OR executive function OR executive OR memory OR attention OR neuropsychological tests) AND (cytokines OR cytokin* OR chemokines OR inflammation OR neurogenic inflammation OR neuroinflammation OR tnf OR interleukins) AND (microglia OR monocytes OR monocyte* OR scd163 OR scd14 OR scd40)

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TS=(HIV OR Acquired Immunodeficiency Syndrome OR "Acquired Immunodeficiency Syndrome" OR AIDS) ANDTS=(HIV associated neurocognitive disorders OR HAND OR neurocognitive OR cogniti* OR Executive Function OR executive OR Memory OR memory OR Attention OR attention OR Neuropsychological Tests) AND TS=(Cytokines OR cytokin* OR Chemokines OR chemokine OR Inflammation OR inflammation OR Neurogenic Inflammation OR neuroinflammation OR TNF OR Interleukins OR interleukins) AND TS=(Microglia OR microglia OR Monocytes OR monocyte* OR sCD163 OR sCD14 OR sCD40)

Table 1: A brief description of immune markers investigated across all reviewed studies

Monocyte activation		**Inflammation**	
Marker	**Function**	**Marker**	**Function**
Neopterin	Neopterin is a CNS immune activation marker (Hagberg et al., 2010), and its dysregulated levels in blood (Krebs et al., 2016) and CSF (Burdo et al., 2013; Ceccarelli et al., 2017) have been associated with HAND.	Chemokine ligand (CCL)2/MCP-1	CCL2 is an important immune marker within the neuropathogenesis of HAND, with both neuro-toxic (Yang et al., 2011) and neuro-protective properties (Eugenin et al., 2007, 2003). CCL2 is a major component for the transmigration of $CD14^{+}CD16^{+}$ monocytes across the blood-brain barrier (Buckner et al., 2011). Elevated CSF (Yuan et al., 2013) and blood (Marcotte et al., 2013) levels have been associated with structural brain changes (Ragin et al., 2006) and neurocognitive impairment in PLWH.
sCD14	sCD14 is an immune marker which is shed from activated monocytes/macrophages and represents an activated immune system (Burdo et al., 2013). sCD14 blood (Ryan et al., 2001) and CSF (Kamat et al., 2012a) levels were associated with HAND.	CCL5/RANTES	CCL5 is a chemotactic cytokine encoded by the CCL5 gene. CCL5 is increased in the CNS after HIV-1 infection and in HIV-associated dementia (HAD) (Kelder et al., 1998). CCL5 has also reported neuroprotective properties *in vitro* (Kaul and Lipton, 1999; Meucci et al., 2000, 1998)
sCD163	CD163 is cleaved from the surface of macrophages and shed as sCD163 following activation and differentiation of monocyte and macrophages (Møller, 2012). Elevated blood (Burdo et al., 2013) and CSF (De Oliveira et al., 2015) levels have been associated with HAND.	Chemokine (C-X-C motif) ligand (CXCL)9/MIG	CXCL9 is a chemokine which plays a role in chemotaxis and immune activation in HIV-1 infection (Kamat et al., 2012b). Limited studies have investigated CXCL9 for its role in HAND.
sCD40 ligand (CD40L)	CD40L is a type II membrane glycoprotein that is predominantly expressed by activated cells (Van Kooten and Banchereau, 2000) and is associated with neurocognitive impairment in people living with HIV (PLWH) (Sui et al., 2007).	C-reactive protein (CRP)	CRP is a homopentameric acute-phase inflammatory protein and a marker indicative of inflammation. Further, CRP levels have been associated with HIV-1 infection (Neuhaus et al., 2010) and HAND (Rubin et al., 2018).
		Cystatin-C	Cystatin-c is a low-molecular-weight protein that is produced by nucleated cells. Cystatin- c has been associated with neurocognitive impairment in selected neurodegenerative populations (Chen et al., 2015; Yaffe et al., 2008) including people with HAND (Sakoda et al., 2017).

		Eotaxin/ CCL11	Eotaxin is an eosinophil-specific chemokine that is associated with the recruitment of eosinophils into sites of inflammation (Teixeira et al., 2018). Despite many studies investigating Eotaxin in HAND, significant findings remain limited at this stage.
		Erythropoietin (EPO)	EPO is a glycoprotein cytokine and has been characterized as a potent anti-inflammatory cytokine in various diseases (Nairz et al., 2012). EPO was found to be neuroprotective in a murine model of HAND (Kang et al., 2010).
		Interferon (IFN)-α2	IFN-α2 is a type I Interferon cytokine involved in activation of the immune system. IFN- α is suggested to be involved in HIV neuropathology, as higher CSF levels were associated with neurocognitive impairment in PLWH (Anderson et al., 2017; Fritz-French and Tyor, 2012; Sas et al., 2009).
		IFN-γ	IFN-γ is a cytokine critical to both innate and adaptive immunity, and functions as the primary activator of macrophages. IFN-γ levels are elevated in PLWH and are associated with HAND (Schrier et al., 2015). Further, IFN-γ has recently been reported to contribute to BBB (Jansson et al., 2016).
		IFN- gamma-induced protein (IP)-10	CXCL10 is an anti-inflammatory marker that exacerbates neurotoxicity and inflammation in PLWH (Williams et al., 2009). CXCL10 has chemoattractant properties that mediate neuronal injury (Williams et al., 2009). Higher peripheral CXCL10 levels were also associated with lower brain volumes in PLWH (Gongvatana et al., 2014).
		Interleukin (IL)-2	IL- 2 promotes inflammatory responses through the generation of Th1 and Th2 effector cells (Hoyer et al., 2008). CSF IL-2 levels were significantly higher in PLWH with a $CD4^+$ count of <200 cells/μL (Abassi et al., 2017). Significant findings for the association of IL-2 with HAND remain limited.
		IL-4	IL-4 is a cytokine that functions as a potent regulator of immunity secreted primarily by mast cells, Th2 cells, eosinophils and basophils (Gadani, Sachin P; Cronk, 2013). Studies of the association of IL-4 and HAND remains limited, however, one study found no significant difference in CSF levels between women living with HIV with and without HAND (Gerena et al., 2019).
		IL-5	IL-5 is an eosinophil-specific regulatory cytokine involved in the differentiation, activation, and survival of eosinophils (Kouro and Takatsu, 2009). Despite many reports, significant findings for the association of IL-5 with HAND remain limited.
		IL-6	IL-6 is a mediator of the acute phase response and can be produced by astrocytes exposed to HIV (Nitkiewicz et al., 2017). Studies show that IL-6 is increased in the CSF

			(Airoldi et al., 2012) as well as blood (Ancuta et al., 2008; Kamat et al., 2012a) of neurocognitively impaired PLWH.
		IL-7	IL-7 is a non-redundant cytokine in T-cell development and function (Elkassar and Gress, 2010). Higher blood IL-7 levels in mothers with HIV were associated with lower composite scores for language in HIV exposed uninfected children (24-28 months) (Sevenoaks et al., 2020).
		IL-8	IL-8 is the primary cytokine involved in neutrophil chemotaxis (Bickel, 1993). IL-8 may be increased by astrocytes in a gp120 mediated pathway (Shah and Kumar, 2010). IL-8 CSF levels were significantly higher in PLWH and HAND (Yuan et al., 2013).
		IL-10	IL-10 is an anti-inflammatory cytokine and maintains the balance of the immune response, allowing the clearance of infection while minimizing damage to the host (Iyer and Cheng, 2012). In brain-derived macrophages infected with HIV-1 and treated with raltegravir, IL-10 was one of the cytokines that remained detectable in samples (Tatro et al., 2014). Blood IL-10 levels were significantly associated with several domains of neurocognitive function (Cohen et al., 2011; Krebs et al., 2016).
		IL-12	IL-12 is a heterodimeric pro-inflammatory cytokine that regulates T-cell and natural killer-cell responses. Further, it induces the production of IFN-γ, favours the differentiation of TH1 cells and is an important link between innate resistance and adaptive immunity (Liu et al., 2005). CSF IL-12 levels were significantly higher in PLWH with a $CD4^+$ count of <200 cells/µL (Abassi et al., 2017), however, significant findings for its association with HAND remain limited.
		IL-13	IL-13 is an immunoregulatory cytokine (Khurana Hershey, 2003). It regulates the function of human B cells and monocytes (McKenzie et al., 1993). At this stage, significant associations of IL-13 and HAND remains limited.
		IL-16	IL-16 is a pleiotropic cytokine acting as a chemoattractive and modulating factor of T cell activation (Hermann et al., 1999). Serum concentrations of IL-16 were significantly associated with slower processing speed, independently of body mass index (Okafor et al., 2017).
		IL-17	IL-17 is a key proinflammatory cytokine that links T cell activation to neutrophil mobilization and activation (Zenobia and Hajishengallis, 2015). In a recent study, no associations were found between IL-17 levels from peripheral blood mononuclear cells and HIV-associated neurocognitive impairment (Swanta et al., 2020).

		IL-18	IL-18 is a pro-inflammatory cytokine and an important simulator of antigen-activated Th1 cells (Dinarello, 2000). Higher IL-18 levels were related to HIV-associated neurocognitive impairment (Correia et al., 2013).
		IL-1β	IL-1β is a potent pro-inflammatory cytokine that is responsible for host defense and responses. IL-1β is increased by HIV infection and aging (Kreuzer et al., 1997; Scully et al., 2016; Wiercińska-Drapalo et al., 2004). A more recent study by Festa and colleagues convincingly suggest that IL-1β functions as a key neuroinflammatory constituent in the pathogenesis of HIV-associated neurocognitive impairment (Festa et al., 2015).
		Macrophage colony-stimulating factor (M-CSF)	M-CSF is a hematopoietic growth factor controlling survival, proliferation, and differentiation as well as other functions of cells of the monocyte/macrophage lineage (Rappaport and Volsky, 2015). Several studies have now investigated the association of M-CSF and HAND, however, majority of the studies have not reported significant associations (Lentz et al., 2010).
		Macrophage inflammatory protein (MIP)-1α (CCL3)	MIP-1α is a chemotactic chemokine secreted by macrophages and functions in the recruitment of cells and inflammation(Bhavsar et al., 2015). In conjunction with HIV Tat, MIP is involved in the pathogenesis of HAND (Bonwetsch et al., 1999).
		MIP-1β (CCL4)	MIP-1β/CCL4 is chemotactic for monocytes, T cells, and Natural Killer cells (Maurer and Von Stebut, 2004). Evidence supports the neuroprotective properties of MIP-1β against gp120-mediated toxicity in rat and murine neuronal cultures (Kaul et al., 2007).
		Matrix metalloproteinases (MMP)-1	Matrix metalloproteinases (MMPs) have found to regulate neuroinflammation, blood-brain barrier (BBB) damage and cell migration(Rempe et al., 2016). MMP-1 levels were associated with HIV-associated neurocognitive impairment (Li et al., 2013), microstructural brain alterations and the degree of atrophy in PLWH (Li et al., 2013; Ragin et al., 2009).
		MMP-2	MMP-2 is a type IV collagenase which is elevated in the CSF of PLWH and HAD (Conant et al., 1999).
		MMP-7	MMP-7 is associated with breakdown of the BBB and leukocyte infiltration of the CNS (Anthony et al., 1998).
		MMP-9	MMP-9 is responsible for remodeling of processes involved in inflammation, degrades extracellular matrix proteins and activates cytokines and chemokines (Yabluchanskiy et al., 2013). MMP9 is increased in HIV infection via pro-inflammatory pathways

			(Missé et al., 2001). It is directly involved in BBB disruption (Takata et al., 2011) and increased levels in serum (Barr et al., 2010) can be used as an indication of BBB damage (Niebroj-Dobosz et al., 2010).
		MMP-10	MMP-10 is expressed by macrophages in numerous tissues after injury. Further, it moderates inflammation by controlling macrophage activation (McMahan et al., 2016) and has been associated with HIV-associated neurocognitive impairment and structural brain changes in PLWH (Li et al., 2013).
		Osteopontin (OPN)	OPN is a cytokine-like phosphoprotein that is increased in the brain, CSF and blood of people with neurodegenerative disorders including Parkinson's (Maetzler et al., 2007), Alzheimer's (Wung et al., 2007), Multiple Sclerosis (Comabella et al., 2005) and HAND (Brown, 2012).
		Progranulin (PGRN)	PGRN is a soluble factor that regulates cell proliferation, motility and inflammation (Suh et al., 2014). PGRN has a potential role in HIV-associated CNS pathologies and neurocognitive impairment (Suh et al., 2014).
		Soluble tumour necrosis factor (TNF) receptor (sTNFR)-I	sTNFR's are the cleaved-off extracellular domains of transmembrane TNF receptors. Blood sTNFR-I is higher in neurocognitively impaired PLWH (Ryan et al., 2001).
		sTNFR-II	sTNFR-II is an inflammatory cytokine that is elevated in blood and CSF of PLWH. Further, its levels are associated with HIV-associated neurocognitive impairment (Krebs et al., 2016; Woods et al., 2006)
		Stromal cell-derived factor (SDF)-1α (CXCL12)	SDF-1α is a chemokine which belongs to the subfamily of CXC-chemokines. It was shown that SDF-1α is present in the HIV brain and offers neuroprotective properties (Langford et al., 2002)
		Transforming growth factor (TGF)-β1	TGF-β1is a potent modulator of immune and glial cells. TGF-β1 exerts anti-inflammatory effects in the HIV-positive brain and play a neuroprotective function (Dhar et al., 2006). An inverse correlation between TGF-β1 and dementia in HIV has been reported (Perrella et al., 2001).
		TGF-β2	TGF-β2 is part of the TGF-β family of cytokines which regulates cell proliferation, differentiation, recognition, and death via serine/threonine kinase activity receptors (Zhang et al., 2017). Thus far, the studies investigating the association of TGF-β2 with HAND remain limited, and those that have done the investigation reports insignificant findings.

		TNF-related apoptosis-inducing ligand (TRAIL)	TRAIL is a type II transmembrane protein belonging to the TNF superfamily (Thorburn et al., 2008). TRAIL is expressed on the surface of natural killer and T cells, macrophages, and dendritic cells (Thorburn, 2007). TRAIL levels were also considered to be specific predictors of cognitive impairment in HIV positive women (Rubin et al., 2018).
		TNF-α	TNF-α is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation and is responsible for a diverse range of signalling events within cells, leading to necrosis or apoptosis (Idriss and Naismith, 2000). TNF has been extensively studied for its role in HAND, with higher levels detected in the brain, CSF (Xing et al., 2017) and blood (Falasca et al., 2017; Sevigny et al., 2004) of cognitively impaired PLWH.

Abbreviations:

BBB: Blood-brain barrier, CCL: Chemokine ligand, CNS: Central nervous system, CRP: C-reactive protein, CSF: Cerebrospinal fluid, CXCL: Chemokine (C-X-C motif) ligand, EPO: Erythropoietin, HAD: HIV-associated dementia, HAND: HIV-associated neurocognitive disorders, IFN: Interferon, IL: Interleukin, IP-10: IFN- gamma-induced protein 10, M-CSF: Macrophage colony-stimulating factor, MIP: Macrophage inflammatory protein, MMP: Matrix metalloproteinases (MMP), OPN: Osteopontin, PGRN: Progranulin, PLWH: People living with HIV, RANTES: Regulated upon Activation Normal T Cell Expressed and Presumably Secreted, sCD: Soluble cluster of differentiation, SDF: Stromal cell-derived factor (SDF), sTNFR: Soluble tumour necrosis factor receptor, TH: T-helper, TGF: Transforming growth factor, TNF: Tumor necrosis factor, TRAIL: TNF-related apoptosis-inducing ligand

Table 2: Quality assessment of studies

Reference	Question 1	Question 2	Question 3	Rating
*(Airoldi et al., 2012)	1	2	2	Intermediate
(Ancuta et al., 2008)	1	2	2	Intermediate
(Brown et al., 2011)	2	2	1	Intermediate
*(Burdo et al., 2013)	2	2	1	Intermediate
(Cohen et al., 2011)	2	2	1	Intermediate
(Correia et al., 2013)	2	0	1	Low
*(D'Antoni et al., 2018)	2	2	2	High
(De Oliveira et al., 2015)	2	2	2	High
(Falasca et al., 2017)	2	2	2	High
(Imp et al., 2017)	2	2	1	Intermediate
(Kamat et al., 2012)	2	2	1	Intermediate
*(Krebs et al., 2016)	2	2	2	High
(Gougeon et al., 2017)	2	2	1	Intermediate
*(Lentz et al., 2010)	1	2	2	Intermediate
(Li et al., 2013)	1	2	1	Intermediate
(Lyons et al., 2011)	2	2	1	Intermediate
*(Marcotte et al., 2013)	2	2	1	Intermediate
*(Monnig et al., 2017)	2	2	1	Intermediate
(Montoya et al., 2019)	2	2	2	High
*(Rubin et al., 2018)	2	2	1	Intermediate
(Ryan et al., 2001)	0	2	1	Low
(Sakoda et al., 2017)	2	2	1	Intermediate
*(Sevigny et al., 2004)	2	2	2	High
(Suh et al., 2014)	2	2	1	Intermediate
(Sui et al., 2007)	0	2	1	Low
(Sun et al., 2010)	2	2	1	Intermediate
*(Valcour et al., 2015)	2	1	2	Intermediate
(Woods et al., 2006)	2	1	1	Intermediate
(Woods et al., 2010)	2	2	1	Intermediate
(Xing et al., 2017)	2	2	1	Intermediate
(Yuan et al., 2015)	1	2	1	Intermediate
(Yu et al., 2017)	2	2	1	Intermediate

*Longitudinal studies. Questions 1-3 were classified as follows: (1) Did the study report on potential confounders such as substance misuse, comorbid conditions (e.g. Hepatitis C (HCV)), neurological conditions and psychiatric disorders and were these controlled for upon statistical analysis? (2) Did the study utilize a test battery of at least 5 domains of function commonly affected by HIV? (3) Did the study report on the current regimen of ART and the duration of treatment before the assays were done for cross-sectional studies? For longitudinal studies, did the study report on the current regimen of ART and the duration of treatment between each time point? Each question was rated for 0=no, 1=partly and 2=yes. Studies that addressed all of the quality questions and had a total rating of 6 were classified as high quality. Studies with a rating between 3 and 5 were considered as intermediate quality and less than 3 as low quality.

Table 3: Studies reporting a relationship with HIV-associated neurocognitive performance when stratified according to CD4 count

Reference	<200 cells/µl	>200 cells/µl	Association with HAND	
			Yes	No
(Ancuta et al., 2008)	✓		✓	
*(Airoldi et al., 2012)	✓			✓
(Brown et al., 2011)	✓			✓
*(Burdo et al., 2013)		✓	✓	
(Cohen et al., 2011)		✓	✓	
(S. Correia et al., 2013)		✓	✓	
*(Michelle L D'Antoni et al., 2018)		✓		✓
(De Oliveira et al., 2015)		✓		✓
(Falasca et al., 2017)		✓	✓	
(Kamat et al., 2012)	✓		✓	
*(Krebs et al., 2016)		✓	✓	
(Gougeon et al., 2017)		✓		✓
*(Lentz et al., 2010)		✓		✓
(Li et al., 2013)		✓	✓	
(Lyons et al., 2011)	✓		✓	
(Montoya et al., 2019)		✓		✓
*(Rubin et al., 2018)		✓	✓	
(Ryan et al., 2001)	✓		✓	
(Sakoda et al., 2017)		✓		✓
(Suh et al., 2014)		✓		✓
(Sui et al., 2007)		✓	✓	
(Sun et al., 2010)		✓		✓
(Woods et al., 2006)		✓	✓	
(Xing et al., 2017)	✓		✓	
(Yuan et al., 2015)	✓		✓	
(Yu et al., 2017)		✓		✓

**longitudinal studies*

Table 4: Studies reporting a relationship with HIV-associated neurocognitive performance when stratified according to viral suppression

Reference	Virally suppressed	Non-virally suppressed	Association with HAND	
			Yes	No
(Ancuta et al., 2008)		✓	✓	
*(Airoldi et al., 2012)	✓			✓
(Brown et al., 2011)		✓		✓
*(Burdo et al., 2013)	✓		✓	
(Cohen et al., 2011)	✓		✓	
(S. Correia et al., 2013)	✓		✓	
*(Michelle L D'Antoni et al., 2018)	✓			✓
(De Oliveira et al., 2015)	✓			✓
(Falasca et al., 2017)	✓		✓	
(Imp et al., 2017)	✓		✓	
(Kamat et al., 2012)		✓	✓	
*(Krebs et al., 2016)		✓	✓	
(Gougeon et al., 2017)	✓			✓
*(Lentz et al., 2010)		✓		✓
(Li et al., 2013)		✓	✓	
(Lyons et al., 2011)		✓	✓	
*(Marcotte et al., 2013)	✓			✓
*(Monnig et al., 2017)	✓		✓	
(Montoya et al., 2019)	✓			✓
*(Rubin et al., 2018)	✓		✓	
(Ryan et al., 2001)		✓	✓	
(Sakoda et al., 2017)	✓			✓
(Suh et al., 2014)		✓		✓
(Sui et al., 2007)		✓	✓	
(Sun et al., 2010)		✓		✓
(Woods et al., 2006)	✓		✓	
(Woods et al., 2010)	✓			✓
(Xing et al., 2017)		✓	✓	
(Yuan et al., 2015)		✓	✓	
(Yu et al., 2017)	✓			✓

**longitudinal studies*

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Chapter 3

The association of immune markers with cognitive performance in South African HIV-positive patients

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Abstract

Dysregulated expression of neuro-immune markers has previously been linked to HIV-associated neurocognitive impairment. We undertook an exploratory approach in a South African cohort, investigating the association between eight immune markers and neurocognitive performance in 99 people living with HIV (PLWH) and 51 HIV-negative participants. Markers were selected on preliminary and putative evidence of their link to key neuro-immune functions. Cognitive performance was established using a battery of tests sensitive to HIV-associated neurocognitive impairment, with domain-based scores utilized in analysis. The markers thymidine phosphorylase (TYMP) and neutrophil gelatinase-associated lipocalin (NGAL) were significantly higher while matrix metalloproteinase (MMP)9 was significantly lower in PLWH. Our results further showed that in PLWH, worse psychomotor processing speed was associated with higher TYMP and NGAL levels and worse motor function was associated with higher NGAL levels. Future studies should explore the underlying mechanisms of these markers in HIV-associated neurocognitive impairment.

3.1. Introduction

HIV-associated neurocognitive impairment occurs in up to 50% of the HIV-infected population (Heaton et al., 2010). HIV-1 invades the brain through a "Trojan Horse" method by crossing the blood-brain barrier (BBB) through infected monocytes that later differentiate into macrophages (González-Scarano and Martín-García, 2005). This triggers an inflammatory response, which is considered to be a crucial contributor to the neuropathogenesis of HIV-associated neurocognitive impairment (Beck et al., 2015; Jones et al., 2016). In human studies, associations of increased pro-inflammatory markers in peripheral blood with cognitive impairments in HIV further support the involvement of a dysregulated immune system in cognitive impairment in HIV (Cohen et al., 2011; Correia et al., 2013; Yuan et al., 2013)

Damage to the BBB is another common hallmark of HIV-associated neurocognitive impairments (Banks et al., 2006). BBB damage allows for the further recruitment of infected cells into the brain (Banks et al., 2006). Differential expression of BBB integrity markers in blood and CSF (e.g. MMP9 and s100-β) correlated with neurocognitive impairment in HIV participants (Abassi et al., 2017; Li et al., 2013; Xing et al., 2017). Therefore, a dysregulated expression of immune markers may contribute to the development of HIV-associated neurocognitive impairments. Efforts in elucidating the neuro-immune response of HIV-associated neurocognitive impairment have to date largely been centered on investigation of monocyte activation markers (Burdo et al., 2013; Imp et al., 2017; Kamat et al., 2012; Lyons et

al., 2011; McGuire et al., 2015) and pro-inflammatory markers (e.g. TNF-α) (Brabers and Nottet, 2006; Wesselingh et al., 1997).

The systematic review (Chapter 2) suggests that an aberrant immune regulation is a key characteristic of HAND. However, low to moderate effect sizes in their associations with HAND and inconsistencies between studies (Cohen et al., 2011; Kamat et al., 2012; Marcotte et al., 2013; Montoya et al., 2019; Rubin et al., 2018; Ryan et al., 2001) suggest that further investigations on new immune markers are warranted. Here, we aimed to explore the associations between several immune markers and neurocognitive performance in HIV (subtype C) participants that were treatment naïve or had only recently started combination antiretroviral therapy (cART) (< 1 month) to elucidate the inflammatory profile without the confounding effects of cART on neuronal health. Specifically, to investigate the association between these markers and neurocognitive domains commonly affected by HIV (Butters et al., 1990). Furthermore, a systematic review and meta-analysis indicate that globally, a large percentage of patients do not adhere to treatment (Uthman et al., 2014), making this population interesting for further investigation. Majority of longitudinal studies also report that with the introduction of cART, the first neuro-immune responses are noted at durations greater than 3 months (Hattab et al., 2014; Krebs et al., 2016; Richert et al., 2017). This population represents participants with a higher risk of developing dementia and therefore this study may provide an opportunity to investigate the immune response in these individuals.

The markers monocyte chemoattractant protein-1/C-C motif ligand 2 (MCP-1/CCL2), transforming growth factor (TGF)-β, matrix metalloproteinase (MMP)9, vascular endothelial growth factor (VEGF), thymidine phosphorylase (TYMP) and neutrophil gelatinase-associated lipocalin (NGAL) were selected for investigation based on their potential involvement in the pathophysiology of neurocognitive impairments in HIV, as identified from the scientific literature. As shown in other neurodegenerative disorders and HIV preclinical studies, these markers are involved in pathways of 1) inflammatory induced neuronal dysfunction and 2) BBB damage (Supplementary Table 1). Similarly, these markers may have a functional role in pathways related to the development of HIV-associated neurocognitive impairments. A literature summary of the potential neurobiological function of these markers are presented in Supplementary Table 1

3.2. Methods

3.2.1. Study participants:

This study included n = 99 HIV-positive and n = 51 HIV-negative participants, which were pooled from two independent studies. Cohort 1 (Joska et al. 2011) included 56 HIV-positive participants from a study which characterized HAND among South African PLWH. Cohort 2 included 43 HIV-positive participants from an ongoing study that is investigating the effects of heavy drinking on HIV-associated neurocognitive impairments. PLWH included in this study were previously recruited from primary health care clinics in Cape Town and the Western Cape region of South Africa. PLWH completed at least one study visit, which included a detailed sociodemographic,

medical and neuropsychological assessment as well as the relevant laboratory measures (including viral load and CD4 count). HIV serostatus was confirmed by two independent rapid tests and confirmed via ELISA analysis. In addition, 51 HIV-negative control participants recruited from local voluntary counseling and testing clinics as part of the ongoing study were included in this study for comparison purposes. Participants included in this study ranged from 18 through 65 years in age with at least 7 years of formal education, across both cohorts. PLWH were treatment naïve or had only recently started cART (< 1 month) prior to neurocognitive measurements and blood collection. Cases were excluded from this study if they had 1) severe psychiatric disorder or presented any other neurological disorder, 2) substance abuse (other than alcohol) and 3) moderate to severe head injury.

Participation was voluntary, and individuals were informed that they could withdraw from the study at any time. Written informed consent was obtained following a thorough explanation of the study procedures. This study was approved by the Human Research Ethics Committee of the Faculty of Health Sciences (University of Cape Town) (Sub-study HREC 213/2018 linked to primary studies: 003/2015, 023/2008 and 263/2007). Individuals received financial compensation for their time. Bloods were obtained at the same visit when measures for neurocognitive performance were evaluated.

3.2.2. Neurocognitive measures:

The presence of HIV-associated neurocognitive impairment was assessed with a detailed battery. All participants were tested in their home language and all instruments had their instructions and content translated into isiXhosa and Afrikaans. Instructions were also back-translated for fidelity. The test battery represents measures of domains typically affected by HIV (Butters et al., 1990) and encompassed several domains of cognitive function. The specific scores used for each domain were acquired from the following neuropsychological measures: Processing speed (WAIS-III Digit symbol, WAIS-III Symbol search, Colour trails I Stroop Colour) (Golden, 1975), verbal (Animal Fluency) (Acevedo et al., 2000), learning (the Hopkins Verbal Learning, Brief Visuospatial Memory Test) (Benedict et al., 1998), memory (HVLT Delayed recall, BVMT Delayed recall), motor functioning (Groove peg board Dominant, Groove peg board Non-dominant) (Klove 1963) and executive functioning (Colour trails II) (D'Elia et al., 1996). The raw neuropsychological test scores were standardized using data from the HIV-negative control participants to calculate *z*-scores. *Z*-scores were then averaged to create 6 composite indices (processing speed, verbal, learning, memory, motor and executive functioning).

3.2.3. Laboratory assessment of blood

Blood samples for all study participants were collected into tubes via venipuncture. Serum tubes were kept at room temperature for 30 minutes to allow for clotting. Plasma EDTA tubes were kept on ice for 30 min until centrifugation. Serum and EDTA tubes were subsequently centrifuged at 2,500 × g. Serum and plasma were then aliquoted into cryo vials and immediately stored at -80 °C until analyses. HIV viral load was determined by the CAP/Roche Cobas Ampliprep (sampled post-2015) and the Abbott M2000SP and M2000RT (sampled prior to 2015) methods. The CD4 count was determined by PanLeucogate (PLG) (Beckman Coulter FC500MPL) method.

All immune markers were measured using Enzyme-linked Immunosorbent Assays (ELISA) (R&D systems, DuoSet ELISA) according to the manufactures instructions. CCL2, VEGF and TYMP were measured in serum. TGF-β1, IL-1β, IFN-γ, MMP9 were measured in plasma. Samples were diluted as follows MCP-1/CCL2: 1:2, TGF-β1: 1:30, IL-1β: no dilution, IFN-γ: no dilution, MMP9: 1:150, VEGF: no dilution, TYMP: 1:10 and NGAL: 1:100. All samples were assayed in duplicate. The intra- and inter-assay coefficients of variation for all tests were within acceptable ranges of < 8% and < 10% respectively.

3.2.4. Other potential covariates

Years of completed formal education and age were included as covariates in models of the association of immune system markers and cognitive function. In addition, given the presence of heavy drinkers in cohort 2, and the observation that chronic excessive alcohol consumption may affect both the immune system and cognitive function in people with HIV (Meyerhoff, 2001), problematic drinking was also included as a covariate. Hazardous drinking was operationalised as more than 5 standard drinks per occasion over a 2-week period for 3 months prior to the marker assays, as assessed using the timeline follow-back calendar questionnaire. A further description of the cohort is described in the supplementary file (Table 2).

3.2.5. Statistical analyses:

All analyses were conducted using SPSS (version 25, IBM, USA). *P* - values were considered statistically significant for all analyses at a value of less than .05. Data distribution for markers NGAL, CCL2 and TGF- β1 were found to be skewed, therefore the data was log transformed prior to statistical analyses. MMP9, TYMP and VEGF demonstrated normal data distribution with acceptable skewness and kurtosis. IL-1β and INF-γ were below the detectable ranges in serum for all samples and omitted from further analyses. Unpaired t-test analyses were performed to investigate differences in the levels of plasma/serum markers between PLWH and HIV-negative study participants. Analyses of covariance (ANCOVA) with levels of

immune markers as dependent variables were subsequently performed to analyze the levels of the immune markers PLWH and HIV-negative study participants, adjusted for age, sex, education, cohort and alcohol use. Exploratory analyses were done with Pearson correlations to investigate associations between the immune markers with the cognitive domains in PLWH and HIV-negative respectively. A Bonferroni correction was accounted for the number of cognitive domains tested (α/n = .05/6 = .008). Subsequently, separate regression models were conducted to examine the associations between cognitive functions and marker concentrations, after adjusting for the following covariates individually: demographics (age, sex and education), cohort (cohort, heavy drinkers) and HIV-positive disease parameters (nadir CD4 count, and viral load).

3.3. Results

3.3.1. Sample characteristics:

The study population included a total of n=150 participants which comprised of n = 99 PLWH and n = 51 HIV-negative controls. PLWH had a mean age of 33.88 years and 36% were female. The HIV-positive group had a median nadir CD4 count of 305 (159 - 414.5) cells/µl and viral load of 3.91 (2.853 - 4.69) log copies/ml. From the PLWH, n = 19 had been on treatment for less than one month upon sample collection, with the remainder cART naïve. The two cohorts differed with respect to mean age, gender composition and current CD4 count. Differences between mean age and gender were controlled for upon statistical analysis. Sample characteristics are

summarized in Table 1. Information on the sample characteristic of the separate cohorts is presented in the supplementary file (Table 2). The effect of drinking habits on cognitive performance in either HIV-negative controls or PLWH in the second cohort was evaluated. No significant differences between the heavy drinkers vs. non/light drinkers in either the HIV-negative control group or the PLWH were found for any of the cognitive domains tested (all *p*-values were greater than .1) (results not shown).

Table 1. Characteristics of HIV-negative and HIV-positive study participants.

	HIV-negative (n=51) **Controls**	**HIV-positive** (n=99) **HIV-positive**	***p*-value**
Age years mean (SD)	37.51 (9.07)	33.88 (6.91)	.01
Sex, female N (%)	27 (52.9)	36 (36.4)	.051
Education, years (SD)	10.28 (1.55)	10.50 (1.30)	.38
CD4 + nadir, median (IQR)	-	305 (159 -414.5)	-
Viral load log copies/mL median (IQR)	-	3.91 (2.853 - 4.69)	-
Heavy drinkers, N (%)	26 (51.0)	18 (18.2)	< .001

Abbreviations: Interquartile range (IQR), Standard deviation (SD)

3.3.2. Immune markers in HIV-positive versus HIV-negative participants.

Bivariate analyses revealed that TYMP ($p < .001$) and CCL2 ($p = .027$) were significantly higher whereas MMP9 ($p = .007$) was lower in PLWH relative to controls (Figure 1). TYMP remained significantly higher ($p < .015$) and MMP9 significantly lower ($p = .005$) in PLWH after controlling for age, sex, education and alcohol use with ANCOVA. CCL2 was no longer significantly higher ($p = .78$) in PLWH after

controlling for these covariates. After controlling for these variables, the difference in NGAL level was statistically significant between the two groups (p = .046).

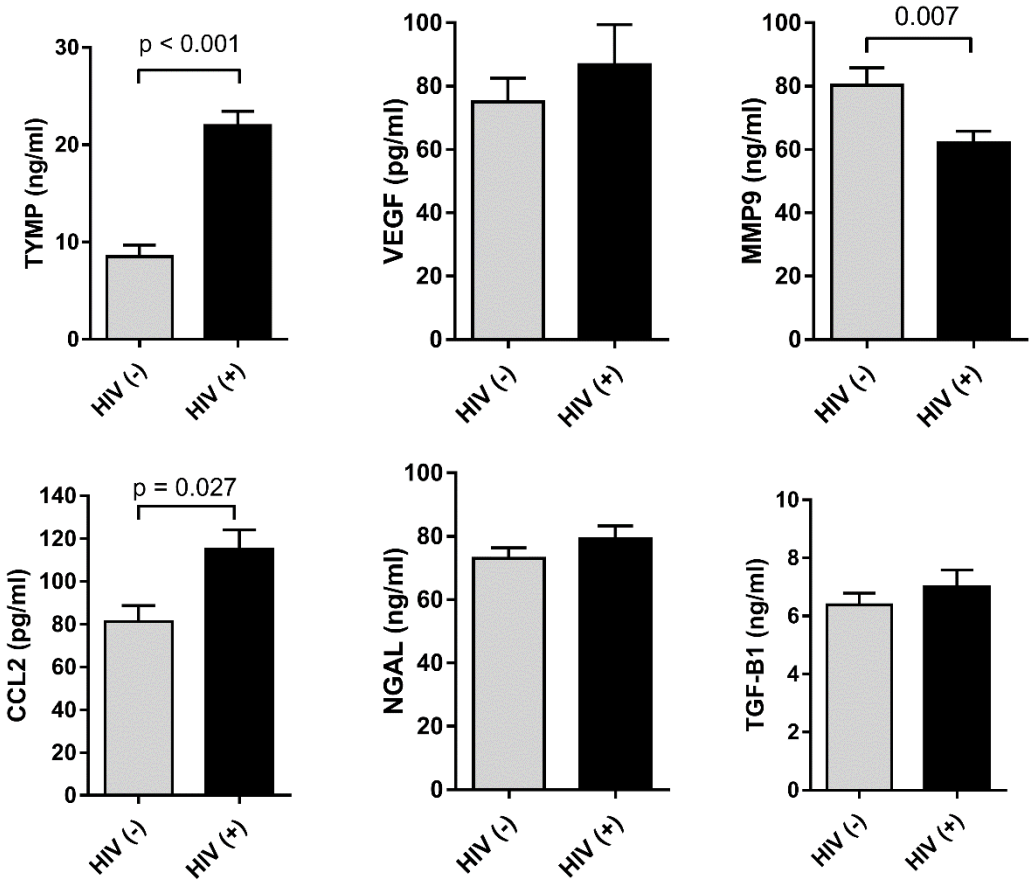

Figure 1. Concentrations of immune markers between HIV-negative and HIV-positive study participants. NGAL, CCL2 and TGF- β1 were ln-transformed and for interpretation purposes presented means were back-transformed. Bars indicate the mean protein concentrations in the different study groups and are expressed as mean ± standard error of the mean (s.e.m.).

3.3.3. Association of immune marker levels and cognitive performance in HIV-negative participants.

After applying Bonferroni corrections of p = .05/6, higher levels of TGF-B1 were associated with better performance in processing speed in HIV-negative participants (p = .008) (Table 2) (unadjusted). Linear regression analyses with covariates; 1) alcohol use and 2) demographics (age, sex and education) were controlled for. After controlling for 1) alcohol use only (B = .367, SE = .149, β =.391, p = .019) and 2) demographics (B =.172, SE =.148, β = .171, p = .25) the association was attenuated.

Table 2. Pearson correlations between immune markers and composite cognitive domain scores (through averaging Z scores of specific within-domain tests) in HIV-negative participants

	TYMP	**VEGF**	**MMP9**	**CCL2**	**NGAL**	**TGF-B1**
Cognitive domain						
Processing speed	-.118	.053	.101	.047	-.189	**.425**[a]
Verbal	-.086	-.250	-.154	.041	-.159	-.007
Learning	-.211	-.048	-.027	.002	-.163	.021
Memory	-.122	-.072	.123	-.142	-.167	.166
Motor Functioning	.187	.119	.085	.210	.117	-.227
Executive function	.098	-.061	.028	.042	.061	-.078

[a] p = .008.

3.3.4. Association of immune marker levels and cognitive performance in PLWH

After applying Bonferroni corrections of p = .05/6, higher TYMP (p = .001) and NGAL (p = .007) levels were associated with worse processing speed and higher NGAL

levels with worse motor functioning (p = .002) in PLWH (Table 3). Further analyses were performed for these markers after adjusting for the covariates, age, sex, education, alcohol use, nadir CD4 count, cohort and HIV viral load (Table 4). The association between TYMP and NGAL with neurocognitive performance remained after correcting for demographics, cohort, alcohol use and HIV disease parameters (Table 4).

Table 3. Pearson correlations between immune markers and composite cognitive domain scores (through averaging Z scores of specific within-domain tests) in PLWH

	TYMP	**VEGF**	**MMP9**	**CCL2**	**NGAL**	**TGF-B1**
Cognitive domain						
Processing speed	**-.340**[a]	-.136	-.025	-.101	**-.285**[b]	.049
Verbal	-.100	-.101	-.046	-.134	-.061	-.034
Learning	-.012	-.257[c]	.208[d]	.176	-.049	-.012
Memory	.014	-.186	.025	.120	-.016	-.015
Motor Functioning	.178	.061	.007	.130	**.329**[e]	-.026
Executive function	.132	.180	.106	.091	.146	.089

[a]p = .001, [b]p = .007, [c]p = .011, [d]p = .043, [e]p = .002.

Table 4. Association of TYMP and NGAL with cognitive performance, including covariates in PLWH.

	TYMP and Processing speed			**NGAL and Processing speed**			**NGAL and motor functioning**		
	B (SE)	β	p	B (SE)	β	p	B (SE)	β	p
Unadjusted	-.013 (.004)	-.34	.001	-.37 (.134)	-.285	.007	.602 (.182)	.329	.002
Model 1	-.014 (.004)	-.37	.001	-.463 (.128)	-.358	.001	.611 (.180)	.350	.001
Model 2	-.013 (.004)	-.33	.003	-.362 (.142)	-.279	.012	.690 (.190)	.377	<.001
Model 3	-.014 (.005)	-.319	.010	-.533 (.172)	-.375	.003	.615 (.269)	.279	.025

Model 1: adjusted for demographics; age, sex and education.
Model 2: adjusted for cohort; cohort, heavy drinkers.
Model 3: adjusted for HIV-positive disease parameters; nadir CD4 count, and viral load

3.4. Discussion

In this exploratory study, we investigated the serum and plasma levels of several immune markers and their associations with domain-based neurocognitive performance. We found significantly higher levels of TYMP and NGAL, and significantly lower levels of MMP9 in PLWH compared to HIV- controls, after controlling for age, sex, education, and alcohol use. Furthermore, in PLWH, we found that after controlling for age, sex, education, alcohol use, CD4 count, cohort and viral load, higher TYMP levels were associated with worse processing speed and higher NGAL levels were associated with worse processing speed and motor functioning.

The current study is the first to our best knowledge to investigate peripheral circulating TYMP levels in PLWH. Changes to the neuroimmmunity in PLWH may contribute to the higher serum levels of TYMP demonstrated in this study. A microarray study of gene expression of TYMP across tissues reported that its expression is particularly high in $CD14^+$ monocytes (Su et al., 2002). The TYMP gene expression was reported to be increased in primary monocytes of HIV patients with viremia (Wu et al., 2013). Moreover, $CD14^+$ monocytes are increased in PLWH (Bowers et al., 2014) regardless of treatment status (Bowers et al., 2014; Mendez-Lagares et al., 2013). $CD14^+$ monocytes may account for the higher TYMP levels in patients with HIV.

Evidence from basic science research may provide insights into the association of TYMP with neurocognitive performance, specifically in the domains of processing

speed. Generally, in a treatment naïve cohort, HIV replication is not controlled and a typical sub-cortical pattern of pathology is seen, with resulting impairment of processing speed and executive functions (Bell, 2004; Moore et al., 2006). A study on post mortem brain tissues from patients with HIV/ Tuberculosis Meningitis (TBM) demonstrated that the TYMP protein expression was further increased when HIV was present in patients with TBM (Kumar et al., 2012). It was further reported that TYMP co-localized with microglia (Kumar et al., 2012), indicating that activated microglia may produce TYMP in the HIV brain. In a study conducted by Chapouly and colleagues, it was demonstrated that TYMP was an important contributor to BBB disruption in brain cell cultures and in an experimental autoimmune encephalitis mouse model (Chapouly et al., 2015). Further studies are needed to elucidate the potential involvement of TYMP in BBB dysregulation in HIV. This is of interest since BBB damage may play an important function in the pathophysiology of neurocognitive impairment in HIV (Banks et al., 2006; Maubert et al., 2017).

Higher plasma NGAL levels in PLWH have recently been reported in a clinical investigation (Morieri et al., 2018) and is supported by the significantly higher levels in PLWH reported in our study. Elevated serum and plasma NGAL has been associated with neurocognitive impairment in patients with mild cognitive impairment in Alzheimer disease (Choi et al., 2011) as well as in patients with acute ischemic cerebrovascular diseases (Elneihoum et al., 1996). This study further contributes to literature indicating that NGAL is an inflammatory marker that is associated with cognitive impairment (Choi et al., 2011). Our findings show that higher NGAL levels are associated with worse processing speed. In this regard, it was

previously demonstrated that higher plasma NGAL levels were associated with impaired verbal memory and processing speed in females with late-life depression (Naudé et al., 2014). Mounting evidence from investigations in cell cultures and animal models suggest that NGAL may contribute to neurobiological mechanisms that are involved in the neuropathology of neurocognitive impairments. NGAL may act on neuronal health by in inhibiting remyelination (Al Nimer et al., 2016) and neuronal survival (Bi et al., 2013). Demyelination or change in myelin structure, in turn, could contribute towards oligodendrocyte damage, resulting in desynchronized neural circuits and impaired information processing speed (Pajevic et al., 2014), which are common pathologies in HIV (Liu et al., 2016). NGAL may also be involved in motor functioning. Animal studies suggest that increased levels of NGAL may interfere with motor functioning (Jang et al., 2013) and BBB integrity (Egashira et al., 2016). However, the neurobiological mechanisms of NGAL in HIV requires further investigation.

Contradictory to previous findings (Lorenzl et al., 2008), here MMP9 was significantly lower in PLWH. This may be due to clinical studies largely investigated MMP9 in extended-duration treatment cohorts and cART may contribute to increased inflammatory levels. A HIV cART treatment cohort demonstrated higher MMP9 levels compared to that of the treatment naïve cohort (Li et al., 2013). In addition, the study by Li and colleagues also found that MMP9 expression levels were very similar for HIV-negative participants (33,385pg/ml ± 16,230) and treatment naïve PLWH (35,073pg.ml ± 43,245) (Li et al., 2013). This may suggest that MMP9 levels may be affected by the extended use of cART.

In addition to the above, Southern Africa is largely a HIV subtype-C population (Wainberg, 2004), which has been linked to lower levels of neurotoxicity (Rao et al., 2013). MMP9 investigations were largely done in subtype-B HIV cohorts (Kim et al., 2014; Li et al., 2013; Sporer et al., 1998), which has been linked to increased prevalence of neurocognitive impairments (Langford et al., 2003) and increased neuroinflammatory levels (Gandhi et al., 2009; Wong et al., 2010). This has been largely attributed to the presence of a dicysteine motif in that Tat protein of subtype-B cohorts, which potentiates increased monocyte chemotaxis and increased neurotoxicity (Rao et al., 2013). It is, therefore, possible that MMP9 levels are affected by subtype differences as well as extended use of cART, potentially explaining the lower MMP9 levels in the PLWH from which our sample was drawn.

A limitation of the present study is its relatively small sample size, which warrants replication in a larger sample size. The cohorts pooled for investigation in this study may have influenced the results. Participants were excluded if they presented with clinical signs of any other co-infections on history and clinical examination. However, potential confounding factors that were not screened out from this study, may in part, explain the outcomes reported in this study. Considering that a subset of participants were heavy drinkers, we performed a sub-analysis to determine the effects of heavy drinking on inflammatory profiles in our HIV-positive population. We demonstrated that there were no significant differences in inflammatory profiles for the HIV-positive non/light drinkers *vs*. HIV-positive heavy drinkers (Supplementary file, Table 3). Participants included in this study were either treatment naïve or only recently initiated treatment (<30 days). cART is also a contributor to dysregulated

inflammatory levels in PLWH(Shah et al., 2016). However, long-term treatment studies reported that in most cases, the first neuro-immune responses are noted at durations greater than 3 months (Hattab et al., 2014; Krebs et al., 2016; Richert et al., 2017). Serum IL-1β and IFN-γ were below the detectable ranges of the assays used in this study and therefore excluded from further statistical analyses. A number of the cognitive domains were only measured with a single neuropsychological test, whereas a larger number of neuropsychological tests per cognitive domain may increase the reliability and external validity of estimates presented here. The findings presented here may not apply to other HIV-1 subtypes (e.g. HIV-1 subtype-B), as inflammatory markers may be affected by the HIV subtype (Gandhi et al., 2009).

3.5. Conclusion

This exploratory study shows that the immune markers TYMP and NGAL may be associated with neurocognitive impairment in PLWH. This is the first study to our knowledge to investigate the associations of TYMP and NGAL in neurocognitive impairments in patients with HIV. The results from this study indicate that TYMP and NGAL potentially are interesting targets for future fundamental and clinical investigations in HIV-associated neurocognitive impairments.

These markers may be involved in the underlying neuropathological mechanisms of HAND. Previous studies have shown an association between peripheral inflammation and reduced brain volumes. However, the association of peripheral inflammation with brain cortical thickness and surface area remains unclear. In the next chapter

(Chapter 4), I aim to establish whether inflammation and in particular, TYMP and NGAL were related to regional cortical brain alterations. Findings in the next chapter may help to explain the associations of these markers with cognitive domains reported in this chapter.

3.6. References

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3.7. Supplementary: Chapter 3

Table 1: Description of inflammatory and BBB integrity markers investigated

Marker	Description
Chemokine ligand 2 (MCP-1/CCL2)	CCL2 is a chemoattractant chemokine. Clinical studies suggest CCL2 increases with age (Blasko et al., 2005) and is associated with faster cognitive decline in dementia (Westin et al., 2012). Blood and CSF CCL2 is related to neurocognitive impairment in HIV (Yuan et al., 2013) and Alzheimer's diseases (Lee et al., 2018). In an in vitro blood-brain barrier (BBB) model, migration of HIV-positive cells across the BBB was largely CCL2 dependent (Eugenin, 2006).
Transforming growth factor (TGF)-β1is	Transforming growth factor (TGF)-β1is potent modulator of immune and glial cells. TGF-β1 exerts anti-inflammatory effects in the HIV-positive brain and play a neuroprotective function (Dhar et al., 2006). An inverse correlation between TGF-β1 and dementia in HIV has been presented (Perrella et al., 2001).
Interleukin (IL)-1β	Interleukin (IL)-1β is a potent pro-inflammatory cytokine that is responsible for host defense and responses. IL-1β is increased by HIV infection and aging (Kreuzer et al., 1997; Scully et al., 2016; Wiercińska-Drapalo et al., 2004). A more recent study by Festa and colleagues convincingly suggest that IL-1β functions as a key neuroinflammatory constituent in the pathogenesis of HIV-associated neurocognitive impairment (Festa et al., 2015).
Interferon (IFN)-γ	Interferon (IFN)-γ is an inflammatory marker that plays a crucial role in the host immunity against viral and bacterial pathogens (Roff et al., 2014). IFN-γ has demonstrated a steady increase in HIV with associations with HIV-associated neurocognitive disorders (Schrier et al., 2015) and extended age (Lee et al., 2014). IFN-γ has recently been reported to contribute to BBB dysfunction through blocking signaling of the Platelet-derived growth factor receptor beta (PDGFRβ) in human brain pericytes. PDGFRβ signaling is crucial in human pericyte function and its removal results in BBB leakage (Jansson et al., 2016).
Neutrophil gelatinase-associated lipocalin (NGAL)	Neutrophil gelatinase-associated lipocalin (NGAL) is a neuroinflammatory mediator that has been identified to influence neuronal health in pre-clinical studies. Specifically, activated astrocytes are able to release NGAL and exert neuronal toxicity (Bi et al., 2013). Animal models have reported that NGAL is upregulated in the brain by induction of lipopolysaccharides as well as after psychological stressors (Ip et al., 2011; Mucha et al., 2011). In a rodent model of cerebral ischemia, it was reported that NGAL is significantly upregulated in the hippocampus. In the same study with cell cultures, it was found that NGAL

	exerted neurotoxic effects on hippocampal neurons and promoted neuroinflammation through activation of microglia (Kim et al., 2017). A clinical study demonstrated that serum NGAL levels are increased in patients with mild cognitive impairment (Choi et al., 2011). However, studies on the association between NGAL with neurocognitive impairment in HIV are still lacking.
Metalloproteinase (MMP)9	Metalloproteinase (MMP)9 is responsible for remodeling of processes involved in inflammation, degrades extracellular matrix (ECM) proteins and activates cytokines and chemokines (Yabluchanskiy et al., 2013). MMP9 is increased in HIV infection via pro-inflammatory pathways (Missé et al., 2001). It is directly involved in BBB disruption (Takata et al., 2011) and increased levels in serum (Barr et al., 2010) can be used as an indication of BBB damage (Niebroj-Dobosz et al., 2010).
Vascular endothelial growth factor (VEGF)	Vascular endothelial growth factor (VEGF) is an angiogenesis growth factor crucial for the growth of blood vessels and the development of the adult nervous system (Toi et al., 1995). Serum VEGF-A is significantly increased in PLWH (Sporer et al., 2004). It also functions in BBB breakdown in neuroinflammatory disease (Argaw et al., 2012) and may function as a marker for BBB injury (Nag et al., 2002)
Thymidine phosphorylase (TYMP)	Thymidine phosphorylase (TYMP) is an endothelial cell growth factor involved in angiogenesis. The combination of increased VEGF-A and TYMP significantly promote BBB breakdown in cell culture and animal models (Chapouly et al., 2015). In a clinical investigation of primary circulating monocytes in PLWH with controlled viremia (below detection level) versus HIV patients on HAART who experience viremia, the TYMP gene expression was upregulated in patients experiencing viremia in comparison to those with below detection level (Wu et al., 2013). HIV may be a contributor to the increased TYMP levels, as reported by an investigation of Tuberculosis Meningitis (TBM) and co-infected TBM/HIV brain tissue. TYMP gene expression was increased by 3-fold in co-infected TBM/HIV tissue in comparison to TBM only tissue samples (Kumar et al., 2012).

Table 2. Sample characteristics of the two cohorts used in this study.

	Cohort 1 (n=56)	**Cohort 2 (n=94)**		
	HIV-positive (n=56)	**HIV-positive (n=43)**	**HIV- control group (n=51)**	
				p-value
Age years, mean (SD)	30.9 (4.73)	37.74 (7.43)	37.51 (9.07)	<.001*
Sex, female N (%)	6 (10.7)	30 (69.9)	27 (52.9)	<.001[#]
Education, years (SD)	10.41 (1.25)	10.68 (1.39)	10.28 (1.55)	N.S.
Current CD4, mean (SD)	273.00 (148.41)	400 (241.46)	-	.006
Viral load, mean (SD)	33799.89 (63991.46)	59486.86 (148238.86)	-	N.S.
Heavy drinkers N (%)	0 (0)	18 (41.86)	26 (51.0)	<.001[$]
HAART N (%)	0 (0)	19 (44.18)	0 (0)	-

For the description statistics of study participant demographics, analysis of variance (ANOVA) was performed for continuous variables (with a Tukey post-hoc test for pair-wise comparisons in case of an overall effect between the three groups for age, sex and education). Unpaired t-test was performed to evaluate differences in CD4 count and viral load. Pearson chi squared tests for categorical variables (heavy drinkers). * Cohort 1 HIV-positive vs. Cohort 2 HIV- and HIV-positive, $p < .001$. [#]Cohort 1 HIV-positive vs. Cohort 2 HIV- and HIV-positive, $p < .001$. [$]Cohort 2 HIV- vs. HIV-positive.

A subgroup of participants included in the study was from a larger study (cohort 2) that investigated the effects of heavy drinking and cognitive performance in HIV participants. These included n=25 HIV-non/light drinkers (self-reported consumption of less than 3 standard (14gm alcohol) drinks per occasion during the 3 months prior to the assay), n=25 HIV-positive non/light drinkers, n=26 HIV-heavy drinkers (> 5 standard drinks per occasion over a 2-week period for 3 months prior to the marker assays) and n=18 HIV-positive heavy drinkers.

Table 3. Comparisons of immune markers in HIV-positive non/light drinkers vs. HIV-positive heavy drinkers.

Inflammatory marker	HIV-positive non/light drinkers (Mean ± SD)	HIV-positive heavy drinkers. (Mean ± SD)	*p*-value
TYMP	14.53 (11.79)	27.78 (28.24)	*p* =.304
VEGF	60.42 (33.00)	90.46 (68.48)	*p* = .728
MMP9	60.30 (29.25)	64.13 (30.99)	*p* = .429
CCL2	68.28 (34.89)	99.15 (55.74)	*p* = .096
NGAL	66.12 (26.72)	70.47 (24.16)	*p* = .103
TGF-B1	6.52 (5.03)	5.58 (3.94)	*p* = .897

Independent sample *t*-test was performed to evaluate immune markers in HIV-positive non/light drinkers *vs*. HIV-positive heavy drinkers.

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Chapter 4

The association of peripheral immune markers with brain cortical thickness and surface area in South African people living with HIV

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Abstract

A spectrum of cognitive impairments known as HIV-associated neurocognitive disorders (HAND) are consequences of the effects of HIV-1 within the central nervous system (CNS). Regardless of treatment status, an aberrant chronic neuro-immune regulation is a crucial contributor to the development of HAND. However, the nature to which inflammation affects brain structures critical for cognitive status remains unclear. The present study aimed to determine associations between peripheral immune markers with cortical thickness and surface area. Participants included 65 treatment-naïve people living with HIV (PLWH) and 26 HIV-negative controls. Thickness and surface area of all cortical regions were derived using automated parcellation of T1-weighted images acquired at 3T. Peripheral immune markers included C-C motif ligand 2 (CCL2), matrix metalloproteinase (MMP)9, neutrophil gelatinase-associated lipocalin (NGAL), thymidine phosphorylase (TYMP), transforming growth factor (TGF)-β1 and vascular endothelial growth factor (VEGF) were measured using enzyme-linked immunosorbent assays. Associations between these markers with thickness and surface area of cortical regions were evaluated. A mediation analysis examined whether associations of inflammatory markers with cognitive functioning were mediated by brain cortical thickness and surface area. After controlling for multiple comparisons, higher NGAL was associated with reduced thickness of the bilateral orbitofrontal cortex in PLWH. The association of NGAL with worse motor function was mediated by the cortical thickness of the bilateral orbitofrontal region in PLWH. Taken together, this study suggests that NGAL plays a potential role in the neuropathophysiology of neurocognitive impairments of HIV.

4.1. Introduction

HIV infection of the central nervous system commonly results in a spectrum of neuropsychological deficits known as HIV-associated neurocognitive disorder (HAND) (Clifford and Ances, 2013). HAND is classified according to the severity of neurocognitive impairment with milder forms occurring in up to 50% of the HIV-infected population regardless of treatment status (Heaton et al., 2010). Neuroimaging studies have indicated that structural brain abnormalities are common features of HAND (O'Connor et al., 2018). Historically, HIV-neuroimaging studies focused on white matter hyperintensities and subcortical atrophy (Aylward et al., 1993; Navia et al., 1986). However, with successful antiretroviral therapy (ART), recent findings have shifted our understanding of the cognitive phenotype of HIV-associated neurocognitive impairments and suggest a mixed subcortical-cortical pattern linked to subcortical damage (Paul, 2019; Sacktor, 2018; Sacktor and Robertson, 2014). Similarly, HIV-1 related effects may directly target subcortical structures. However, cortical structures may be indirectly affected via several (inflammatory) mechanisms and observed as changes in cortical thickness and surface area. This coincides with an interest in understanding the mechanisms of HIV-1 cortical dysfunction.

Despite the growing body of evidence, existing HIV-1 studies largely investigated cortical volumes (Ances et al., 2012; Cohen et al., 2010; Janssen et al., 2015; Kallianpur et al., 2013), with limited investigations into thickness and surface area. In the pre-ART era, a magnetic resonance imaging (MRI) study indicated that cortical

thickness is affected, in acquired immunodeficiency syndrome (AIDS) participants having 15% thinner primary sensory, motor and premotor cortices (Thompson et al., 2005). A reduction in the prefrontal and parietal tissue correlated with cognitive-motor deficits (Thompson et al., 2005). Therefore, investigations of cortical thickness and surface area may be a useful tool to study the associations of biological anomalies e.g. an increased inflammatory environment, with changes in brain structures that are affected by the neuropathophysiology of HIV-1.

Previous investigations on HIV-1 subtype B (HIV-1B) infection have shown that peripheral inflammatory (C-C chemokine ligand (CCL)2, interleukin (IL)-1β, IL-6, IL-16, IL-18, interferon gamma-induced protein (IP)-10, macrophage inflammatory protein (MIP)-1β, and stroma cell-derived (SDF)-α) and monocyte activation marker (soluble cluster of differentiation (sCD)14 and sCD163) levels were associated with reduced brain volumes in several regions (Gongvatana et al., 2014; Ragin et al., 2010). However, associations of peripheral immune markers with thickness and surface area were not previously assessed in HIV-1 infection. This may be useful as it may provide insight into inflammatory mechanisms responsible for structural brain changes and cognitive status. Peripheral inflammation was found to mediate the relationship of cortical thickness and cognitive performance with metabolic disorders (Corlier et al., 2018; Kaur et al., 2015). Further, inflammation was related to cortical thinning in older age and schizophrenia patients (Jacomb et al., 2018; McCarrey et al., 2014). Similarly, inflammation may influence cortical thickness and surface area in HIV-1 infection and may provide insight into indirect inflammatory mechanisms

responsible for structural changes and cognitive status in people living with HIV (PLWH).

Studies of immune markers and brain alterations primarily included study participants with HIV-1B and it is known that the neurovirulence of HIV-1 may differ between various subtypes (Santerre et al., 2019; Tyor et al., 2013). Pre-clinical cell culture studies suggest that inflammatory responses are higher in HIV-1B infection than in HIV-1 subtype C (HIV-1C) infection (Campbell et al., 2007; Gandhi et al., 2009; Ruiz et al., 2019; Wong et al., 2010). Therefore, there is a need to investigate the nature to which inflammatory markers may influence brain cortical thickness and surface area of PLWH in geographical locations that are primarily HIV-1C infected. This may provide insights into the underlying mechanisms of HAND for these geographical regions.

CSF markers may be a more accurate representation of neuroinflammation and the CNS pathophysiology that is associated with or lead to HAND. Considering that to a certain extent, 1) peripheral inflammation may provide an indication of inflammation within the CNS ((Capuron and Miller 2011) and 2) the ease and limited invasiveness of obtaining peripheral blood, this creates a practical option to investigate peripheral immune markers that are associated with structural brain alterations and the development of HAND. Therefore, the identification of such peripheral immune markers may provide insights of mechanisms that may be active within the CNS, potentially those related to the development of HAND.

In a previous study (Chapter 3), we found significantly higher levels of thymidine phosphorylase (TYMP) and neutrophil gelatinase-associated lipocalin (NGAL), and significantly lower levels of matrix metalloproteinase (MMP)9 in PLWH compared to HIV-negative controls, after controlling for age, sex, education, and alcohol use (Williams et al. 2019). TYMP and NGAL were associated with worse processing speed and motor function in PLWH (Williams et al. 2019). TYMP (Chapouly et al., 2015) and NGAL (Choi et al., 2011; Elneihoum et al., 1996; Morieri et al., 2018; Naudé et al., 2013) are immune markers that may be involved in the neuropathophysiology of neurodegenerative diseases. TYMP gene expression is increased in circulating monocytes of PLWH (Wu et al., 2013) and may be increased in the HIV-infected brain (Kumar et al., 2012). Furthermore, preliminary evidence suggests that TYMP may have a functional role in BBB damage (Chapouly et al., 2015). NGAL promotes a pro-inflammatory phenotype and apoptosis in microglia (Lee et al., 2007), astrocytosis (Lee et al., 2009) and sensitizes neurons to neurotoxicity (Bi et al., 2013; Naudé et al., 2012). Therefore, TYMP and NGAL show promise in furthering our understanding of the neuropathophysiology of HAND (Williams et al. 2019).The present study builds on these initial findings, and for this reason, we selected these cognitive domains (processing speed and motor function) for further investigation. We, therefore, hypothesized that these markers may be associated with reduced cortical thickness and surface area in the brain regions that are involved with these cognitive functions (processing speed and motor function). In addition to TYMP and NGAL, we investigated additional peripheral markers based on putative evidence of their associations with HIV-1 neuropathology in both pre-clinical and clinical studies. Therefore, the current study aim was to determine the association of peripheral

immune markers (CCL2, TGF-β1, MMP9, VEGF, TYMP and NGAL) with the cortical brain integrity, quantified using 3T MRI

4.2. Methods

4.2.1. Study participants

A subset of participants that had undergone neuroimaging analysis from the overall cohort (Chapter 3) were included in this study. This included n = 65 HIV-positive and n = 26 HIV-negative participants, which were pooled from two independent studies. Cohort 1 (Joska et al. 2011) included 48 HIV-positive participants without comorbid alcohol or illicit substance use disorder. Cohort 2 included 17 HIV-positive and 26 HIV-negative participants, 23 of whom (15 HIV-negative and 8 HIV-positive) met the diagnostic criteria for alcohol use disorder. A subset of participants was included in a prior study of peripheral immune dysregulation and neurocognitive performance (Williams et al. 2019). PLWH were recruited from primary health care clinics in Cape Town and the Western Cape region of South Africa. Participants were aged 18 through 65 years with at least 7 years of formal education. PLWH were treatment-naïve or had only recently started cART (< 1 month) before blood collection and neuroimaging analysis. The HIV-negative control participants were recruited from local voluntary counselling and testing clinics. Demographical variables were collected for all participants and the relevant laboratory measures (viral load and CD4 count) were collected for PLWH. HIV status was confirmed by two independent rapid tests and confirmed via ELISA analysis. Participation was voluntary and written

informed consent was obtained following a thorough explanation of the study procedures. Individuals received financial compensation for their time. This study was approved by the Human Research Ethics Committee of the Faculty of Health Sciences (University of Cape Town) (Sub-study HREC 213/2018 linked to primary studies: 003/2015, 023/ 2008 and 263/2007). Exclusionary criteria included: 1) severe psychiatric disorders or presented any other neurological disorders, 2) illicit substance abuse, 3) moderate to severe head injury (history of head injury with a loss of consciousness exceeding 30 minutes) or 4) HIV-positive participants with clinical signs of any other co-infections on history and clinical examination. The MINI v.6 (Sheehan et al. 1998) and Kreek–McHugh–Schluger–Kellogg (KMSK) (Kellogg et al. 2003) were administered to screen for substance abuse in the past 12 months. Alcohol use was classified as non/light drinkers (self-reported consumption of 2 or fewer standard (<14gm alcohol) drinks per occasion in a day over a period of 3 months prior to the assay) and heavy drinkers (> 5 standard drinks per occasion over a 2-week period over a period of 3 months prior to the marker assays).

4.2.2. Neurocognitive measures

Neurocognitive performance was assessed with a detailed battery as described previously (Joska et al., 2011; Williams et al., 2019). Briefly, the test battery included processing speed (WAIS-III Digit symbol, WAIS-III Symbol search, Colour Trails I, Stroop Colour) (Golden, 1975), verbal (Animal Fluency) (Acevedo et al., 2000), learning (the Hopkins Verbal Learning Test-Revised, Brief Visuospatial Memory Test-Revised) (Benedict et al., 1998), memory (HVLT-R Delayed recall, BVMT-R Delayed

recall), motor functioning (Groove pegboard Dominant, Groove pegboard Non-dominant) (Klove 1963) and executive functioning (Colour Trails2) (D'Elia et al., 1996) that were used to create 6 composite indices (processing speed, verbal, learning, memory, motor and executive functioning) as previously described (Joska et al., 2011; Williams et al., 2019)

4.2.3. Laboratory assessment of blood

Blood samples were collected via venipuncture at a study visit within a period of 1 month prior to the scan visit. Blood samples for each participant were separated, and plasma and serum samples were immediately frozen and stored at -80°C. HIV viral load was determined by the CAP/Roche Cobas Ampliprep (sampled post-2015) and the Abbott M2000SP and M2000RT (sampled prior to 2015) methods. The CD4 count was determined by PanLeucogate (PLG) (Beckman Coulter FC500MPL) method. Peripheral immune markers were selected for investigation based on putative evidence of their associations with HIV-1 neuropathology in both pre-clinical and clinical studies. These included C-C motif ligand 2 (MCP-1/CCL2) (Cohen et al., 2011; Eugenin, 2006), matrix metalloproteinase (MMP)9 (Barr et al., 2010; Xing et al., 2017), neutrophil gelatinase-associated (NGAL) (Williams et al., 2019), thymidine phosphorylase (TYMP) (Williams et al., 2019), transforming growth factor (TGF)-β1 (Dhar et al., 2006; Perrella et al., 2001), and vascular endothelial growth factor (VEGF) (Argaw et al., 2012; Nag et al., 2002; Sporer et al., 2004). Blood concentrations of CCL2, TGF-β1, MMP9, VEGF, TYMP and NGAL were measured using the enzyme-linked immunosorbent assay (ELISA) (R&D systems, DuoSet ELISA)

according to the manufacturer's instructions. CCL2, VEGF, and TYMP were measured in serum. TGF-β1, NGAL and MMP9 were measured in plasma. Samples were diluted as follows CCL2: 1:2, TGF-β1: 1:30, MMP9: 1:150, VEGF: no dilution, TYMP: 1:10 and NGAL: 1:100. All samples were assayed in duplicate. The intra- and inter-assay coefficients of variation for all tests were within acceptable ranges of <8% and <10% respectively.

4.2.4. Structural neuroimaging acquisition and processing

Imaging was performed on a 3T Siemens Skyra with 32 channel head coil (Siemens AG, Erlangen Germany). The structural images were acquired using a T1-weighted 3-dimensional magnetization-prepared rapid acquisition gradient echo (MPRAGE) sequence: TE[1]: 1.53ms, TE[2]: 3.21ms, TE[3]: 4.89ms, TE[4]: 6.57ms, fov: 256mm, flip angle = 20, 128 slices and voxel size: 1x1x15.mm.

Quantification of cortical thickness and surface area with certain regions was performed using the Freesurfer software suite (v5.3) (Martinos Center, Harvard University, Boston, MA; http://surfer.nmr.mgh.harvard.edu). Technical post-processing details have been described previously (Desikan et al., 2006; Fischl et al., 2002). In brief, Freesurfer is a validated automated software program which transforms the MPRAGE scan of an individual into a template space with the skull stripped and the brain segmented. Brain regions were parcellated into subcortical and cortical regions of interest (ROI). For the primary aim, the analysed ROIs included the cortical lateral orbitofrontal, precentral, caudal anterior cingulate,

paracentral, superior parietal, inferior parietal, supramarginal and precuneus. These cortical regions were chosen based on prior studies (Kallianpur et al., 2012; Netto et al., 2011; Sanford et al., 2017; Thompson et al., 2005). As an exploratory aim, the analysed ROIs included all cortical regions of the whole brain (Supplementary file). Two trained raters independently confirmed segmentation of these ROIs (MEW and ARA). Bilateral estimates were used for all analyses for each brain region.

4.2.5. Statistical analysis

All analyses were conducted using SPSS (version 25, IBM, USA). Data distribution for the markers MMP9, TYMP, VEGF, NGAL, CCL2 and TGFβ1 were found to be skewed, therefore the data was natural log-transformed, which resulted in acceptable skewness (<-1.2) and kurtosis (<1.7). For description statistics of study participant demographics, Wilcoxon *t*-test was performed for continuous variables and Pearsons chi-squared tests for categorical variables.

For the primary aim, an exploratory analysis was done with Pearson correlations to investigate the associations between the inflammatory markers and the cortical thickness/surface areas of specific ROIs in PLWH and HIV-negative participants respectively. Statistical significance for all analyses was set at *p* less than .05.

Bonferroni correction accounted for the number of brain regions tested (α/n = .05/8 = .00625) in the group comparisons (primary aim). Covariates that may affect marker levels included; sex (Krebs et al., 2016; Mathad et al., 2016), age (De Oliveira et al.,

2015), comorbidities (Keating et al., 2017) and alcohol use (Meyerhoff, 2001). First, we evaluated the correlations between immune marker concentrations and thickness/surface area of cortical ROIs. Thereafter, for significant findings, separate regression models were conducted to examine the associations between cortical thickness/surface area and marker concentrations, after adjusting for the following covariates in individual models: demographics (age, sex and education), cohort (cohort, heavy drinkers) and HIV-positive disease parameters (nadir CD4 count, and viral load). A post-hoc analysis of covariance (ANCOVA) determined the interaction between HIV status and immune marker with cortical thickness/surface area as the dependent variable.

As a secondary aim, a mediation analysis was conducted using the Baron and Kenny approach (Hayes, 2009). This was conducted to establish the mediating role of brain cortical thickness or surface area on the associations of inflammatory markers with cognitive functioning. Linear regression analysis was used to evaluate the associations between the independent variable (inflammatory marker) and the outcome variable (cognitive functioning). This direct association was referred to as relation *c*. The association between the independent variable (inflammatory marker) with the possible mediator (brain thickness/surface area) (referred to as association *a*) and the association between the possible mediator with the outcome variable (cognitive functioning) (referred to as relation *b*) was also evaluated. According to the guidelines of Baron and Kenny (Hayes, 2009), the associations of *a*, *b* and *c* needs to be significant in order to evaluate possible mediation by the mediator. Finally, the amount of change in the regression coefficient for the associate *c*, after adjustment

for the mediator was evaluated (referred to as *c'*). The mediation effect was calculated as the difference in the regression coefficient between *c* and *c'* (referred to as *ab*).

As an exploratory analysis, Pearsons correlations were conducted to investigate associations between the inflammatory markers and the cortical thickness/surface areas ROIs of the whole-brain (including those investigated in the primary aim) in PLWH and HIV-negative participants respectively. *P* values were considered statistically significant for all analyses at a value of less than .05 (Supplementary Table 1-Table 4).

4.3. Results

4.3.1. Sample characteristics

The study sample included a total of 91 participants which comprised of 65 PLWH and 26 HIV-negative controls. PLWH had a mean age of 33.32 (6.66) years and 32.3% were female. The HIV-positive group had a median nadir CD4 count of 307 (169 - 417) cells/µl and viral load of 3.95 (3.15 - 4.51) log copies/ml. PLWH were treatment-naïve or had only recently started cART (< 1 month). PLWH had a higher mean age (p = .01) and a lower percentage of female participants (p = .025) when compared to HIV-negative controls. There were no significant differences for the 6 composite cognitive indices between HIV-positive and HIV-negative participants (p > .05). Sample characteristics are summarized in Table 1.

Table 1: Characteristic of HIV-positive and HIV-negative study participants

	HIV– controls (n = 26)	**HIV+** (n = 65)	***p*-value**
Age years mean (SD)	37.54 (7.66)	33.32 (6.66)	.01
Sex, female N (%)	15 (57.7)	21 (32.3)	.025
Education, years (SD)	10.88 (1.12)	10.38 (1.37)	.12
CD4 + nadir, median (IQR)	-	307 (169- 417)	-
Viral load log copies/mL median (IQR)	-	3.95 (3.15 - 4.51)	-
Heavy drinkers, N (%)	15 (57.7)	8 (12.3)	< .001
Cognitive domain			
Processing speed, mean (SD)	0.14 (0.50)	0.06 (0.53)	0.506
Verbal, mean (SD)	-0.15 (0.94)	-0.01 (1.03)	0.556
Learning, mean (SD)	-0.01 (0.89)	0.16 (0.56)	0.402
Memory, mean (SD)	-0.05 (0.84)	0.05 (0.90)	0.634
Motor functioning, mean (SD)	0.01 (1.01)	-0.15 (0.84)	0.334
Executive functioning, mean (SD)	-0.02 (1.09)	0.12 (1.10)	0.580

Abbreviations: Interquartile range (IQR), Standard deviation (SD)

4.3.2. Association of immune marker levels with the cortical thickness

In PLWH, higher CCL2 levels were associated with larger thickness of the precentral gyrus (p = .019) and higher NGAL levels were associated with reduced thickness of the lateral orbitofrontal region (p = .004). After applying Bonferroni corrections of p = .05/8, higher NGAL levels remained associated with reduced thickness of the lateral orbitofrontal region *(*p = .004) (Table 2). To further evaluate if the outcome was specific for HIV status, an ANCOVA was performed post-hoc to determine the interaction between HIV status and NGAL levels, with the lateral orbital frontal cortex thickness as the dependent variable. The ANCOVA failed to detect evidence for an interaction (p > .05). The association between higher NGAL levels with the reduced lateral orbitofrontal thickness remained significant after controlling for demographic variables (age, sex and education) *(*p = .01), cohort (cohort, heavy

drinkers) (p = .002) and HIV-positive disease parameters (nadir CD4 count, and viral load) (p=.006) in separate models. Before and after applying Bonferroni corrections p = .05/8, no associations were found between any of the investigated immune markers and the respective regions of brain cortical thickness in HIV-negative participants (Table 3).

Table 2: The associations of brain cortical thickness with peripheral immune markers in PLWH

Bilateral brain ROIs	**TYMP**	**VEGF**	**MMP9**	**CCL2**	**NGAL**	**TGFβ1**
Lateral orbitofrontal	-.112	-.149	.033	.094	**-.365**[a]	.072
Precentral	.182	-.208	-.132	.353[b]	-.093	.093
Caudal anterior cingulate	-.195	.063	-.058	.050	-.202	-.045
Paracentral	.003	-.070	-.076	.194	-.078	.152
Superior parietal	-.067	.096	.003	.192	-.012	.037
Inferior parietal	-.149	.093	-.040	.164	-.182	-.154
Supramarginal	-.199	.108	-.109	.211	.065	-.016
Precuneus	-.088	.000	-.104	.184	-.162	.035

Results displayed as standardized β (unadjusted). Results with significant p values are presented in bold, [a] p = .004, [b] p = .019

Table 3: The associations of brain cortical thickness with peripheral immune markers in HIV-negative participants

Bilateral brain ROIs	**TYMP**	**VEGF**	**MMP9**	**CCL2**	**NGAL**	**TGFβ1**
Lateral orbitofrontal	-.323	-.121	.089	-.078	-.158	-.025
Precentral	-.248	.011	.070	.017	-.240	.061
Caudal anterior cingulate	-.128	.007	.249	.037	-.033	.138
Paracentral	-.214	.013	.217	.001	-.014	-.182
Superior parietal	-.336	.058	.110	-.058	.120	-.219
Inferior parietal	-.339	-.205	-.339	.273	-.340	-.138
Supramarginal	-.443	-.125	.021	-.048	.066	-.189
Precuneus	-.369	.090	.202	-.051	.100	-.163

Results displayed as standardized β (unadjusted).

The mediating role of lateral orbitofrontal thickness in the association of NGAL with processing speed was not determined because no significant association (p = .44) was found between the lateral orbitofrontal thickness and processing speed. Higher NGAL levels were associated with impaired motor functioning (c; β = .292, p = .02). This association was mediated by the thickness of the lateral orbitofrontal cortex (c'; β = .200, p = .12; ab; β = .09) (Figure 1).

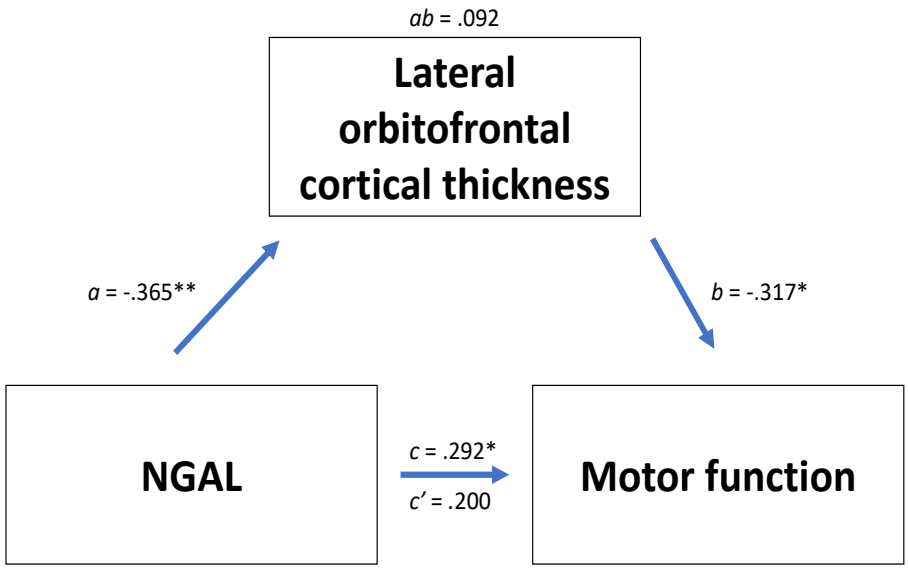

Figure 1: Schematic illustration of the mediation paths. Results displayed as standardized β. *p < .05, **p < .01

4.3.4. Association of immune marker levels with the surface area

In PLWH, higher TYMP levels were associated with the larger surface area of the supramarginal cortex (p = .01). In HIV-negative participants, higher NGAL levels were associated with a larger surface area of the lateral orbitofrontal (p = .032) and higher TYMP levels were associated with reduced surface area of the paracentral (p = .044).

After applying Bonferroni corrections of $p = .05/8$, no significant associations were found between the investigated immune markers and the respective regions of brain cortical surface area for either group (Table 4 and Table 5).

Table 4: The associations of brain surface area with peripheral immune markers in PLWH

Bilateral brain ROIs	**TYMP**	**VEGF**	**MMP9**	**CCL2**	**NGAL**	**TGFβ1**
Lateral orbitofrontal	-.099	.019	-.063	-.175	.124	-.045
Precentral	-.126	-.112	.015	-.145	.175	.010
Caudal anterior cingulate	-.003	.036	.092	.241	.148	-.060
Paracentral	-.154	.062	-.005	-.063	.010	-.059
Superior parietal	.075	.003	-.089	.096	.099	.100
Inferior parietal	-.211	.106	-.089	.003	-.087	.253
Supramarginal	.391[a]	.013	-.042	.210	.223	.156
Precuneus	.159	-.023	-.062	.111	.185	.035

Results displayed as standardized β (unadjusted). [a] $p = .01$

Table 5: The associations of brain surface area with peripheral immune markers in HIV-negative participants

Bilateral brain ROIs	**TYMP**	**VEGF**	**MMP9**	**CCL2**	**NGAL**	**TGFβ1**
Lateral orbitofrontal	.177	.344	.242	.138	.420[a]	-.089
Precentral	-.163	.225	-.050	-.149	.118	.059
Caudal anterior cingulate	-.021	.037	-.075	-.168	.023	-.220
Paracentral	-.407[b]	-.091	-.183	.001	.062	-.310
Superior parietal	-.153	.294	.055	.121	.255	-.207
Inferior parietal	.073	.204	-.349	.303	.026	.253
Supramarginal	-.302	.071	-.303	.080	.131	.058
Precuneus	-.031	.315	-.067	.072	.234	-.178

Results displayed as standardized β (unadjusted). [a] $p = .032$, [b] $p = .044$

4.3.5. Exploratory analysis: Association of Immune markers with the cortical thickness and surface area

Higher NGAL levels were associated with reduced thickness in the highest number of ROIs (n=14), followed by TYMP (n=6). These associations were largely found in the regions of the frontal cortex in PLWH (Supplementary Table 1). The most significant findings (considered as $p \leq .004$) observed were the associations of higher NGAL levels with the reduced thickness of the frontal pole, inferior temporal and lateral orbitofrontal (all $p = .004$) and higher TYMP levels with the reduced thickness of the frontal pole ($p = .002$) (Supplementary Table 1). These associations were not observed in HIV-negative participants (Supplementary Table 2). The most significant findings for surface area were higher TYMP levels and a larger surface area of the supramarginal ($p = .01$) and higher TGFβ1 levels with the larger surface area of the postcentral gyrus ($p = .007$) in PLWH (Supplementary Table 3). These associations were not found in the HIV-negative participants (Supplementary Table 4).

4.4. Discussion

The results from this study demonstrate that higher plasma NGAL levels may be associated with a reduced thickness of the bilateral orbitofrontal cortex and that that cortical thickness of the bilateral orbitofrontal may mediate the association of NGAL with worse motor function in PLWH. An exploratory analysis revealed that higher NGAL and TYMP levels had several associations with ROIs in the frontal cortex of PLWH.

To the best of our knowledge, this is the first study to describe the associations of circulating immune markers with brain structural abnormalities in South African patients that are geographically located to regions which are primarily infected with HIV-1C. Only two other existing studies with HIV-1B infected participants described associations of plasma cytokines with brain volumes (Gongvatana et al., 2014; Ragin et al., 2010).

In the current study, we report an association of higher NGAL levels with reduced thickness of the lateral orbitofrontal cortex in PLWH. The reduced thickness of the lateral orbitofrontal region in HIV-1 infection has been reported in several studies (Kallianpur et al., 2012; Netto et al., 2011; Sanford et al., 2017). We further report an association between peripheral immune dysregulation, reduced cortical thickness and cognitive performance in HIV-1 infection. However, ANCOVA analyses did not show a significant interaction between NGAL and HIV status, with lateral orbitofrontal thickness as the dependent variable. Therefore, this finding may not be dependent on HIV-status. For the mediation analysis, we describe that the association of higher NGAL levels with worse motor function (Williams et al., 2019) was mediated by the reduced cortical thickness of the lateral orbitofrontal region in PLWH. Even though the orbitofrontal region is not formally considered to be involved in motor function, prior studies have found an association between prefrontal (Thompson et al. 2005) and orbitofrontal cortical thinning and worse motor function (Kallianpur et al. 2012). A previous study with treatment-naïve participants showed that the reduced thickness of the prefrontal cortex was

associated with cognitive/motor deficits in HIV-1 infection (Thompson et al., 2005). More specifically, our findings are in line with a study that reported an association between the reduced lateral orbitofrontal thickness and worse motor function in PLWH (Kallianpur et al., 2012). Further, two parcels in the frontal gyrus, pars orbitalis (8 and 7) have strong connectivity with the lateral orbitofrontal cortex and also with several movement-related areas, including the supplementary motor area, insula, midcingulate and supracallosal anterior cingulate, supramarginal left and right. These inferior frontal gyrus-pars orbitalis areas may relate the lateral orbitofrontal cortex to brain areas involved in movement initiation (Du et al. 2020). It remains unclear whether these associations reflect neuronal death due to HIV-related toxins or perhaps to a diaschisis effect resulting from compromised sub-cortical brain structures (Kallianpur et al., 2012). Based on our findings, there is a possibility that the lateral orbitofrontal region may be affected by immune responses in HIV-1 infection, however, this warrants further investigation.

Frontal cortical dysfunction may be particularly vulnerable to indirect immune-related mechanisms. Findings from the exploratory analysis revealed that from all the investigated markers, NGAL and TYMP showed the majority of associations with the regions of cortical thickness in PLWH. These associations were largely found with reduced thickness in regions of the frontal cortex. This is of interest since previous studies have shown associations between reduced thickness in the frontal cortex with worse processing speed and motor function in HIV-1 infection (Kallianpur et al., 2012; Netto et al., 2011; Sanford et al., 2017; Thompson et al., 2005), supporting the findings from the current study. A recent study has reported that peripheral

monocyte activation markers; sCD14 and sCD163 were associated with reduced volumes in regions of the frontal lobe in treated women with HIV (Kamkwalala et al. 2020). Neuroinflammation is increased in the frontal cortical tissue of participants with HAND (Boven et al. 1999; Guha et al. 2018). Despite this being an exploratory analysis and not controlling for multiple comparisons, the association shown by NGAL and TYMP are in line with the association in specific domains shown in our previous study (Williams et al., 2019). Higher TYMP and NGAL levels were associated with worse neurocognitive performance in the domains of processing speed and motor function. The associations of these markers were largely found in the regions involved in these cognitive functions.

The exact mechanism of NGAL and TYMP in the neuropathophysiology of HIV is not clearly understood. However, evidence from existing studies on the neurobiological functions of NGAL and TYMP in other neurological diseases can provide a hypothetical explanation for our findings. NGAL or lipocalin-2 (LCN2), is an acute-phase protein that is secreted in response to a wide range of pathological stimuli (Chakraborty et al., 2012). NGAL reportedly promotes a pro-inflammatory phenotype and apoptosis in microglia (Lee et al., 2007), astrocytosis (Lee et al., 2009) and sensitize neurons to neurotoxicity (Bi et al., 2013; Naudé et al., 2012). NGAL was significantly higher in the frontal cortical tissue (including the orbitofrontal cortex) of patients with Alzheimer's disease compared to non-demented controls (Dekens et al., 2017). In an HIV gp120 transgenic mouse model, it was shown that NGAL was one of the most upregulated immune factors in the brain of an HIV gp120 transgenic mouse model (Maung et al., 2014). Moreover, NGAL could contribute to neuronal

injury in gp120 transgenic mice (Maung et al., 2014). Therefore, NGAL may be present in the frontal cortex of PLWH and potentially have negative effects on neurons and immune cells of the CNS, resulting in structural cortical changes. Findings from the current study further support the potential role for NGAL in HIV-associated neurocognitive impairments and this may be an important marker for future investigations.

TYMP (or ECGF1) is an endothelial cell growth factor with a functional role in angiogenesis. The TYMP gene expression is increased in circulating monocytes of PLWH with viremia (Wu et al., 2013) and its protein expression may be increased in the CNS of people with HIV-1 (Kumar et al., 2012). Furthermore, TYMP was shown to contribute to blood-brain barrier breakdown in cell culture and animal models (Chapouly et al., 2015). Studies investigating the association of TYMP with HIV-associated neurocognitive impairments remain limited and to our best knowledge, no other study has investigated these markers PLWH. Future studies should investigate the underlying mechanisms of both NGAL and TYMP in HIV-associated neurocognitive impairments. Specifically, investigations of human post-mortem brain tissues and gene expression will provide further insights into the expression levels of these immune markers in HIV infection.

Higher CCL2 levels were associated with a larger thickness of the precentral gyrus. Even though this association did not survive multiple comparisons, this is a contradictory finding from previous studies linking higher CCL2 levels with lower volumes (Ragin et al. 2010a) and with worse neurocognitive performance (Woods et

al. 2006; Ancuta et al. 2008). However, higher peripheral CCL2 levels were also associated with higher volumes of the basal ganglia and amygdala volumes in HIV-positive participants (Gongvatana et al. 2014). The basis for this positive association cannot be ascertained from this study. The positive relationship between peripheral CCL2 levels and brain structures may be due to hypertrophy resulting from fluid accumulation secondary to inflammation of these brain structures (Gongvatana et al. 2014).

Our results further indicate that TYMP, NGAL and TGF-β1 were associated with differences in cortical surface area, although these findings did not survive corrections for multiple comparisons. The associations and biological effects of immune markers on brain surface area are still largely unknown. Cortical surface area is believed to be determined by the number of ontogenetic columns that run perpendicular to the surface of the brain (Hogstrom et al. 2013). The radial unit hypothesis of cortical development stipulates that cells within the cortical column share a common origin and migrate from the subventricular zone to their location within the cortex (Rakic 1988). The tension-based morphogenesis hypothesis (Van Essen 1997) further suggest that abnormal mechanical tension along the axons may affect the cortical surface area. We speculate that TYMP, NGAL and TGF-β1 levels may be associated with neurobiological processes such as synaptogenesis, dendritic arborization and intracortical myelination that may continue to affect cortical surface area. Their associations with the brain surface area may depend on the underlying neuropathological environment.

Our study sample differed from previous studies, with regards to geographical region (HIV-1 subtype) and treatment status. All studies investigating cortical thickness/surface area were done in HIV-1 subtype B infection (Kallianpur et al., 2012; Netto et al., 2011; Sanford et al., 2017; Thompson et al., 2005). It is known that the inflammatory phenotype may differ between HIV-1 subtypes (Gandhi et al., 2009), therefore findings presented here may differ for HIV-1B infection. Interestingly, when comparing the effect of HIV-1B and HIV-1C on structural volume declines, HIV- 1B and HIV-1C were associated with similar structural volume differences compared to their relative controls. However, no significant differences were found in the structural volume decline when directly comparing HIV-1B and HIV-1C (Ortega et al., 2013). This underscores the need for further investigation into the direct and indirect mechanisms for geographical regions which are primarily HIV-1C infected.

We investigated a treatment-naïve cohort. Majority of previous studies investigating the effect of HIV-1 on cortical thickness was done in treatment-experienced participants (Kallianpur et al., 2012; Netto et al., 2011; Sanford et al., 2017), with limited studies investigating treatment-naïve participants (Thompson et al., 2005). It was shown that in both treatment-naïve and treatment-experienced HIV-1 positive participants, there is an association between a reduced frontal cortex and worse neurocognitive performance. Similarly, as shown in the current study, associations of immune markers were largely found in the regions of reduced frontal cortex.

Inflammation may persist in the brain of both treatment-naïve and treatment-experienced participants as shown in studies with post-mortem human brain tissues. A study by Shapshak et al., reported an increase of pro-inflammatory cytokines in post mortem brain tissue of treatment-naïve cognitively impaired PLWH (Shapshak et al., 2004). Interestingly, it was found that when comparing brain tissue of HIV-positive participants with and without HIV encephalitis, similar features of immune activation in the CNS was recorded, regardless of ART at the time of death (Tavazzi et al., 2014). Furthermore, brain alterations may persist despite viral suppression. In a longitudinal treatment study, at both baseline and follow-up, virally suppressed PLWH had greater white matter volume loss compared to HIV-negative controls (Cardenas et al., 2009). Similarly, in a 24-month longitudinal treatment study, volume decline of the basal ganglia was reported in young adults despite the initiation of ART within the first 4 weeks of infection (Kallianpur et al., 2019). This is suggestive of an ongoing structural brain dysfunction despite the introduction of ART, indicating persistent underlying neurobiological mechanisms that are involved in the neuropathophysiology of HIV.

Several studies that investigated ART experienced participants with (Cohen et al. 2011; Correia et al. 2013; Falasca et al. 2017; Imp et al. 2017) and without viral suppression (Ancuta et al. 2008; Kamat et al. 2012; Krebs et al. 2016; Xing et al. 2017) reported an association between inflammation and HAND. Similarly, studies investigating cohorts with varying levels of CD4 count (Ancuta et al. 2008; Cohen et al. 2011; Kamat et al. 2012) showed associations between inflammation and HAND (Williams et al. 2020). Even though ART may reduce the viral load and subsequently

the level of inflammation in the CNS, low-grade neuroinflammation may still be present. Therefore, the effect of low-grade inflammation on brain structures and the development of HAND may be independent of ART. We hypothesize that findings presented in the current study may similarly be reflected in investigations of treatment-experienced participants.

Several study limitations should be emphasized. First, this study included a limited number of study participants, so replication in a larger cohort is warranted. Second, some of the study participants were heavy alcohol users. Heavy episodic drinking affects brain cortical thickness and structure (Morris et al., 2019). To minimize these effects, we controlled for alcohol use in our statistical analysis. Further, we have previously shown that both 1) inflammatory marker levels and 2) neurocognitive performance was not significantly different between the heavy-drinkers and non-heavy drinkers (all *p* values were greater than .1) (Williams et al., 2019). It is also relevant to note, the findings presented here may not apply to HIV-1B infection, as inflammatory levels may be influenced by the HIV-1 subtype (Gandhi et al., 2009). Further, when adjusting for multiple comparisons, an adjustment was only made for the number of brain regions tested. Therefore, these findings should be considered as preliminary and the interpretation of findings requires caution. Other potential confounding factors that were not screened out from this study, may in part, explain the outcomes reported in this study.

4.5. Conclusion

This study shows that the immune marker NGAL was associated with the reduced thickness of the lateral orbitofrontal cortex and that the association of NGAL with worse motor function may be mediated by the thickness of the lateral orbitofrontal cortex. Further, NGAL and TYMP may be important markers of frontal cortex structural dysfunction in South African participants, which are predominantly HIV-1C infected. Therefore, in the context of regions geographically located to HIV-1C, NGAL and TYMP may play a role in the neurobiological mechanisms underlying the neuropathophysiology of HIV-1 associated neurocognitive impairment. NGAL and TYMP are promising markers for further investigations into their potential as anti-inflammatory therapies for HIV-associated neurocognitive impairments.

Here, I show that peripheral immune markers are associated with structural brain changes as well as HIV-associated neurocognitive impairment in South African participants. Several pre-clinical studies have suggested that inflammation may be higher in HIV-1B infection. However, this has not been investigated in clinical studies of PLWH. Recent evidence has reported that the HIV viral Tat protein differs between HIV-1 subtypes. These Tat differences may influence the differential inflammatory levels between HIV-1 subtypes. In the next chapter, I review the current literature of the differential effects of Tat sequence variation on the underlying mechanisms of HAND, including neuroimmune regulation.

4.6. References

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4.7. Supplementary: Chapter 4

Exploratory analysis

As an exploratory aim, the analysed ROIs of the whole brain included the superior frontal, rostral and caudal middle frontal, pars opercularis, pars triangularis, pars orbitalis, lateral and medial orbitofrontal, precentral, paracentral, frontal pole, superior parietal, inferior parietal, supramarginal, postcentral, precuneus, superior, middle, and inferior temporal, banks of the superior temporal sulcus (STS), fusiform, transverse temporal, entorhinal, temporal pole, parahippocampal, occipital, lateral occipital, lingual, cuneus, pericalcarine and cingulate.

Table 1: Association of Immune markers with the cortical thickness of all brain regions in HIV-positive participants

Brain ROIs	**TYMP**	**VEGF**	**MMP9**	**CCL2**	**NGAL**	**TGFβ1**
Temporal pole	.097	**-.313***	-.013	-.201	-.109	-.201
Frontal pole	**-.383****	.072	-.124	-.006	**-.362****	.007
Banks sts	-.107	.059	.229	-.017	-.069	.272
Superior temporal	.081	.014	.227	.061	.060	.203
Middle temporal	**-.331***	-.159	-.154	**-.337***	**-.433****	-.167
Precentral	.182	-.208	-.132	**.353***	-.093	.093
Postcentral	-.150	.093	-.077	.175	.030	.289
Supramarginal	-.199	.108	-.109	.211	.065	-.016
Superior parietal	-.067	.096	.003	.192	-.012	.037
Precuneus	-.088	.000	-.104	.184	-.162	.035
Cuneus	-.122	.090	-.112	.125	.030	-.121
Pericalcarine	-.099	-.154	.14	.010	-.232	.007
Lingual	-.075	-.189	-.104	.035	-.246	-.039
Superior frontal	-.180	-.085	-.057	.179	**-.264***	-.034
Rostral anterior cingulate	-.074	-.068	-.19	.042	**-.280***	-.115
Caudal anterior cingulate	-.195	.063	-.058	.050	-.202	-.045
Posterior cingulate	-.024	.085	-.194	.041	-.162	.035
Isthmus cingulate	-.224	-.093	-.243	-.081	**-.252***	-.154
Medial orbitofrontal	-.155	.036	.046	.076	**-.282***	-.050
Inferior temporal	**-.346****	**-.260***	-.037	**-.316***	**-.374****	-.106
Lateral occipital	**-.298***	-.060	-.008	-.069	-.231	.048
Inferior parietal	-.149	.093	-.04	.164	-.182	-.154
Caudal middle frontal	-.037	**-.252***	-.185	.143	**-.304***	-.019
Rostral middle frontal	**-.256***	-.083	-.07	-.016	**-.341****	-.038
Lateral orbitofrontal	-.112	-.149	.033	.094	**-.365****	.072
Pars orbitalis	**-.274***	-.160	-.031	.032	**-.338****	.038
Pars triangularis	-.041	-.145	-.06	.051	**-.261***	-.090
Pars opecularis	-.119	**-.256***	-.059	.094	**-.276***	.064
Insula	-.05719	-.157	-.065	.164	-.256	-.151
Transverse temporal	.01369	-.038	-.084	.077	-.137	.061
Entorhinal	-.05009	-.070	**.304***	-.035	-.064	-.132
Paracentral	.003372	-.070	-.076	.194	-.078	.152
Fusiform	-.262*	-.154	-.017	-.050	**-.277***	-.133
Para hippocampal	.138	-.227	-.182	-.065	-.155	-.240

Results displayed as Pearsons *r.* Results with significant *p*-values are presented in bold. * $p < .05$, ** $p < .01$

Table 2: Association of Immune markers with the cortical thickness of all brain regions in HIV-negative participants

Brain ROIs	TYMP	VEGF	MMP9	CCL2	NGAL	TGFβ1
Temporal pole	-.044	-.117	.100	-.272	.270	-.225
Frontal pole	-.294	-.046	.184	-.104	.023	-.169
Banks sts	**-.683****	-.093	-.025	.022	-.097	-.274
Superior temporal	.025	.026	**-.466***	.255	-.195	**.531***
Middle temporal	-.221	-.079	.147	.163	.062	.124
Precentral	-.248	.011	.070	.017	-.240	.061
Postcentral	-.213	**.428***	.186	.229	.266	.211
Supramarginal	-.443	-.125	.021	-.048	.066	-.189
Superior parietal	-.336	.058	.110	-.058	.120	-.219
Precuneus	-.369	.090	.202	-.051	.100	-.163
Cuneus	.057	-.100	-.306	**-.489***	-.207	-.413
Pericalcarine	-.378	-.256	-.064	-.316	.000	-.468
Lingual	**-.624****	-.027	-.067	.011	.104	-.368
Superior frontal	.024	.003	.292	-.305	-.011	-.179
Rostral anterior cingulate	-.061	.126	**.435***	-.159	.096	-.145
Caudal anterior cingulate	-.128	.007	.249	.037	-.033	.138
Posterior cingulate	-.363	-.177	.105	.037	-.146	-.098
Isthmus cingulate	**-.624****	-.383	-.085	-.004	-.215	-.216
Medial orbitofrontal	-.283	-.188	.199	.118	-.096	-.023
Inferior temporal	-.088	.041	.351	-.016	.225	-.217
Lateral occipital	-.165	.085	-.283	.146	.100	-.149
Inferior parietal	-.339	-.205	-.339	.273	-.340	-.138
Caudal middle frontal	-.388	.101	.162	-.080	.064	-.290
Rostral middle frontal	-.306	.104	.175	-.139	.029	-.113
Lateral orbitofrontal	-.323	-.121	.089	-.078	-.158	-.025
Pars orbitalis	-.070	.123	.178	-.058	.175	.002
Pars triangularis	-.259	.116	.050	-.083	.055	-.027
Pars opecularis	-.237	.121	.077	-.226	-.006	-.220
Insula	-.234	-.152	.371	-.132	-.072	-.145
Transverse temporal	-.297	-.094	.091	.012	-.130	.048
Entorhinal	.083	-.112	-.149	.117	-.267	**.600***
Paracentral	-.214	.013	.217	.001	-.014	-.182
Fusiform	-.262	-.044	.169	-.187	.058	-.083
Para hippocampal	-.169	.076	.006	-.232	-.083	.059

Results displayed as Pearsons *r.* Results with significant *p*-values are presented in bold. $^{*}p < .05$, $^{**}p < .01$

Table 3: Association of Immune markers with the surface area of all brain regions in HIV-positive participants

Brain ROIs	TYMP	VEGF	MMP9	CCL2	NGAL	TGFβ1
Temporal pole	.044	.165	-.076	.077	.142	-.112
Frontal pole	-.082	-.047	-.166	-.138	.066	-.084
Banks sts	-.005	**.286***	.071	.096	.164	.107
Superior temporal	**.318***	.030	.067	.023	.293	.038
Middle temporal	-.001	.229	.084	-.018	.130	**.276***
Precentral	-.126	-.112	.015	-.145	.175	.010
Postcentral	-.150	.029	.036	-.096	.209	**.385****
Supramarginal	**.391****	.013	-.042	.210	.223	.156
Superior parietal	.075	.003	-.089	.096	.099	.100
Precuneus	.159	-.023	-.062	.111	.185	.035
Cuneus	.189	-.074	-.169	.058	.138	.076
Pericalcarine	.008	-.189	-.118	-.010	.157	.073
Lingual	-.107	-.014	-.091	.140	.168	-.095
Superior frontal	-.140	.038	-.030	-.140	.135	.001
Rostral anterior cingulate	-.048	.029	.072	.119	.126	-.096
Caudal anterior cingulate	-.003	.036	.092	.241	.148	-.060
Posterior cingulate	.087	.047	.077	.142	.121	-.083
Isthmus cingulate	.131	.077	.144	.118	**.303***	.139
Medial orbitofrontal	-.065	-.010	-.017	-.121	.131	-.038
Inferior temporal	-.001	.131	.010	-.088	.061	.115
Lateral occipital	-.016	.243	-.009	.107	.244	-.060
Inferior parietal	-.211	.106	-.089	.003	-.087	.253
Caudal middle frontal	-.028	.089	.096	.027	.192	.053
Rostral middle frontal	.005	-.105	-.041	.061	.146	.015
Lateral orbitofrontal	-.099	.019	-.063	-.175	.124	-.045
Pars orbitalis	.048	-.032	-.118	-.084	.031	-.093
Pars triangularis	.120	.028	-.118	.204	.098	-.049
Pars opecularis	-.064	-.011	.045	-.066	.058	-.013
Insula	-.107	.030	.143	-.061	.168	.040
Transverse temporal	.090	-.040	.195	.037	.183	-.026
Entorhinal	.184	-.094	-.084	-.081	-.091	-.043
Paracentral	-.154	.062	-.005	-.063	.010	-.059
Fusiform	.059	-.081	-.118	-.077	.160	-.175
Para hippocampal	-.222	.164	.053	-.081	.059	.006

Results displayed as Pearsons *r.* Results with significant *p*-values are presented in bold. * $p < .05$, ** $p < .01$

Table 4: Association of Immune markers with the surface area of all brain regions in HIV-negative participants

Brain ROIs	TYMP	VEGF	MMP9	CCL2	NGAL	TGFβ1
Temporal pole	-.141	.161	-.116	.407	.087	.161
Frontal pole	.228	-.084	-.354	.132	-.033	.265
Banks sts	-.221	.054	**-.588***	.441	-.027	.135
Superior temporal	-.274	.102	-.532	**.537***	-.060	.226
Middle temporal	-.196	.212	**-.698****	.144	.119	-.037
Precentral	-.163	.225	-.050	-.149	.118	.059
Postcentral	-.225	.291	-.211	.139	.148	.081
Supramarginal	-.302	.071	-.303	.080	.131	.058
Superior parietal	-.153	.294	.055	.121	.255	-.207
Precuneus	-.031	.315	-.067	.072	.234	-.178
Cuneus	.356	.345	-.302	-.371	.342	-.098
Pericalcarine	-.208	-.138	-.023	-.440	.109	-.365
Lingual	.208	.008	-.046	-.214	.222	-.159
Superior frontal	-.045	.149	-.317	.023	.128	-.095
Rostral anterior cingulate	-.024	.056	-.228	-.097	.133	-.054
Caudal anterior cingulate	-.021	.037	-.075	-.168	.023	-.220
Posterior cingulate	-.113	.128	-.246	-.051	.133	-.195
Isthmus cingulate	.364	**.567****	-.144	-.041	.354	.107
Medial orbitofrontal	.103	.017	-.176	-.170	-.148	.059
Inferior temporal	-.135	-.004	**-.463***	.226	-.088	.181
Lateral occipital	-.046	.306	-.328	.051	.202	.133
Inferior parietal	.073	.204	-.349	.303	.026	.253
Caudal middle frontal	-.061	.122	-.167	.015	.327	-.095
Rostral middle frontal	.124	.169	-.116	.124	.082	.017
Lateral orbitofrontal	.177	.344	.242	.138	**.420***	-.089
Pars orbitalis	-.006	.326	-.027	.005	.212	-.174
Pars triangularis	.292	.387	-.161	-.160	.196	.140
Pars opecularis	.303	.292	**-.565****	-.227	.200	.123
Insula	-.002	.205	**.530***	-.110	.248	-.285
Transverse temporal	.157	.307	-.275	-.012	.264	.013
Entorhinal	.192	.270	-.338	-.229	-.086	.372
Paracentral	**-.407***	-.091	-.183	.001	.062	-.310
Fusiform	-.160	.010	-.270	.124	-.103	.363
Para hippocampal	-.301	.054	-.128	.318	.110	.013

Results displayed as Pearsons *r.* Results with significant *p*-values are presented in bold. * $p < .05$, ** $p < .01$

Chapter 5

Signatures of HIV-1 subtype B and C Tat proteins and their effects in the neuropathogenesis of HIV-associated neurocognitive impairments

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Abstract

HIV-associated neurocognitive disorders (HAND) are a spectrum of neurological disorders due to the effects of HIV-1 in the central nervous system (CNS). The HIV-1 subtypes; HIV-1 subtype B (HIV-1B) and HIV-1 subtype C (HIV-1C) are responsible for the highest prevalence of HAND and HIV infections respectively. The HIV transactivator of transcription (Tat) protein is a major contributor to the neuropathogenesis of HIV. The effects of the Tat protein on cells of the CNS is determined by the subtype-associated amino acid sequence variations. The extent to which the sequence variation between Tat-subtypes contribute to underlying mechanisms and neurological outcomes are not clear. In this review of the literature, we discuss how amino acid variations between HIV-1B Tat (Tat-B) and HIV-1C Tat (Tat-C) proteins contribute to the potential underlying neurobiological mechanisms of HAND. Tat-C is considered to be a more effective transactivator, whereas Tat-B may exert increased neurovirulence, including neuronal apoptosis, monocyte infiltration into the brain, (neuro)inflammation, oxidative stress and blood-brain barrier damage. These findings support the premise that Tat variants from different HIV-1 subtypes may direct neurovirulence and neurological outcomes in HAND.

5.1. Introduction

HIV-1 is well known for its effects on the immune system and the development of Acquired Immunodeficiency Syndrome (AIDS). However, the presence of HIV-1 in the central nervous system (CNS) is also responsible for a spectrum of neurological disorders known as HIV-associated neurocognitive disorders (HAND). HIV-associated neurocognitive impairment ranges in severity from asymptomatic neurocognitive impairment (ANI) to mild neurocognitive disorder (MND) to the most severe form, HIV-associated dementia (HAD) (Antinori et al., 2007).

The neuropathogenesis of HAND is complex and is characterized by events such as oxidative stress, neuroinflammation, synapse pruning and neuronal death. The development of HAND is affected by several factors, which may include the HIV-1 subtype (Constantino et al., 2011; Gandhi et al., 2009; Rao et al., 2008; Samikkannu et al., 2015). Further, the neurovirulence and neurological outcomes of HIV-1 subtypes were shown to be affected by Trans-activator of transcription (Tat)-subtype variation (Gandhi et al., 2009; Rao et al., 2013). However, the extent to which Tat and HIV subtypes contribute to the neurovirulence and neurological outcomes remains largely unclear.

HIV-1 is divided into four groups M, O, N and P. Group M is the "major" group responsible for the global human HIV epidemic. Further, Group M is subdivided into 9 subtypes (A, B, C, D, F, G, H, J and K) as well as at least 51 circulating recombinant forms (CRFs) (de Arellano et al., 2010; Hemelaar, 2012). The HIV-1 subtype-B (HIV-

1B) is present in America, Western Europe, and Australasia and represents about 12% of all HIV infections (Taylor et al., 2008). In contrast, the dominant HIV-1 subtype-C (HIV-1C) is present in countries of Southern Africa and India and represents about 50% of the world's HIV infected population (Geretti, 2006). The current knowledge of HIV-1 neuropathogenesis is derived largely from studies on the HIV-1B subtype (Taylor et al., 2008). The onset and severity of HAND are affected by HIV subtype differences (Tyor et al., 2013), with HIV-1B and HIV-1C having different effects (Santerre et al., 2019). Whether genetic variations between HIV-1 subtypes affect HIV transmission, disease progression and neurological outcomes remain controversial.

Pre-clinical studies suggest an increased neurovirulence for HIV-1B compared to HIV-1C (Mishra et al., 2008; Rao et al., 2008). Initial investigations report a lower prevalence for HAND in countries where HIV-1C is the dominant subtype (Clifford et al., 2007) compared to countries where HIV-1B is dominant (Sacktor et al., 2001). More specifically, studies of HIV-1B reported HAND prevalence rates of 36%-52% (Heaton et al., 2011, 2010). In the pre-antiretroviral therapy (ART) era patients with HIV-1B were characterized by deficits in motor skills, cognitive speed, and verbal fluency, while in the post-ART era, deficits were more pronounced in memory (learning) and executive function (Heaton et al., 2011, 2010). Studies of HIV-1C reported HAND prevalence rates of 33%-53% (Ghate et al., 2015; Joska et al., 2011; Kamat et al., 2017; Mogambery et al., 2017). Patients were characterized by deficits in motor and information processing speed (Ghate et al., 2015; Kamat et al., 2017).

Evidence from more recent studies provide contradictory findings and suggest that there are no significant differences in the prevalence of HAND between HIV-1B and HIV-1C (Day et al., 2016; de Almeida et al., 2013). A recent study in China showed that the prevalence of HAND may be slightly higher in HIV-1B compared to HIV-1C (31,8% *versus* 23.9%) (Wu et al., 2017) but remain statistically insignificant. Recently, it has been shown that even though CSF/serum ratios of amyloid-β protein isoforms were significantly lower in HIV-1C than in HIV-1B, there were no significant differences in global deficit scores between HIV-1B and HIV-1C patients (de Almeida et al., 2019). Similarly, HIV-1B and HIV-1C were associated with similar structural volume declines compared to their relative controls. However, no significant differences were found in the structural volume decline when directly comparing HIV-1B and HIV-1C (Ortega et al., 2013).

A challenge when comparing the neurocognitive outcomes between two different geographical regions [e.g. Europe and America (HIV-1B) and Southern African and India (HIV-1C)] is addressing differences in education as well as other socio-cultural dissimilarities. The interpretation of findings from such comparative studies, therefore, become more challenging. At this stage, it is difficult to clearly understand the role of the HIV-1 subtype on clinical outcomes. The limited number of clinical studies available underscores the need for further research in this field.

The increased neurovirulence of HIV-1B may be attributed to the activity of HIV viral proteins, including; glycoprotein (gp) 120, Tat and Viral protein r (Vpr). This review will focus on the HIV Tat protein. Although other viral proteins such as Vpr and

gp120 have been shown to cause neurotoxicity (Jones et al., 2007; Rao et al., 2014), there is a strong research interest in understanding the neuropathological mechanisms induced by Tat because its secretion persists despite viral suppression (Johnson et al., 2013; Mediouni et al., 2012). A clearer understanding of the possible differences in the pathogenic potency of Tat variants from different HIV-1 subtypes may provide insight into the underlying characteristics of subtype-specific HAND. Due to limited studies on how Tat subtype differentially affects underlying mechanisms of HAND, here, we reviewed the differences by both Tat and HIV-1 subtype.

5.2. HIV Tat protein sequence and structure

The HIV-1 subtype B Tat protein has approximately 101 amino acid residues which differ for each HIV strain and consists of two exons with six functional regions (Siddappa et al., 2006). The first exon contains residues 1-72 and second exon has residues 73-101. In Tat-B, region 1 (residues 1-21) is a Proline-rich region with a conserved Tryptophan (W_{20}). Region 2 (residues 22-37) is a Cysteine-rich region, with seven well-conserved Cysteines at position 22, 25, 27, 30, 34, and 37. Region 3 (residues 38-48) contains conserved residues at F_{38} and at $_{43}$LGISYG$_{48}$. The 4th region (residues 49-57) contains basic residues and a conserved region $_{49}$RKKRRQRRRPP$_{57}$. This is also known as the TAR binding domain or the cell-penetrating peptide (CPP). Region 5 (residues 58 to 72) is a Glutamine-rich region and considered as the region with the highest rate of sequence variation. Region 6 (residues 73 to 101) consists of

the C-terminal domain that contains the ESKKKVE motif known as the RGD domain and is encoded by the second exon (Figure 1). In HIV-1B and HIV-1D, a conserved RGD motif is present in this region (Siddappa et al., 2006; Smith et al., 2003).

Tat sequences from most clinical HIV-1 isolates are 101 amino acids in length. Several clinical isolates also display Tat sequences with 86-106 amino acids (Jeang et al., 1999). Pre-clinical and *in vitro* studies largely make use of the 86 amino acid configuration. However, it is considered as a truncated protein compared to that found in clinical isolates. Several *in vitro* studies utilize the HIV-1B (BRU strain) (Barré-Sinoussi, Chermann et al., 1983; Rosen et al., 1986) or a closely related HXB2 HIV-1B molecular clone (Rosen et al., 1986). Studies suggest that Tat sequences from clinical isolates preserve the full 101 amino acid form, which indicates the functional relevance of the second exon in an *in vivo* setting (Strazza et al., 2011).

A sequence alignment (Figure 1) of the full-length Tat-B and Tat-C indicates several residue variants. Tat-C contains several polymorphisms which have been previously shown to be involved in neurological outcomes in both pre-clinical and clinical studies (Rao et al., 2013, 2008; Ruiz et al., 2019). As reviewed here, key polymorphisms include a Serine substitution at residue 31 ($C_{31}S$), a Serine substitution at residue 57 ($R_{57}S$) and a Glutamate substitution at residue 63 ($Q_{63}E$). With these residue changes, initial biochemical studies speculated Tat-C to have a relatively higher-ordered structure and be less flexible than Tat-B (Siddappa et al., 2006). However, recent findings from additional biochemical studies speculated the opposite with Tat-C having a more flexible structure which allows for greater

flexibility in binding and resulting in greater transactivation efficiency (Bachu et al., 2012; Desfosses et al., 2005; Johri et al., 2015). Further, Tat protein structural studies are needed to support these findings.

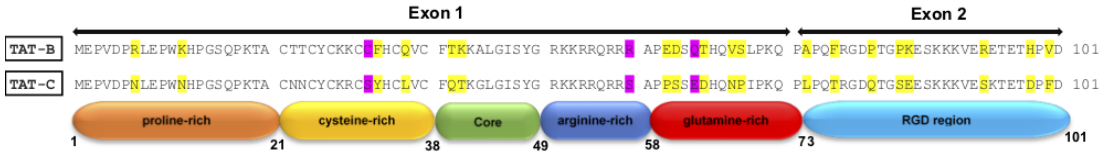

Figure 1: Schematic presentation of HIV Tat-B (subtype B, Isolate MN) and HIV Tat-C (subtype C, Isolate 92BR025) full amino acid sequence alignment including Exon 1 and 2. Yellow highlighted residues present sequence variance. Purple highlighted residues represent key sequence polymorphisms with a reported effect on neurological outcomes. Functional regions of Tat: region I (residues 1 to 21) is an acidic/Pro-rich region; region II (residues 22 to 37), a Cysteine-rich region; region III (residues 38 to 48) containing conserved “core” region; region IV is arginine-rich (residues 49–57); region V (residues 58–72) a Glutamine-rich region and region VI (residues 73–101) is encoded by exon 2 and known as the RGD region.

Tat functions by binding to a 59-bp stem-loop RNA structure, known as the transactivation response element (TAR). The TAR is located at the 5' end of nascent HIV-1 transcripts. Tat, therefore, acts as an RNA specific viral transcription factor via its interaction with the TAR RNA element (Desfosses et al., 2005). The effective binding of Tat to TAR allows HIV to achieve a high-level transcription. Specifically, sequence variation between Tat-B and Tat-C has been shown to affect the transactivation capacity, immune modulation, neuronal damage, and pathways related to neuronal damage.

5.3. Transcriptional capacity

It is known that Tat brings about a strong transactivation response from a long terminal repeat (LTR) promoter through binding to TAR. The binding affinity between Tat and TAR determines the transcriptional capacity, which may be affected by residue polymorphisms present in Tat variants from different HIV-1 subtypes. Studies showed a greater transactivation capacity for Tat-B (Siddappa et al., 2006), whereas others have found a greater transactivation capacity for Tat-C (Bachu et al., 2012; Desfosses et al., 2005). Several aspects contribute to the level of Tat transactivation which includes the stability of the Tat-TAR complex, number of LTR NF-κB binding sites, and its subsequent immune regulation.

Tat-C has a more flexible structure, which allows for more effective binding to partners, compared to Tat-B. Therefore, Tat-C-TAR forms a more stable complex with a higher binding affinity (Johri et al., 2015). In addition, it has been reasoned that HIV-1C LTR has a greater number of NF-κB binding sites and subsequently allows for greater LTR activation (Bachu et al., 2012). HIV-1 subtype-specific mutations are present in regulatory regions within the LTR and this results in HIV-1C presenting three NF-κB binding sequences and HIV-1B presenting two NF-κB binding sequences (de Arellano et al., 2010). This may have contributed to the fact that when HIV-1 variants and CRFs were investigated for their transcription activities, HIV-1C LTR had the highest basal and phorbol myristate acetate induced transcription activities related to the HXB2 subtype B promoter (de Arellano et al., 2010). Compared to Tat-B, Tat-C had stronger transactivation potentials, and a higher affinity for the Tat RNA

element in a Tat-TAR electrophoretic mobility shift assay (de Arellano et al., 2010; Desfosses et al., 2005).

The transcriptional capacity of Tat is linked to specific residue polymorphisms between Tat-B and Tat-C. A Glutamic acid mutation ($Q_{63}E$) present in Tat-C was shown to contribute to greater transcriptional activation in human CD4 T-Cells (Kurosu et al., 2002). Another well studied polymorphism $C_{31}S$ is present in Tat-C. The Cysteine at position 31 is not considered as critical for LTR activation, but it may be more critical for inducing monocyte chemotaxis (Ranga et al., 2004) as discussed in the latter section of this review. Interestingly, HIV-1B is considered to have a greater neurovirulence, but a less potent transactivator. This may suggest that in addition to HIV transactivation, several other mechanisms may contribute to the development of HAND.

5.4. Neuronal cell damage

The Tat protein is a key viral protein in the development of HAND. Tat-mediated neuronal cell damage occurs through various mechanisms which include but not limited to the dysfunction and activation of the kynurenine pathway (KP), glutamatergic system, and the upregulation of key synaptic plasticity genes in neuroblastoma cells. The level of neuronal damage may also be influenced by Tat protein sequence variation.

It has been shown that Tat-induced neurotoxicity may be influenced by specific protein signatures present in Tat-B. The level of neurotoxicity may be due to the presence of the dicysteine motif ($C_{30}C_{31}$) within the Tat-B protein than attributed to the HIV subtype (Table 1). Tat-C with an intact dicysteine motif proved to be more neurotoxic to human neuronal cultures compared to Tat-C lacking this motif (Rao et al., 2013). Therefore, Tat-B with its key protein signatures may exert its neurotoxic effects by contributing to several potential underlying mechanisms and pathways of neuronal damage.

5.4.1. Tat dysregulates the Kynurenine Pathway (KP)

The KP is responsible for catabolizing tryptophan to quinolinic acid (QUIN) using several enzymatic reactions. Activation of indoleamine-2,3-dioxygenase (IDO) (rate-limiting enzyme of the kynurenine pathway) leads to increased tryptophan catabolism and the generation of neurotoxins such as kynurenine (KYN) (Samikkannu et al., 2009). This process is important in energy production, protein production, protein synthesis, neurotoxicity, and immune tolerance. Several studies have reported an association between QUIN and severity of HAND (Heyes et al., 2001; Valle et al., 2004).

KP activation may be present in the brain, even with the introduction of combination antiretroviral therapy (cART) (Drewes et al., 2015). QUIN/Tryptophan ratios are early predictors of neurological diseases in untreated SIV-infected macaques and were considered to outperform all other KP metabolites in predicting neurological disease

(Drewes et al., 2015). In cART treated macaque models, KP metabolites did not restore to control levels in the striatum even though levels were normalized in the CSF of most animals (Drewes et al., 2015). These suggest that cerebral KP activation may only be partially resolved with cART (Drewes et al., 2015). QUIN production may be specific to stimulation by HIV viral proteins, including Tat.

Macrophages treated with Tat (subtype not reported) for 72 hours yielded significantly higher levels of QUIN compared to negative controls (Smith et al., 2001). The levels of QUIN produced was above or equal to that related to neuropathological damage *in vitro* (Kerr et al., 1998, 1995). Increased IDO mRNA expression was also found in Tat treated macrophages in a dose-dependent manner (Smith et al., 2001). This level of expression may also be affected by the HIV Tat variants from different HIV-1 subtypes. In HIV-1B treated astrocytes, IDO enzyme activity, as well as KYN concentration, were significantly upregulated compared to HIV-1C treated cells (Samikkannu et al., 2009). At this stage, it remains unclear as to which amino acid polymorphism within the Tat protein may influence differential activation of the KP. However, introducing a $C_{30}S$ mutation in Tat-C results in a potentially ineffective upregulation of IDO mRNA expression from Tat treated monocytes (Campbell et al., 2007).

5.4.2. Tat disrupts calcium ion regulation in the glutamatergic system

The dysfunction of the glutamatergic system and N-methyl-D-aspartate receptors (NMDAR) are key neuropathological contributors to neuronal damage. The presence

of HIV-1 in the CNS induces the recruitment of infected cells (monocytes) into the brain (González-Scarano and Martín-García, 2005) (Figure 2). Neurotoxic and viral products present in the brain overexcites the NMDAR which may lead to neuronal damage. Overexcitation of NMDAR has been linked to several neurodegenerative disorders (Lin et al., 2014). HIV-1 and in particular the HIV-1 Tat protein can induce neuronal apoptosis by over-excitation of NMDAR (Haughey et al., 2001; Hu, 2015; O'Donnell, 2006).

Glutamate is an excitatory neurotransmitter and plays an important function in learning and memory. However, excessive glutamate causes excitotoxicity by increasing calcium ion influx, which leads to neuronal dysfunction and cell-death (Vázquez-Santiago et al., 2014). Similar findings were observed *in vivo*, whereby Tat-B induced hippocampal neural degeneration and was associated with learning and memory impairment in an animal model of HAND (Fitting et al., 2008; Harricharan et al., 2015).

In Tat-B and Tat-C treated human neuroblastoma cells, glutamate was significantly increased in Tat-B treated cells and glutamine was significantly increased in Tat-C treated cells (Samikkannu et al., 2015). Increased glutamate subsequently reduces glutamine and this results in an increased level of nitric oxide synthase (NOS) (Prast and Philippu, 2001). NOS has been linked to several cellular dysfunctions in the CNS (Prast and Philippu, 2001), including neurotoxicity (Samikkannu et al., 2015). The HIV Tat protein has also directly been linked to inducing NOS production in human

astrocytes (Liu et al., 2002) and increased NOS levels have previously been associated with HAD in people living with HIV (PLWH (Pocernich et al., 2005).

Moreover, the HIV Tat may act as an agonist of the NMDAR and can induce neuronal excitotoxicity (Li et al., 2008). In an *in vivo* experiment, Tat-B ventricular infusion in C57BL/6J mice leads to synapse loss by altering the GluN2B subunit containing NMDAR signaling (Raybuck et al., 2017). The polymorphisms in the HIV Tat sequence, particularly the $C_{31}S$ mutation is suggested to have no effect of the binding of Tat to the NMDAR but may rather be more critical for activation of NMDA (Li et al., 2008). It had been shown that once Tat is bound to the cell membrane by the Arginine-rich domain, C_{31} would bind with C_{744} on the NR1 subunit forming a disulfide bond (Li et al., 2008). This results in a free thiol group on C_{798} which may cause persistent activation of the NMDA. This may explain why Tat-B treated neuronal cultures may have greater neurotoxicity compared to Tat-C (Li et al., 2008). Tat may also indirectly activate the NMDAR by activation of the low-density lipoprotein receptor-related protein (LRP). Tat-B interacts with the LRP leading to activation of Src-kinase, which in turn leads to increased NMDA-evoked (Ca^{2+}) responses (Krogh et al., 2014), but it is not known whether there are subtype-specific differences in LRP activation.

In addition to the dysregulation of NMDA, abnormal intracellular calcium (Ca^{2+}) influx can be induced by dysfunction of the L-type voltage-gated calcium channels (L-channels). Several pre-clinical studies suggest that the HIV Tat protein increases Ca^{2+} through over-activation of L-channels (Bansal et al., 2000; Brailoiu et al., 2008). There are also associations between Tat-induced Ca^{2+} dysregulation and neuronal

dysfunction (Nath et al., 2000b, 2000a). Tat-B (dicysteine motif) appears to be more effective than Tat-C in activating L-channels, and this may account for the differences in neurotoxicity (Campbell et al., 2007; Wong et al., 2010).

Tat protein is also responsible for disrupting calcium-regulating systems in the endoplasmic reticulum (ER) and mitochondria in neurons. The ER and mitochondria are important storage organelles for calcium and vital in maintaining Ca^{2+} homeostasis. Increased mitochondrial injury has been associated with worse neurocognitive function in PLWH (Var et al., 2016). In mouse striatal neuron cultures, Tat-B protein induced instability in the mitochondrial inner membrane resulting in excessive Ca^{2+} and synaptic injury (Fitting et al., 2014). Moreover, due to the dicysteine motif in Tat-B, Tat-B significantly depolarizes the mitochondrial membrane in human neuronal cultures, whereas in Tat-C, there is little or no depolarization of mitochondrial membranes (Mishra et al., 2008).

5.4.3. Tat alters synaptic plasticity and neuron morphology

Tat-B and Tat-C differentially impact synaptic plasticity. An investigation of the expression of 84 key human synaptic plasticity genes of Tat treated neuroblastoma cells (SK-N-MC) yielded differential results. Tat-B treated cells upregulated 69/84 synaptic plasticity genes and 36 genes were upregulated more than ≥3 fold (Samikkannu et al., 2015). Whereas in the case of Tat-C, 46/84 synaptic plasticity genes were upregulated with 25 genes being upregulated more than ≥3 fold compared to control cells. Even though a direct comparison of the gene expression

levels induced between Tat-B and Tat-C has not been made, the Tat-B protein induced a greater fold change in several gene expression levels than Tat-C. These included genes of synaptic plasticity, immediate-early response, late response, long-term potentiation, long-term depression, cell adhesion, cAMP response element-binding protein (CREB) factors, neuronal receptors, postsynaptic density and others. This further correlates with Tat-B treated cells displaying a greater decrease in spine density compared to Tat-C treated cells (Samikkannu et al., 2015). This study suggests that Tat-B substantiates neuronal toxicity and dysregulates synaptic plasticity genes in SK-N-MC, which may contribute to the severe neuropathogenesis linked to HAND.

It was also shown that Tat-B reduced synaptic proteins and neuronal dendritic spines in a mouse model of HAND (Fitting et al., 2010; Hammond et al., 2018; Lu et al., 2011). Synaptodendritic injury may be related to the presence of the dicysteine motif present in Tat-B proteins. When human hippocampal neuronal cell cultures were exposed to either Tat-C or Tat-B for 24 hours, early reduction in F-actin puncta was more in Tat-B than in Tat-C. The activity of Tat-B on synaptodendritic damage was associated with the dicysteine motif. Reduction in F-actin puncta is a key feature of synaptodendritic injury, and this suggests that the dicysteine motif of Tat-B may be critical for synaptodendritic damage (Bertrand et al., 2013).

The Tat protein may indirectly induce neuronal damage. Monocyte-derived macrophages (MDM) were infected with either HIV-1B or HIV-1C. As a method of evaluating indirect macrophage-mediated neurotoxicity, conditioned medium (i.e.

cell culture medium taken from HIV infected cells) from infected MDMs were used to treat human and rat primary neuronal cell cultures. A greater level of neuronal toxicity was found in cultures exposed to HIV-1B conditioned medium compared to cultures exposed to HIV-1C conditioned medium (Constantino et al., 2011). HIV-1 isolates used in this study were derived from peripheral blood mononuclear cells of HIV infected patients. The lower levels of neurotoxicity by HIV-1C was due to the slower replication kinetics of HIV-1C in MDMs. This may be attributed to the viral genotype, specifically Tat and not the viral load. In a study conducted by Rao and colleagues, it was shown that in MDMs infected with an equal viral load, HIV-1B infected MDMs recruited more monocytes than HIV-1C infected MDMs (Rao et al., 2008). Furthermore, monocyte recruitment was HIV-1 Tat and CCL2 dependent. The results from this study, therefore, show that the differential effects of HIV-1B and HIV-1C on mechanisms of neurotoxicity may be attributed to the viral genotype, specifically Tat (Rao et al., 2008).

Previous research has shown that subtype-specific differences between HIV-1C and HIV-1B may influence HIV-1 fitness (replicative capacity). Higher replicative capacity of HIV-1B isolates compared to HIV-1C isolates were mediated by the gp120 coding region of the *env* gene and an increased binding/fusion of the CD4/CCR5 on host cells (Marozsan et al., 2005). Further, it has been reported that HIV-1B and HIV-1C differ in the sequence of the Gag polyprotein. HIV-1B viruses contain a LYPX motif whereas HIV-1C viruses lack the LY dipeptide in the late domain II of the Gag polyprotein (Ajasin et al., 2019; Kiguoya et al., 2017). The presence of the LYPX motif in HIV-1B influences increased virion release in the presence of CCL2 due to greater

access to ALIX (an adapter protein which enhances viral particle production). In the case of HIV-1C, the absence of the LYPX domain results in HIV-1C being unable to utilize ALIX and subsequently results in the lower replicative capacity of HIV-1C (Ajasin et al., 2019). Future studies should investigate whether Tat protein signatures may influence HIV-1 replicative capacity.

An additional protein signature present in Tat-B may influence neuroinflammatory levels, which can subsequently affect the morphology and integrity of neurons and astrocytes. In primary human neuron-astrocyte co-cultures that were incubated with media containing equivalent amounts of Tat variant, the Tat-B (R_{57}) and mutated Tat-C ($S_{57}R$) presented with the shorter, thinner and more beaded appearance of neurons compared to Tat $S_{57,}$ which are suggestive of neuronal damage (Ruiz et al., 2019). Axonal/dendritic integrity was more significantly compromised in co-cultures treated with conditioned microglial media with Tat-R_{57} compared to Tat-S_{57} variants and these are further suggestive of synaptic compromise in HAND (Ruiz et al., 2019; Takeuchi et al., 2005). These suggest that the $R_{57}S$ mutation that is largely found in Tat-C may influence the lower levels of inflammation and subsequent neuronal damage (Table 1). In addition to these mechanisms, Tat and specifically Tat protein signatures may influence several indirect mechanisms in the development of HAND.

5.5. Monocyte activation and recruitment

Monocyte activation is one of the initiating steps in the neuropathology of HAND (González-Scarano and Martín-García, 2005). Monocyte activated phenotypes result in the increased recruitment of peripheral cells into the CNS, contributing to the neuropathology of HAND (González-Scarano and Martín-García, 2005). Limited studies exist for the effect of HIV subtype and Tat variation on the level of monocyte activation itself. However, HIV Tat proteins can activate microglia and this activated state presents with a release of inflammatory chemokines and cytokines (Gandhi et al., 2009) (Figure 2).

The HIV Tat protein is responsible for several chemotactic events within the neuropathophysiology of HAND. The Tat protein has been correlated with increased CCL2 levels in human astrocytes (Khiati et al., 2010), and increased monocyte transmigration across a BBB model (Weiss et al., 1999). This is supported in C578BL/6 mice that were injected with Tat_{1-72} into the right hippocampus, which resulted in significantly higher CCL2 levels compared to control animals. The changes were also associated with substantial infiltration of monocytes into the brain (Pu et al., 2003). Increased levels of chemotactic immune marker CCL2 have been implicated in the migration of HIV-positive cells across the BBB (Eugenin, 2006) and worse neurocognitive performance in PLWH (Ancuta et al., 2008; Kamat et al., 2010; Woods et al., 2006).

The level of monocyte chemotaxis may also be attributed to the specific HIV subtype and the signature in the HIV Tat protein. MDMs infected with HIV-1B recruited 64% more monocytes than HIV-1C infected MDMs (Rao et al., 2008). Monocyte recruitment was HIV-1 Tat and CCL2 dependent. This suggests that the increased level of monocyte recruitment of HIV-1B may, therefore, be influenced by the Tat protein and its relevant protein sequence. This has been linked specifically to the dicysteine motif present in Tat-B proteins. When comparing MDMs infected with HIV-1C (Tat) with an intact dicysteine motifs and MDMs infected with HIV-1C (Tat) lacking the dicysteine motif, the presence of the motif accounted for the increased monocyte chemotaxis (Rao et al., 2013). The $C_{31}S$ mutation found in Tat-C renders it defective for monocyte chemotactic activity without a loss in the transactivation property (Ranga et al., 2004). Therefore, Tat-B may be more effective in increasing the transmigration of infected monocytes in the brain, increasing inflammatory markers, neurotoxic and viral products, which essentially may be a reason for the increase neurovirulence of HIV-1B compared to HIV-1C.

5.6. (Neuro)inflammation: Cytokines and Chemokines

Cytokines and chemokines are small proteins which function in cell signaling (Leonard and Lin, 2000). A dysregulated cytokine and inflammatory profile are major contributors to the developments of HAND (Burdo et al., 2013; Lyons et al., 2011; Yuan et al., 2013). Dysregulated levels of cytokines/chemokines in the peripheral

blood, CSF and brain have been associated with HAND (Burdo et al., 2013; Lyons et al., 2011; Shapshak et al., 2004; Yuan et al., 2013).

Primary CNS cells [human astrocytes (HA) and neuronal cells (SK-N-MC)] treated with HIV-1B produced higher levels of IL-33/ST2L compared to HIV-1C treated cells (Yndart et al., 2015). IL-33 is an inflammatory marker with an association to myocyte enhancer factor 2C (MEF2C) expression, which is a transcription factor that regulates synaptic function. In HIV-B, increase IL-33 is also associated with an increase in apoptosis, nucleotide-binding oligomerization domain containing 2 (NOD2) and solute carrier family 11 (SLC11A1). These are markers related to dysregulated synaptic function and apoptosis and are common hallmarks of HAND (Yndart et al., 2015). More particularly, the change in the inflammatory response may be related to the HIV Tat protein and its relative protein sequence.

In pre-clinical investigations, Tat-B treated monocytes induced a significant upregulation of proinflammatory cytokines IL-6 and TNF-α compared to Tat-C treated monocytes (Gandhi et al., 2009). IL-6 and TNF-α are common pro-inflammatory markers that are key regulators of the inflammatory environment and can contribute to the development of HAND when chronically increased (Harezlak et al., 2011). In primary human neuronal cell cultures, induction of neuronal apoptosis was maximized when cells were exposed to a combination of TNF-α and Tat, compared to exposing cells to either TNF-α or Tat separately (Shi et al., 1998). TNF-α mediated neuronal apoptosis is induced by increasing oxidative stress (Shi et al., 1998).

Furthermore, in human and rat Tat_{1-86} treated neuronal cultures, neuronal apoptosis was induced by TNF-α and activation of Non-N-methyl-D-aspartate receptors by an NF-κB-independent mechanism (New et al., 1998). When treating CHME3 cells with Tat-B and Tat-C, contradictory findings to the above reported that Tat-C had greater potential in inducing higher levels TNF-α and IL-6 at the mRNA level (Johri et al., 2015). However, a major shortcoming of this study is that the CHME3 cell line has been recently shown to be derived from CHME5 which is a rat derived cell line (Garcia-Mesa et al., 2017). Species differences in chemokine/cytokine subnetworks in neurons (Du et al., 2017) may help explain why the findings may differ from many other studies of human neuronal cell cultures (Campbell et al., 2007; Ruiz et al., 2019; Wong et al., 2010). In another study, injecting Tat-C into the hippocampus increased IL-1β expression but not TNF-α in rats (Makhathini et al., 2018). Interpretation of these contradictory findings may be challenging due to the lack of supportive evidence of the Tat variant, as inflammatory responses may be related particularly to their Tat sequence (Campbell et al., 2007; Ruiz et al., 2019).

Anti-inflammatory molecules and IL-4 and IL-10 were found to be higher in Tat-C treated monocytes compared to Tat-B treated monocytes (Gandhi et al., 2009). This is contradictory to previous findings, whereby Tat-C induced lower levels of IL-10 in monocytes compared to Tat-B (Wong et al., 2010). This discrepancy can be attributed to the different Tat-C proteins used in these studies. Tat-C proteins lacking the dicysteine motif ($C_{31}S$) could not induce an intracellular calcium flux through L-type channels and this resulted in lower levels of IL-10 (Wong et al., 2010).

Therefore, the decrease in IL-10 production by Tat-C has been suggested to be partly due to the lack of L-type calcium channel activation in this subtype (Campbell et al., 2007; Wong et al., 2010). Due to the lack of Tat-C's ability to induce a calcium influx, it is less effective in upregulating the markers TNF-α, IL-6, CCL2, and IDO in comparison to that of Tat-B (Campbell et al., 2007) (Table 1).

The natural polymorphism found in Tat-C ($R_{57}S$) may be an additional influencer of the lowered inflammatory response. This polymorphism may be responsible for the reduced uptake of Tat by bystander cells and subsequently reduced neuroinflammation (Ruiz et al., 2019). In the study by (Ruiz et al., 2019), Tat proteins were mutated to investigate its effects on the inflammatory response. Tat-B containing the R_{57} yielded higher levels of inflammatory gene transcripts of IL-6, IL-8, IL-1β, and CXCL1 compared to Tat-B with an $R_{57}S$ (found in Tat-C) substitution. Conversely, the same was noted in mutated Tat-C $S_{57}R$ (found in Tat-B) variants that yielded higher levels of these inflammatory markers compared to Tat-C ($R_{57}S$). This emphasizes the importance of this polymorphism in Tat uptake and neuroinflammation. Also, only wild type Tat-B (R_{57}) and Tat-C ($S_{57}R$) mutant were able to stimulate TNF-α production in treated cells, compared to Tat-B ($R_{57}S$) mutant and wild type Tat-C (S_{57}) (Ruiz et al., 2019). Genetic variation can modulate the ability of HIV-1 Tat to systematically disseminate (neuro)inflammation (Ruiz et al., 2019). Tat variants from different HIV-1 subtypes may therefore affect inflammatory responses.

5.7. Tat and amyloid-beta production

HIV patients may present intraneuronal amyloid beta 1-42 ($A\beta_{1-42}$) accumulation or perivascular diffuse $A\beta_{1-42}$ depositions, whereas extracellular amyloid plaques are predominant features in Alzheimer's disease (András and Toborek, 2013; Ortega and Ances, 2014). Under pathological conditions, $A\beta_{1-42}$ is generated from amyloid precursor protein (APP) and catalysed by two proteases, β-secretase and γ-secretase a process known as amyloidogenesis. It was recently shown that in 65 PLWH from Brazil, CSF/serum ratios of Aβ40, Aβ42, and Aβ-total were lower in the HIV-1C group than in the HIV-1B group (de Almeida et al., 2019). Growing evidence suggests that Tat upregulates $A\beta_{1-42}$ production and Tat-B specifically has been marked as the major contributor to increased $A\beta_{1-42}$ deposition in HIV patients (Aksenov et al., 2010; Hategan et al., 2017).

In a study conducted on cultured hippocampal neurons, it was shown that Tat-B induced $A\beta_{1-42}$ generation, whereas this effect was not found for Tat-C (Aksenov et al., 2010) (Table 1). In another study with a human primary glioblastoma cell line and a transgenic mouse model of Alzheimer's disease, it was shown that Tat in extracellular space can directly stimulate $A\beta_{1-42}$ generation by binding and recruiting APP into lipid rafts, a site where β- and γ-secretase activity is high (Kim et al., 2013). Tat at the extracellular space may gain entry into the cell cytoplasm through a process facilitated by heparan sulfate proteoglycans and LRPs (Krogh et al., 2014; Liu et al., 2000; Tyagi et al., 2001). The LRP is a large endocytic and signaling receptor

that is widely expressed in tissues, including neuronal cells involved in the uptake and degradation of various physiological ligands including APP and Aβ. Binding of Tat to LRP competitively inhibits the uptake and clearance of Aβ (Kim et al., 2013).

The $R_{57}S$ mutation in Tat-C significantly reduces its cellular uptake (Ruiz et al., 2019), which may account for reduced $A\beta_{1\text{-}42}$ generation, compared to Tat-B (R_{57}). Tat can disrupt $A\beta_{1\text{-}42}$ clearance, interfering with neprilysin, a major Aβ-degrading enzyme in the brain (Shirotani et al., 2001). Tat-B was shown to directly inhibit neprilysin cleaving activity by 80% and the Cysteine-rich domain of the Tat protein is essential for binding to the neprilysin active site, as peptides without Cysteine residues fail to bind to neprilysin (Daily et al., 2006; Rempel and Pulliam, 2005).

In primary cultured rat hippocampal neurons, it was demonstrated that internalized Tat is rapidly engulfed into the endosome which fuses with the lysosome to form endolysosome. Tat engulfment enlarges and increases endolysosome pH (Chen et al., 2013; Hui et al., 2012). Both these events have been linked to the disruption of Aβ metabolism in Alzheimer's disease (Cataldo et al., 2004, 2000). Comparative evidence on whether Tat-B and Tat-C endosome engulfment and endolysosome processing are subtype-specific is lacking. However, Tat-B may be more efficient in cellular uptake compared to Tat-C (Ruiz et al., 2019) and therefore may possess enhanced endolysosome distraction. The majority of our current understanding of the effects of Tat on Aβ production is largely based on *in vitro* experimentation. Thus, further investigations with human post-mortem brain tissues are needed to

evaluate Aβ localisation and levels of different Aβ species in the brains of HIV-1B and HIV-1C patients.

5.8. Blood-brain barrier damage

The blood-brain barrier (BBB) is a diffusion barrier that functions in regulating the microenvironments for protecting the neural tissues from infectious agents and toxins within the circulating system (Atluri et al., 2015). BBB dysfunction is a common hallmark of HAND (Saylor et al., 2016). The HIV viral Tat protein contributes to BBB disruption by several mechanisms including; Tat-mediated apoptosis of human brain microvascular endothelial cells (hBMVECs), alteration of multiple signaling pathways related to BBB disruption (McRae, 2016), increasing oxidative stress, increasing the production of metalloproteinases (MMPs) and altering expression of tight junction proteins (Banerjee et al., 2010; McRae, 2016). Collectively, these Tat-mediated mechanisms contribute to the increased BBB damage in HIV neuropathogenesis.

Tat-mediated apoptosis of hBMVECs is a product of activating signaling pathways of BBB disruption and oxidative stress. Treatment of hBMVECs with recombinant Tat (subtype not mentioned) resulted in the activation of vascular endothelial growth factor receptor-2 (VEGFR-2) and FMS-related tyrosine kinase 4 (FTK-4) (Kim et al., 2003). Activation of VEGFR-2 and FTK-4 resulted in the dysregulated release of NOS and subsequent cellular apoptosis (Kim et al., 2003). Another study suggested that in

Tat (101 amino acid configuration) treated hBMVECs, apoptosis was a result of ER stress and mitochondrial dysfunction (Ma et al., 2016). Further, Tat alters multiple signaling pathways related to BBB disruption that includes interaction and activation of Mitogen-activated protein kinase (MEK) ½, Phosphoinositide 3-kinases (PI-3K), NF-κB, CREB, Ras, caveolin-1, COX-2 and Rho (McRae, 2016).

Studies investigating the differential effects of Tat variants from different HIV-1 subtypes on Tat-mediated apoptosis of hBMVECs remains scarce. Current findings are largely based on Tat-B mediated apoptosis of hBMVECs. Further investigations of the effect of Tat-C mediated apoptosis of hBMVECs may provide insight into the neuropathogenesis of HAND within the context of HIV-1C infection. However, current literature suggests that Tat-B and Tat-C differ in their ability to produce reactive oxygen species (ROS).

ROS are highly reactive, toxic oxygen moieties which can lead to oxidation of proteins and DNA, peroxidation of lipids, and ultimately cell death (Ivanov et al., 2016). It is known that HIV infection and the Tat protein can activate several key pathways, induce ROS production, alter redox regulation which collectively contributes to apoptosis and peroxidative damage (Aukrust et al., 2003). HAD has been associated with markers for oxidative stress in the brain and CSF of HIV patients (Pocernich et al., 2005).

A tripeptide knows as glutathione (GSH) alters the redox balance and also leads to the production of ROS and subsequently functions in the development of HAND.

(Samikkannu et al., 2014). Depending on the Tat variant, Tat proteins exert differential effects on monocyte (MC)-derived immature dendritic cells (IDC) and neuroblastoma cells (SK-N-MC) by redox activation. Tat-B treated cells significantly increases the production of ROS, down-regulates gene expression and protein modification of glutathione synthetase (GSS), glutathione peroxidase 1 (GPx1), superoxide dismutase 1 (SOD1) and catalase (CAT), compared to Tat-C treated cells (Samikkannu et al., 2014). This further suggests that Tat-B induces oxidative stress and exacerbated immuno-neuropathogenesis compared to Tat-C (Samikkannu et al., 2014). Even though Tat-C may exert damage to a lesser degree, Tat-C induces NADPH oxidase (NOX)2 and NOX4 expression and intracellular ROS levels in Tat-C treated hBMVECs (Mishra and Singh, 2014). However, studies performing a direct comparison of the effects of these subtypes remains limited. In combination, the presence of Tat, ROS and inflammatory markers are contributors to the level of BBB dysfunction (Price et al., 2005).

In combination with oxidative stress induced by Tat, Tat may also upregulate inflammatory markers, complement components and MMPs. MMPs have shown evidence for its association with BBB damage (Xu et al., 2012) as well as neurocognitive impairment in PLWH (Li et al., 2013). In a study investigating the recombinant Tat-C, (CRF)02_AG (West and Central Africa) and Tat-B on hBMVECs, (CRF)02_AG reported minimal effects, whereas Tat-B induced transcriptional upregulation of 90 genes in hBMVECs. These included pro-inflammatory chemokines, complement components C3, C7, complement factor B and MMP-3, MMP-10, and MMP-12 (Woollard et al., 2014). These findings suggest that Tat

proteins from these subtypes may differentially affect molecular and cellular functioning in hBMVECs and may affect BBB dysfunction. Tat may also alter the expression of key proteins which maintain the integrity of the BBB (i.e. tight junctions).

Tat alters the expression of tight junction proteins, with reductions in zona occuldens (ZO)-1, ZO-2, occludin, claudin, and increases in junctional adhesion molecule (JAM)-2 (Banerjee et al., 2010; McRae, 2016). In hBMVECs, redox-sensitive kinase (PYK2) activation leads to increased tyrosine phosphorylation of VE-cadherin and β-catenin, which results in a disruption of junctional assembly of hBMVECs, and ultimately increased endothelial permeability (Mishra and Singh, 2014). Tat-C induces disruption of VE-cadherin mediated by miRNA- 101 in hBMVECs (Mishra and Singh, 2013). Furthermore, Tat-B can produce an aggravated disruption of BBB integrity and greater altered expression of tight junction, ZO-1 and JAM-2, compared to Tat-C (Gandhi et al., 2010). These may explain the reason for a greater neurovirulence of Tat-B compared to Tat-C (Mishra et al., 2008).

Tat-B may also influence the functionality of important drug transporters such as P-glycoprotein (P-gp) and multi-drug resistance protein (MRP)-1, which are present on endothelial cells. Efflux function, expression and promoter activity of P-gp and MRP-1 is upregulated by Tat-B (Hayashi et al., 2006, 2005; Zhong et al., 2010). However, it is not clear as to how subtype variation and particularly Tat sequence variation may affect P-gp and MRP-1. This should be investigated in future studies as this may provide insight into subtype-specific treatment strategies.

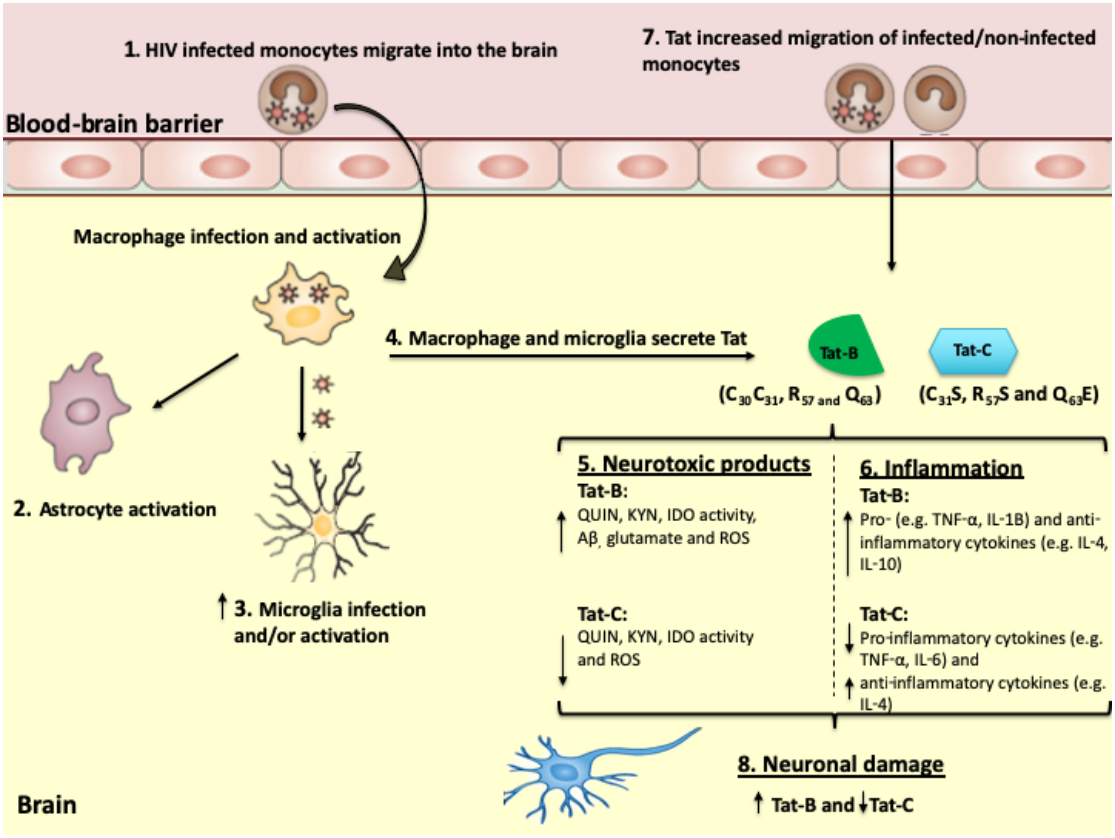

Figure 2: Differential effects of Tat-B and Tat-C in the neuropathophysiology of HAND. (1) Infected monocytes are able to cross the BBB which later differentiate into macrophages. (2-3) Infected macrophages within the brain can infect and activate microglia and further activate astrocytes. (4) These infected macrophages release viral Tat proteins that may directly further activate neuroimmune cells including microglia and astrocytes. (5) Tat-B induces a higher level of oxidative stress, KP metabolites, Aβ and glutamate which essentially affects neuronal integrity. (6) Tat-B induces a higher inflammatory response compared to Tat-C. (7) Tat-B may be responsible for higher CCL2 levels, greater BBB damage and essentially higher monocyte transmigration across the BBB. This was largely found to be attributed to the dicysteine motif present in Tat-B. (8) In combination, Tat-B may exert its neurotoxic effects to a greater degree than Tat-C and subsequently a greater level of neuronal damage.

Table 1: Amino acid signatures associated with differential effects of HIV-1 Tat-B and Tat-C proteins

Subtype/ sequence	Effect	Reference	Subtype/ sequence	Effect	Reference
1. Transcriptional capacity					
Tat-B	Lower transactivation capacity	(Bachu et al., 2012; de Arellano et al., 2010; Desfosses et al., 2005)	Tat-C	Higher transactivation capacity	(Bachu et al., 2012; de Arellano et al., 2010; Desfosses et al., 2005)
Tat-B $C_{30}C_{31}$	Not critical for transactivation	(Ranga et al., 2004)	Tat-C $C_{30}S$	No significant effect on the transactivation capacity	Ranga et al. 2004)
Tat-B Q_{63}	Lower transactivation capacity	(Kurosu et al., 2002)	Tat-C $Q_{63}E$	Higher transactivation capacity	(Kurosu et al., 2002)
2. Neuronal damage					
Tat-B	Higher glutamate levels in treated neuroblastoma cells	(Samikkannu et al., 2015)	Tat-C	Higher glutamine levels in treated neuroblastoma cells	(Samikkannu et al., 2015)
	Upregulation of (69/84) synaptic plasticity genes in treated neuroblastoma cells	(Samikkannu et al., 2015)		Upregulation of (46/84) synaptic plasticity genes in treated neuroblastoma cells	(Samikkannu et al., 2015)
Tat-B $C_{30}C_{31}$	Higher neurotoxicity to human neuronal cells	(Li et al., 2008; Rao et al., 2013)	Tat-C $C_{30}S$	Lower neurotoxicity to human neuronal cells	(Rao et al., 2013)
	Higher activation of the NMDA	(Li et al., 2008)		Does not affect NMDAR binding but attenuates NMDA activation and neurotoxicity in HEK-NMDAR cells	(Li et al., 2008)
	Higher activation of L-type channels	(Campbell et al., 2007; Wong et al., 2010)		Lower activation of L-type channels	(Campbell et al., 2007; Wong et al., 2010)
	Greater depolarization of mitochondrial membranes in neurons	(Mishra et al., 2008).		Little or no depolarization of mitochondrial membranes in neurons	(Mishra et al., 2008).
	Critical for synaptodendritic injury in hippocampal neuronal cells	(Bertrand et al., 2013)		No significant synaptodendritic injury in hippocampal neuronal cells	(Bertrand et al., 2013)

	Higher IDO mRNA expression in monocytes	(Campbell et al., 2007)		Less effective at upregulating IDO mRNA expression in monocytes	(Campbell et al., 2007)
Tat-B R_{57}	Higher inflammatory-induced dysfunction in human neuron-astrocyte cultures	(Ruiz et al., 2019)	Tat-C $R_{57}S$	Lower inflammatory-induced dysfunction in human neuron-astrocyte cultures	(Ruiz et al., 2019)
3. Monocyte activation and recruitment					
Tat-B $C_{30}C_{31}$	Higher monocyte chemotaxis	(Ranga et al., 2004; Rao et al., 2013, 2008)	Tat-C $C_{30}S$	Defective for monocyte chemotaxis	(Ranga et al., 2004)
4. (Neuro)inflammation: Cytokines and Chemokines					
Tat-B	Higher inflammation (IL-6, TNF-α) in monocytes	(Gandhi et al., 2009)	Tat-C	Lower inflammation (IL-6, TNF-α) in monocytes	(Gandhi et al., 2009)
Tat-B $C_{30}C_{31}$	Higher inflammation (TNF- α, IL-6, IL-10 and CCL2) in monocytes	(Campbell et al., 2007; Wong et al., 2010)	Tat-C $C_{30}S$	Does not induce a calcium influx which results in lower levels of IL-10 in monocytes	(Wong et al., 2010)
				Less effective in upregulating markers TNF- α, IL-6 and CCL2 in monocytes	(Campbell et al., 2007)
				Higher levels of IL-4 in monocytes	(Gandhi et al., 2009)
Tat-B R_{57}	Higher expression of inflammatory gene transcripts (IL-6, IL-8, IL-1β, and CXCL1)	(Ruiz et al., 2019)	Tat-C $R_{57}S$	Reduced uptake of Tat by bystander cells and subsequently reduced neuroinflammation (IL-6, IL-8, IL-1β, and CXCL1)	(Ruiz et al., 2019)
	Stimulate TNF-α production in treated cells	(Ruiz et al., 2019)		Unable to stimulate TNF-α production in treated cells	(Ruiz et al., 2019)
5. Tat and amyloid-beta production					
Tat-B	Higher induction of $Aβ_{1-42}$ production in hippocampal neurons	(Aksenov et al., 2010)	Tat-C	Less effective in inducing $Aβ_{1-42}$ production in hippocampal neurons	(Aksenov et al., 2010)
Tat-B $C_{30}C_{31}$	Higher binding to the neprilysin active site	(Daily et al., 2006; Rempel and Pulliam, 2005)	Tat-C $C_{30}S$	Tat lacking the dicysteine motif fail in binding neprilysin	(Daily et al., 2006; Rempel and Pulliam, 2005)

6. Blood-brain barrier damage					
Tat-B	Higher ROS production in MC-derived IDC and SK-N-MC	(Samikkannu et al., 2014)	Tat-C	Less effective in upregulating ROS in IDC and SK-N-MC	(Samikkannu et al., 2014)
	Higher expression of genes related to pro-inflammatory chemokines, complement components C3, C7, complement factor B and MMP-3, MMP-10, and MMP-12 in hBMVECs	(Woollard et al., 2014)		Less effective in upregulating genes of BBB integrity in hBMVECs	(Woollard et al., 2014)
	Altered expression of tight junction, ZO-1 and JAM-2 in hBMVECs	(Gandhi et al., 2010)		Less effective in altering the expression of tight junction, ZO-1, and JAM-2	(Gandhi et al., 2010)

Where Tat-B/Tat-C are indicated, these are studies that did not include sequence data and only reported the effect of Tat-B/Tat-C on underlying mechanisms

5.9. Key observations

The HIV Tat protein is a major viral determinant in the development of HAND. Tat-B and Tat-C differ biologically, and this affects the neuropathophysiology and clinical outcomes of HAND. The extent to which Tat sequence variation affects neurovirulence and neurological outcomes requires further investigation. This is particularly evident with pre-clinical studies reporting contradictory findings to that reported in clinical studies.

Firstly, it is noted that this area of research remains scarcely investigated with a limited number of clinical studies being investigated within the last ten years. The current knowledge of Tat-mediated neuropathogenesis is largely derived from Tat-B/HIV-1B, with a limited amount of studies investigating Tat-C. The lack of clinical studies in this area underscores the need for further research. Therefore, future investigations into Tat-C and the effect of Tat sequence variation may further explain the effect of Tat on clinical outcomes. Due to the limited number of studies investigating Tat-B and Tat-C variation, for this review, we have also included studies on the variation of HIV-1B and HIV-1C and their contribution to neurological outcomes. However, it should be noted that neurological outcomes from these studies cannot be solely attributed to the Tat protein, as several other viral proteins differ by subtype and subsequently may have also affected neurological outcomes (Samikkannu et al., 2011).

Secondly, several studies did not report the HIV Tat variant and HIV-1 subtype in their relevant investigations. It is assumed that Tat-B is the universal strain for the neuropathogenesis of HAND. Future studies should specify the Tat variant and HIV-1 subtype investigated, as many of the neuropathological effects are influenced by specific residues within the Tat protein and not specifically by the HIV-1 subtype.

Thirdly, this review identified several key protein sequence differences which may account for the altered neuropathogenesis. This includes a Serine substitution at residue 31 ($C_{31}S$), a Serine substitution at residue 57 ($R_{57}S$) and a Glutamate substitution at residue 63 ($Q_{63}E$). These polymorphisms present in Tat-C accounts for the more potent transactivation capacity and a lower level of monocyte recruitment, neuroinflammation, and neuronal damage. Future studies should aim in investigating these signatures across larger cohorts to support its relevant role in HAND. Furthermore, key questions remain with as to how these cross-subtype polymorphisms contribute to 1) monocyte activation 2) monocyte recruitment 3) neuroinflammation and 4) BBB dysfunction.

Fourthly, amino acid sequences of other viral proteins (i.e. gp120) differ between HIV-subtypes (Gnanakaran et al., 2007; Rao et al., 2014). Future studies should sequence additional viral proteins (gp120, Nef, Vpr, and p24) and determine to which extent protein signatures from these proteins are involved in neuropathological processes of HAND. An understanding of the differences in the pathogenesis of these subtypes and protein signatures may provide insight into the

development of HAND and potential avenues for improved therapeutic development.

5.10. Conclusions

HIV-1B and HIV-1C present with several key biological differences which may affect the development of HAND. The extent to which Tat sequence variation contributes to underlying mechanisms and neurological outcomes remain unclear. Here we reviewed and synthesized the current literature in the aim of elucidating the effect of Tat variation in the neuropathogenesis of HAND. Despite limited studies and the lack of Tat sequence data, we report from fundamental research studies, key signatures that may be involved in the underlying mechanisms of HAND. These include sequence polymorphisms $C_{31}S$, $R_{57}S$ and $Q_{63}E$ present in Tat-C. Based on these polymorphisms, studies collectively indicated that Tat-C may be a more effective transactivator. Tat-B may have a greater effect on neuronal damage by neuronal pathways, increasing monocyte recruitment, (neuro)inflammation, oxidative stress and BBB dysfunction. This review highlights important protein signatures that may be crucial in the underlying mechanism for HAND.

Based on the findings from this prospective review, in the next chapter, I report data for the influence of Tat sequence variation on inflammatory levels. In particular, how Tat sequence variation at positions 31 and 57 may influence immune markers in HIV-1C infection.

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Chapter 6

Impact of the HIV Tat $C_{31}S$ and $R_{57}S$ mutations on peripheral immune marker levels in subtype-C infection: An exploratory study

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Abstract

The HIV transactivator of transcription (Tat) protein is an important viral protein and has a functional role in the neuropathophysiology of HIV-associated neurocognitive disorders (HAND). Previous pre-clinical studies have identified the $C_{30}S$ and $R_{57}S$ mutations of Tat as major contributors to the reduced immune activity and neurovirulence in HIV-1 subtype-C (HIV-1C) infection. However, the number of available studies investigating the association between immune marker levels and Tat sequence variation in HIV-1C infection remains limited. This exploratory study investigated the association between several peripheral immune markers and Tat sequence variation. Peripheral Immune markers were selected on putative evidence of their link to key neuro-immune function. Immune markers were measured using enzyme-linked immunosorbent assays (ELISA). Thirty-six HIV-1 subtype-C infected participants were included with all participants investigated for the $C_{31}/C_{31}S$ mutation and thirty-five participants investigated for the $R_{57}/R_{57}S$ mutation. Peripheral immune marker levels did not differ across the $C_{31}S$ and C_{31} groups. Peripheral thymidine phosphorylase levels (TYMP) were lower in the $R_{57}S$ group than in the R_{57} group (p = .05). Although this study did not have sufficient power to rule out false negatives, this exploratory study provides evidence that the Tat sequence variant which is largely present in Tat-C ($R_{57}S$) may account for the lower TYMP levels in HIV-1C infection. Future studies should investigate these signatures across larger cohorts to support the findings presented here.

6.1. Introduction

HIV-associated neurocognitive disorder (HAND) is a common feature of HIV-1 infection of the central nervous system (CNS). The neuropathogenesis of HAND is multidimensional and is affected by several factors including the HIV-1 subtype (Rao et al., 2013, 2008). HIV-1 is characterized by genotypical variation which is distributed across different geographical regions. HIV-1 subtype-C (HIV-1C) is responsible for the highest prevalence of HIV infection and is geographically associated with Southern Africa and India (Geretti, 2006). HIV-1 subtype-B (HIV-1B) is related to a higher reported prevalence of HAND and is the dominant subtype in areas of America and Europe (Taylor et al., 2008).

A major viral determinant of neurological outcomes in HIV infection is attributed to the HIV Transcription of transactivation (Tat) protein (Gandhi et al., 2009; Rao et al., 2013). HIV-1 Infected monocytes are able to migrate across the blood-brain barrier (BBB), and subsequently infect and activate cells of the CNS (González-Scarano and Martín-García, 2005). This leads to the secretion of the HIV Tat protein from microglia and macrophages. Amongst other mechanisms, Tat can stimulate the release of inflammatory markers (Chen et al., 2017). The chronic release of inflammatory markers affects neuronal health. Previous studies have shown associations between an aberrant immune regulation and HAND (Cohen et al., 2011; Falasca et al., 2017). Further, HIV-1 subtype/Tat sequence variation may directly influence the levels of inflammatory markers (Gandhi et al., 2009).

Recent studies have found that Tat protein sequence mutations at position 31 (Campbell et al., 2007; Rao et al., 2013; Ruiz et al., 2019; Wong et al., 2010) and 57 (Ruiz et al., 2019) had an association with the levels of inflammation, neurovirulence, and neurocognitive performance (Williams et al., 2020). HIV-1C and HIV-1B differ in their Tat sequence. Tat-B generally has a Cysteine at position 31 (C_{31}) and an Arginine at position 57 (R_{57}), whereas Tat-C generally has a Serine mutation at position 31 (S_{31}) [prevalence of 74%-99% (Rao et al., 2008)] and 57 (S_{57}) [prevalence of 82% (Ruiz et al., 2019)]. Therefore, Tat-C largely presents with the important mutations $C_{31}S$ and $R_{57}S$.

It is not clear to what extent Tat protein sequence variation may influence inflammatory levels in HIV-1C infection. Further, no study has directly examined immune marker levels among individuals based on the presence or absence of the $C_{31}S$ and $R_{57}S$ variant in individuals with HIV-1C infection. Therefore, this study aimed to determine the effect of Tat sequence variation ($C_{31}S$ and $R_{57}S$) on selected peripheral immune marker levels in HIV-1C infection.

6.2. Methods

6.2.1. Study Participants

This study included a sub-sample of a previously published dataset (Joska et al., 2011). All participants with genotyping data from the primary study (Joska et al., 2011) were included in this study. A total of 36 HIV-1C infected participants were

included in this study. All participants had measures for the $C_{31}/C_{31}S$ mutation, and 35 participants had measures for the $R_{57}/R_{57}S$ mutation. One participant had a $R_{57}K$ mutation and was therefore excluded from the study.

People living with HIV (PLWH) were previously recruited from primary health care clinics in Cape Town and the Western Cape region of South Africa. PLWH completed at least one study visit, which included a detailed sociodemographic, medical and neuropsychological assessment as well as the relevant laboratory measures (including genotyping, viral load and CD4 count). HIV serostatus was confirmed by two independent rapid tests and confirmed via ELISA analysis. Participants included in this study ranged from 18 through 65 years in age. PLWH were treatment naïve or had only recently started cART (< 1 month) before blood collection. This was to investigate the levels of Tat without the potential confound of A Cases were excluded from this study if they had 1) severe psychiatric disorder or presented any other neurological disorder, 2) substance abuse (other than alcohol) and 3) moderate to severe head injury. Written informed consent was obtained following a thorough explanation of the study procedures and individuals received financial compensation for their time. This study was approved by the Human Research Ethics Committee of the Faculty of Health Sciences (University of Cape Town) (Sub-study HREC 213/2018 linked to primary studies: 003/2015, 023/2008 and 263/2007).

6.2.2. Laboratory Assessment of Blood

Blood samples were prepared as described in a previous study (Chapter 3) (Williams et al., 2019). All immune markers were measured using Enzyme-linked Immunosorbent Assays (ELISA) (R&D systems, DuoSet ELISA) according to the manufacturer's instructions. For this study, the immune markers matrix metalloproteinase (MMP)9, neutrophil gelatinase-associated lipocalin (NGAL), transforming growth factor (TGF)-β1 and thymidine phosphorylase (TYMP) were selected for investigation. These markers were selected for investigation based on their 1) positive outcomes in the primary study (Williams et al., 2019) as well as their 2) potential involvement in the pathophysiology of Tat-mediated neuropathogenesis and HAND, as reviewed from the scientific literature (Choi et al., 2011; Dhar et al., 2006; Kumar et al., 2012; Wu et al., 2013; Xing et al., 2017). TYMP was measured in serum. MMP9, NGAL, and TGF-β1 were measured in plasma. Samples were diluted as follows MMP9: 1:150, NGAL: 1:100, TGF-β1: 1:30 and TYMP: 1:10. All samples were assayed in duplicate. The intra- and inter-assay coefficients of variation for all tests were within acceptable ranges of < 8% and < 10% respectively.

6.2.3. HIV-subtyping

Tat exon 1 region (HXB2 position 5831–6045) was amplified by polymerase chain reaction (PCR) using the Promega GoTaq Flexi Kit (Promega, Madison, WI). The primer pair Tat-1_OF (5'-AAAGCCACCTYTGCCTAG) / Tat-1_OR (5'-CTCATTGCCACTGTCTTCTGC), and Tat-1_IF (5'-GTAGARGATMGATGGAACRA) / Tat-

1_IR (5'-CYCTAATTCTTTYAAYTAACC) were used for pre-nested and nested PCR, respectively. Amplification conditions were held at 94°C for 2 min, followed by 40 cycles of denaturing (94°C; 30s), annealing (55°C; 30s), extension (68°C; 1 min) and a final extension step for 10 min at 68°C. The PCR product was stored at 4 °C until visualized by agarose gel electrophoresis

Purification of PCR products was performed with the Nucleospin® Gel and PCR clean-up kit, according to manufacturer instructions (Machery-Nagel GmbH & Co.KG, Germany). All PCR products were sequenced by BigDye Terminator v.3.1 Cycle Sequencing Ready Reaction Kit (ThermoFisher Scientific) and analysed on the ABI Prism 3130xl automated DNA sequencer (Applied Biosystems, Foster City, CA). Sequences were analysed and the overlapping DNA fragments were assembled using Sequencer version 5.2.4 (Gene Codes Corporation, Ann Arbor, MI). Nucleotide sequences were translated into amino acid sequences and the $C_{30}C_{31}$ motif or $C_{31}S$ mutation and R_{57} or $R_{57}S$ mutation was recorded. The Tat exon 1 subtype was determined using online subtyping tools Jumping profile of Hidden Marko Model (jphmm.gobics.de) and REGA HIV-1 (www.bioafrica.net/subtypetool/html/subtyinghiv.html)

6.2.4. Statistical analysis

All analyses were conducted using SPSS (version 25, IBM, USA). *P*-values were considered statistically significant for all analyses at a value of less than .05. Data distribution for markers NGAL and TGF- β1 were found to be skewed, therefore the

data was log-transformed prior to statistical analyses. MMP9 and TYMP demonstrated normal data distribution with acceptable skewness and kurtosis. Pearson's chi-squared tests were used to test group differences for sex between Tat variants. The Wilcoxon signed-rank test was used to determine differences in the study characteristics and levels of plasma/serum markers between Tat variants C_{31} *versus* $C_{31}S$ and R_{57} *versus* $R_{57}S$ respectively.

6.3. Results

6.3.1. Sample characteristics

The pilot study population included a total of 36 PLWH, which included 36 participants investigated for the $C_{31}/C_{31}S$ mutation and 35 participants investigated for the $R_{57}/R_{57}S$ mutation. For the $C_{31}/C_{31}S$ mutations, 28 participants had the $C_{31}S$ and 8 had the C_{31}. For the $R_{57}/R_{57}S$ mutations, 31 participants had the $R_{57}S$ and 4 had the R_{57}. A full sample description is provided in Table 1.

Table 1: Characteristics of PLWH

	31 (n=36)		*p*-value	57 (n=35)		*p*-value
Amino Acid	C	S		R	S	
n	8 (22.2%)	28 (77.8%)		4 (11.4%)	31 (88.6%)	
Sex (male) n, %	8 (100%)	24 (85.7%)	.26	4 (100 %)	27 (87.0%)	.44
Age, mean (SD)	30.25 (5.52)	30.29 (4.91)	.99	329.50 (4.20)	30.55 (4.91)	.72
Viral load Log copies/mL, median (IQR)	3.41 (3.11 - 4.56)	4.09 (3.13 - 4.69)	.61	4.05 (3.20 - 4.76)	4.04 (3.02 - 4.51)	.64
CD4+ Nadir, median (IQR)	334.5 (236 - 520)	222.5 (129.25 - 384.5)	.11	337.5 (237 - 497.75)	222.5 (148 - 381.5)	.58

Abbreviations: Interquartile range (IQR), Standard deviation (SD)

6.3.2. Peripheral Immune marker levels and Tat C_{31}/C_{31}S variation

C_{31}/C_{31}S data were available for 36 participants. Marker levels were similar between all C_{31}/C_{31}S variations and no significant differences were found. The largest difference in immune marker levels was found for MMP9 (p = .26). The C_{31}S group reported lower MMP9 levels, however, this was insignificant (p = .26). NGAL, TYMP and TGF-β1 levels were also insignificant between C_{31}/C_{31}S variations (p > .05) (Figure 1).

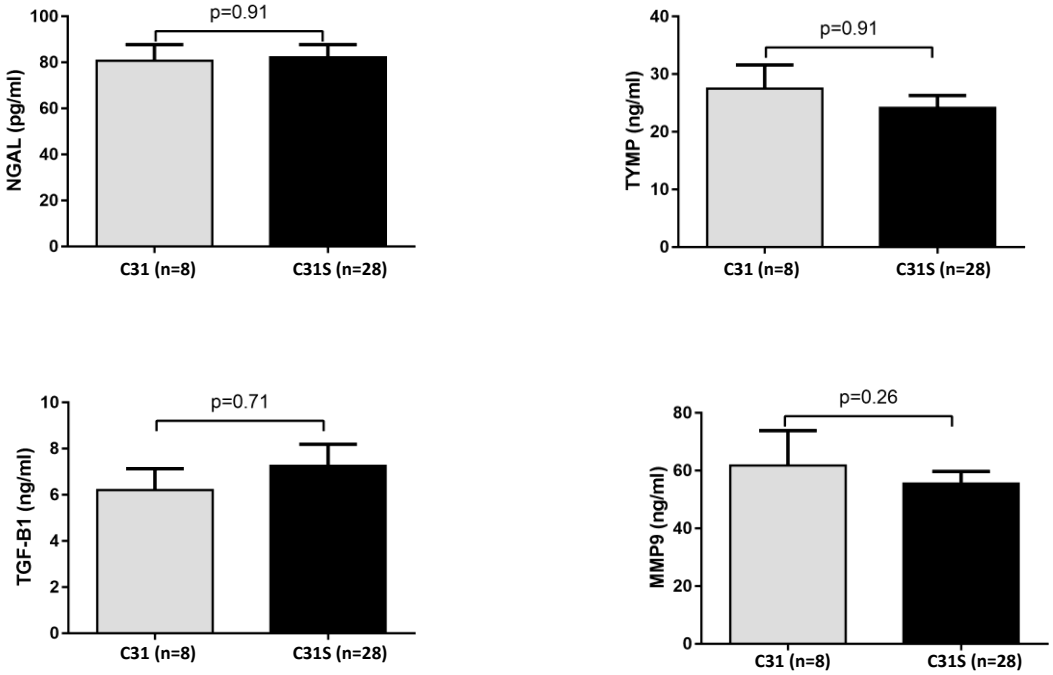

Figure 1: Differences in peripheral immune marker levels between Tat-C C_{31} and $C_{31}S$. The bars indicate mean protein concentrations in the different study groups and are expressed as mean±standard error of the mean (SEM).

6.3.3. Peripheral Immune marker levels and Tat R_{57}/$R_{57}S$ variation

R_{57}/$R_{57}S$ data were available for 35 PLWH. The $R_{57}S$ group reported lower TYMP levels in PLWH (p = .05). NGAL, TGF-β1 and MMP9 levels were insignificant between R_{57}/$R_{57}S$ variations (p > .05) (Figure 2).

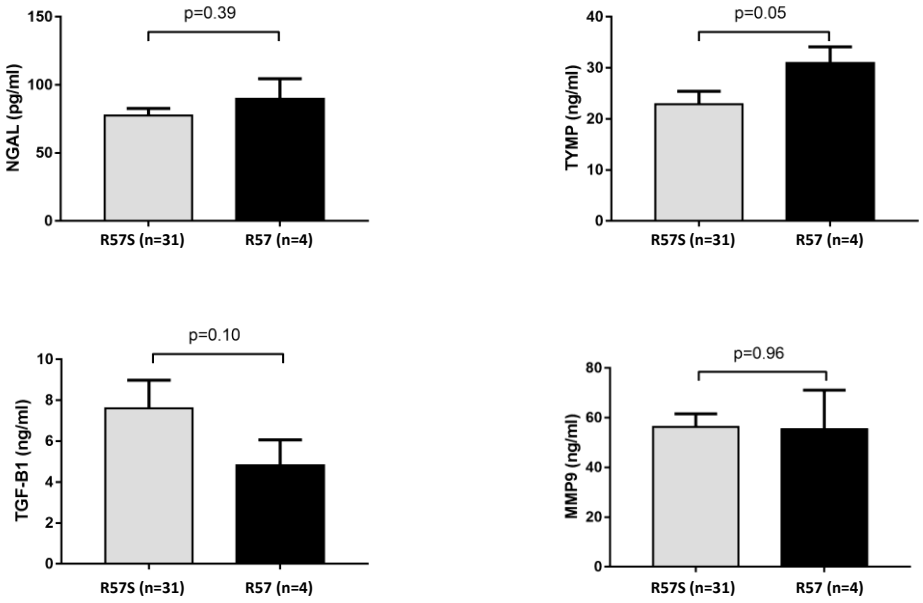

Figure 2: Differences in peripheral immune marker levels between Tat-C R_{57} and $R_{57}S$. The bars indicate mean protein concentrations in the different study groups and are expressed as mean±standard error of the mean (SEM).

6.4. Discussion

The main findings from this pilot study were that 1) there were no significant differences in immune marker levels between $C_{31}S$ and C_{31} and 2) lower TYMP levels were found in $R_{57}S$ compared to R_{57} (p = .05).

The $C_{31}S$ Tat mutation which is largely found in HIV-1C infection (present in 74%-99% of HIV-1C infections) is arguably the most widely studied in Tat-mediated neuropathogenesis (Campbell et al., 2007; Li et al., 2008; Ranga et al., 2004; Rao et al., 2013). Here, it is seen that there were no significant differences in immune marker levels between $C_{31}S$ and C_{31}. Previously, pre-clinical studies have shown that monocyte cultures treated with Tat $C_{31}S$ resulted in lower levels of TNF-α, IL-6, IL-10

and MCP-1 compared to Tat $C_{30}C_{31}$ (Campbell et al., 2007; Wong et al., 2010). The limited number of study participants in the current study may explain the lack of statistical power for comparisons between $C_{31}S$ and C_{31}. We have also investigated different immune markers compared to previous studies, and this, too, could explain the differences in findings.

Recently, another important mutation ($R_{57}S$) present in Tat-C has been suggested to influence immune marker levels and neurovirulence (Ruiz et al., 2019). The current study reported lower TYMP levels for $R_{57}S$ compared to R_{57} (p = .05). These findings are in line with a recent pre-clinical study which suggested that the $R_{57}S$ mutation (largely found in Tat-C) may be responsible for lower immune marker levels (Ruiz et al., 2019). Further, in a previous study done in our group (Williams et al., 2019), TYMP was significantly increased in South African PLWH (HIV-1C). The R_{57} is largely present in Tat-B and it is possible that this marker may be higher in HIV-1B infection compared to HIV-1C infection (Williams et al., 2019). Further, TYMP was associated with worse psychomotor processing speed in HIV-1C infection (Williams et al., 2019). However, the exact mechanism for TYMP is not yet known. TYMP gene expression is increased in primary monocytes in PLWH (Chapouly et al., 2015) and TYMP levels in the brain may be further increased by the presence of HIV (Kumar et al., 2012). Moreover, It was proposed that TYMP has a functional role in BBB dysfunction (Chapouly et al., 2015). TYMP should be investigated for its potential role in a Tat-mediated pathway of HAND.

This study is limited by a sample size that is insufficiently large to exclude false-negative findings. However, this exploratory study does provide evidence for the association between Tat variation and clinical immune marker levels. Future studies should investigate these sequence variants in larger cohorts as well as in HIV-1B cohorts to elucidate the potential underlying mechanisms of Tat-mediated neuropathogenesis.

6.5. Conclusions

Despite the limited number of participants, this pilot study provides evidence for the relationship between the $R_{57}S$ variant and lower peripheral TYMP levels in HIV-1C infection. Future studies should investigate these Tat sequence variations in larger cohorts. The next final chapter summarizes and provides overall conclusions for all of the findings of my investigations.

6.6. References

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Chapter 7

Summary and conclusions

7.1. Summary of findings

In this thesis, I investigated the associations of peripheral inflammation (immune markers) with brain structural alteration and neurocognitive impairment. Further, I investigated the significance of Tat protein sequence variation on immune marker levels. These research questions were addressed in five manuscripts. The key findings of the thesis were as follows:

1) Based on a systematic search of the current literature, it was concluded that PLWH have altered immune marker levels, including elevated markers of monocyte activation (neopterin, sCD14, sCD163) and inflammation (CCL2, IL-8, IL-18, IP-10, IFN-α, sTNFR-II and TNF-α). These elevated levels persist in PLWH despite viral suppression. Further, the majority of studies found associations of HAND with immune markers, including those linked to monocyte activation (sCD14 and sCD163) and inflammation (IL-18 and IP-10) (Chapter 2-manuscript 1)
2) In an exploratory investigation in a South African cohort of 99 PLWH and 51 HIV-negative participants, TYMP and NGAL levels were significantly higher, while MMP9 levels were significantly lower in PLWH compared to HIV-negative controls. Further, my results showed that in PLWH, worse psychomotor processing speed was associated with higher TYMP and NGAL levels and worse motor function was associated with higher NGAL levels in PLWH. This is the first study to my knowledge to investigate the associations of TYMP and NGAL with neurocognitive performance PLWH (Chapter 3-manuscript 2).

3) In a subset of South African PLWH (Chapter 3), I examined the associations of peripheral immune markers with cortical thickness/surface area in 65 PLWH and 26 HIV-negative controls. Results indicate that higher NGAL levels were associated with the reduced thickness of the lateral orbitofrontal cortex. The association of NGAL with worse motor function was mediated by the cortical thickness of the lateral orbitofrontal cortex (Chapter 4-manuscript 3).
4) In a prospective review, I summarized the differences between Tat-B and Tat-C to elucidate the effect of Tat sequence variation on the underlying neuropathophysiology of HAND. Key sequence signatures $C_{31}S$, $R_{57}S$ and $Q_{63}E$ are largely present in Tat-C. With these protein signatures, Tat-C is considered to be a more effective transactivator, whereas Tat-B may exert increased neurovirulence, including neuronal apoptosis, monocyte infiltration into the brain, (neuro)inflammation, oxidative stress and blood-brain barrier damage. (Chapter 5-manuscript 4)
5) In an exploratory study, I investigated the association between peripheral immune markers and Tat sequence variants $C_{30}S$ and $R_{57}S$ in HIV-1C infection. Peripheral immune marker levels did not differ across the $C_{31}S$ and C_{31} groups. However, peripheral thymidine phosphorylase levels (TYMP) were lower in the $R_{57}S$ group than in the R_{57} group (Chapter 6-manuscript 5).

7.2. Final recommendations

The overall conclusion from this thesis supports the premise that inflammation contributes to the development of HAND and that Tat sequence variation may

influence inflammatory levels in PLWH. The major conclusions from this thesis can be divided into three major components.

Firstly, from a systematic search of the existing literature, I found that peripheral inflammation persists despite the introduction and successful use of ART. This suggests that other strategies besides simply suppressing the viral load, are necessary to address the ongoing peripheral inflammation in PLWH. Long-term treatment studies suggest that there is an ongoing immune activation in PLWH, and this may explain the persistent development of milder forms of HAND in the ART era. It is also noted that heterogeneity is evident in the current studies investigating the association of general peripheral immune markers and HAND. Therefore, greater uniformity in all studies investigating general markers and cognitive performance will allow for improved comparability of findings into the future. HIV Tat sequence variation may also be a contributor to the heterogeneity of inflammatory marker levels and cognitive impairments in the general HIV population.

Secondly, in an evaluation of blood immune markers in a South African treatment naïve cohort, two immune markers; TYMP and NGAL may be promising targets for future investigation in samples of treatment-experienced participants. To my best knowledge, this study is the first to investigate the associations between TYMP and NGAL with HIV-associated neurocognitive impairments. This thesis reported the association of these markers with domain-based HIV-associated neurocognitive impairments as well as alteration of the frontal cortex in South African PLWH. Further, since inflammation is reportedly lower in HIV-1C infection, these markers

may possibly have a greater functional role in HIV-1B infection. Future studies should investigate the role of these markers in the underlying mechanisms of HAND. With this investigation done in treatment-naïve participants, findings of these markers may represent HIV-related effects only and not findings which may be confounded by the use of ART. The effect of inflammation on brain structures and HIV-associated neurocognitive impairment may be independent of ART, and we hypothesize that findings presented in the current study may similarly be reflected in investigation of treatment-experienced participants. However, further investigation is warranted. Therefore, these markers may be important in HIV-1C infection and should be investigated in 1) larger cohorts, 2) HIV-1B infected and 3) treatment-experienced participants.

Thirdly, in a review of the existing literature, it was shown that Tat sequence variation influences the neurovirulence of HIV-1. Two sequence variants ($C_{31}S$ and $R_{57}S$) in particular, were implicated in the differential effects of neuronal apoptosis, monocyte recruitment and BBB damage. The more recent $R_{57}S$ Tat variant was considered important in neuroinflammation. Similarly, in the investigated cohort, lower TYMP levels were found in the $R_{57}S$ group than in the R_{57} group. Despite the limited sample size, I reported evidence that Tat sequence variation may influence inflammatory levels. These signatures may also influence other immune markers not investigated in my pilot study. This may be a plausibility for the different immune marker levels in HIV-1B and HIV-1C infection. These signatures need to be investigated across larger cohorts as well as in HIV-1B infection. Studies of HIV-1C in general, as well as comparative studies between HIV-1C and HIV-1B, remains

scarcely investigated, with limited studies within the last ten years. The lack of studies in this area underscores the need for further research. Amino acid sequences of other viral proteins (i.e. gp120) also differ between HIV-subtypes (Gnanakaran et al., 2007; Rao et al., 2014). Future studies should sequence additional viral proteins (gp120, Nef, Vpr, and p24) and determine to which extent protein signatures from these proteins are involved in neuropathological processes (immune responses) of HAND. An understanding of the differences in the pathogenesis of these subtypes and protein signatures may provide insights into the development of HAND and potential avenues for improved therapeutic development.

7.3. Limitations

This study has several limitations which deserve emphasis. Firstly, in a systematic review of the literature, heterogeneity of the included studies was evident, which made the interpretation of findings much more challenging. A meta-analysis was not conducted due to the heterogeneity of the studies pooled for analysis (Chapter 2).

Secondly, a limitation of the empirical chapters (Chapters 3, 4 and 6) were the limited sample sizes. Therefore, findings are to be replicated in larger prospective and ART-experienced cohorts. A larger sample size is needed to understand the association between immune markers and neurocognitive performance, brain imaging and Tat sequence variation.

Thirdly, a subset of the included participants were heavy drinkers which may have influenced the results. It has been shown that heavy drinking may affect neurocognitive performance, immune system (Meyerhoff, 2001) and brain cortical structure (Morris et al., 2019). However, I adjusted for various covariates in statistical analysis. It was also reported that there were no significant differences in 1) immune marker profiles and 2) neurocognitive performance for the HIV-positive non/light drinkers *vs*. HIV-positive heavy drinkers.

Fourthly, a small subset of participants started ART (<30 days) before blood collection, neurocognitive testing and neuroimaging. ART is also a contributor to dysregulated inflammatory levels in PLWH (Shah et al., 2016). However, long-term treatment studies reported that in most cases, the first neuro-immune responses are noted at durations greater than 3 months (Hattab et al., 2014; Krebs et al., 2016; Richert et al., 2017).

Finally, findings presented here may not apply to other HIV-1 subtypes (e.g. HIV-1B), as inflammatory markers may be affected by the HIV subtype (Gandhi et al., 2009). Since inflammation is reportedly higher in HIV-1B infection, the peripheral immune markers identified in this study may be higher in HIV-1B infected participants.

7.4. Conclusion

The key findings from this thesis indicate that peripheral monocyte activation and inflammation plays a key role in HAND despite viral suppression, as shown in a

systematic search of the literature. Further, in a South African context which is primarily HIV-1C infected, TYMP and NGAL may be important markers in the neuropathophysiology of HAND. Finally, Tat protein sequence variation may influence certain immune marker levels and subsequently the phenotype of HAND. Taken together, the findings presented here further the understanding of the associations of inflammation with HAND, particularly in the South African context where HIV-1C predominates. Future studies should investigate these identified immune markers and Tat protein signatures across larger and various HIV-1 subtype cohorts.

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