

**The influence of HIV-1 viral protein R sequence
variation on immune activation, inflammation,
and metabolism: implications for neurocognitive
impairment**

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the degree *Master of Science in Biochemistry* at the North-West
University

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DECLARATION

I, Levanco Keegan Asia, do hereby declare that this dissertation is based on three journal manuscripts: one of which has been published (Chapter 2) and two of which are under review (Chapter 3 and 4). Building on the research outlined in Chapter 3, a portion of this work was showcased at the International Congress of Immunology in 2023. These manuscripts have been formatted for the purpose of this dissertation regarding the referencing, style, coherence, and the use of terms. Although the content of each manuscript is unchanged from that which has been published and/or submitted to journals for publication; the conclusions of each manuscript have been edited for the coherence of the entire dissertation i.e., how each chapter connects to the next/other chapters. Where co-authorships are involved, co-authors have given permission to include the publication(s) and manuscripts that have been submitted to journals for publication. Below is a list of the manuscripts with a description of my contribution to each.

Chapter 2

Asia LK, Jansen Van Vuren E, Williams ME. The influence of viral protein R amino acid substitutions on clinical outcomes in people living with HIV: A systematic review. Eur J Clin Invest. 2023 May;53(5):e13943. doi: 10.1111/eci.13943. Epub 2023 Jan 10. PMID: 36579370.

This systematic review compiled and summarized clinical research on the association of HIV Vpr amino acid substitutions with clinical outcomes. This manuscript indicated the inconsistencies in the literature and highlighted the need to investigate HIV Vpr amino acid substitutions in clinical studies. Dr Monray Williams and I conceptualized the study, generated the first draft of the paper, and conducted all database scans and selected the articles for inclusion. All tables and figures were generated by me. Dr Esmé Jansen van Vuren provided conceptual input and reviewed all drafts of the manuscript. All authors are aware of, and approved of, the submission of this manuscript.

Chapter 3

Asia LK, Jansen Van Vuren E, Kruger IM, Williams ME. A pilot investigation of the association between Vpr amino acid substitutions and peripheral immune marker levels in people living with HIV: implications for neurocognitive impairment.

Under review in *Open Forum Infectious Diseases* (OFID-D-23-01181).

This data manuscript investigated the influence that HIV Vpr sequence variation has on peripheral inflammation in people living with HIV. Here, I investigated inflammation levels for immune markers (sCD163, NGAL, suPAR, IL-6, and hsCRP), which are associated with HIV-1 pathogenesis and the development of HIV associated neurocognitive disorders (HAND) to determine their association with HIV Vpr amino acids at neurological associated positions 22, 41, 45, and 55. Here, suPAR levels were nearing significance for higher levels in participants with the G41 amino acid variant compared to those with the 41S variant. Amino acid variations at position 41 (between 41G and 41S) exhibited a significant associating with suPAR. This study was performed using samples obtained from the Prospective Urban and Rural Epidemiology (PURE) study. Dr Monray Williams and I generated the concept of the manuscript. I performed the laboratory work for ELISA assays. Dr Monray Williams and Dr Esmé Jansen van Vuren assisted with the statistical analysis. Dr Monray Williams and I drafted the manuscript, while I wrote the literature background, introduction, methodology, and discussion sections of the manuscript. All authors, Dr Monray Williams, Dr Esmé Jansen van Vuren, Prof Iolanthe Kruger, and I, provided conceptual and intellectual contributions to the final drafts of the manuscript.

Peer reviewed conference proceedings (poster presentation):

Asia, L.K., Jansen Van Vuren, E., Williams, M.E (2023, November) The influence of HIV-1 Viral Protein R (Vpr) sequence variation on C-reactive protein (CRP) peripheral levels. The International Congress of Immunology (IUIS), Cape Town, South Africa.

This conference paper explores the impact of HIV Vpr sequence variation (high-risk vs. low-risk groups) on peripheral CRP levels in people living with HIV. Dr. Monray Williams and I conceptualized the conference paper. The laboratory ELISA measurements of CRP levels were conducted earlier by a colleague from the Hypertension in Research in Africa team. Dr. Monray Williams and Dr. Esmé Jansen van Vuren provided support with the statistical analysis.

Chapter 4

Asia LK, Jansen Van Vuren E, Williams ME. A pilot investigation of the association between HIV-1 Vpr amino acid sequence diversity and the tryptophan-kynurenine pathway as a potential mechanism for neurocognitive impairment.

Under review in *Virology Journal* (c713cda8-ebc2-4311-8776-790b6a8867ee).

This second data manuscript investigated tryptophan metabolism, with focus on the kynurenine pathway and their associations with HIV Vpr sequence variation in people living with HIV. Alterations in the levels of metabolic profiles tryptophan, kynurenine, kynurenic acid, and quinolinic acid are associated with HIV-1 pathogenesis and neurological outcomes. The amino acid variant 41G and 55A was nearing significance for higher levels of QUIN compared to 41S and 55T groups. Multiple regression analysis displayed significant associations with QUIN after adjustment. Furthermore, higher QUIN levels seen in the 41G group were found to be correlated with suPAR. This study was performed using samples obtained from the Prospective Urban and Rural Epidemiology (PURE) study. For this chapter, a total of 32 participants were used due to depleted samples upon completion of Chapter 3. Dr Monray Williams and I generated the concept of the manuscript. I performed the metabolomics laboratory work. Prof Zander Lindeque provided LC-MS/MS training and provided insight into the statistical analysis. Dr Monray Williams and Dr Esmé Jansen van Vuren assisted with the statistical analysis. Dr Monray Williams and I drafted the manuscript, while I wrote the literature background and completed the introduction, methodology, and discussion sections of the manuscript. All authors, Dr Monray Williams; Dr Esmé Jansen van Vuren; Prof Zander Lindeque; and I, provided conceptual and intellectual contributions to the final drafts of the manuscript.

Financial support

This work was supported by the National Research Foundation of South Africa (NRF, Grant UID number 140524), the Poliomyelitis Research Foundation (PRF) grant (23/24), and North-West University (NWU). The conclusions drawn from this work are not necessarily those of the NRF, PRF, or NWU, but rather those of the authors.

Ethics approval

The ethics number for this sub-study is N W U – 0 0 1 0 6 – 2 2 – A 1 – 0 1.

Author permission

As a co-author/researcher, I hereby approve and give consent that the mentioned articles and data may be used for the MSc dissertation of Mr. L.K. Asia. I declare that my role in the study is an accurate representation of my contribution.

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I extend my deepest gratitude to my supervisor, Dr Monray Williams, whose unwavering support, invaluable guidance, and encouragement have been the cornerstone of this journey. Your dedication to fostering both my academic and personal growth has been truly inspiring.

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Mr. Gatora, Junior, Karabo, Wes, and Janice thank you for being supportive and always checking in on me during this journey. I appreciate you.

A special thank you to my parents and wonderful sister for always encouraging me to pursue my dreams. I know you always have my back and will do anything to support me. This achievement is as much yours as it is mine. I love and appreciate you.

ABSTRACT

Title: The influence of HIV-1 viral protein R sequence variation on immune activation, inflammation, and metabolism: implications for neurocognitive impairment

Background

Human immunodeficiency virus (HIV) affects millions of people around the world and was shown to affect the central nervous system (CNS), resulting in HIV-associated neurocognitive disorder (HAND). Despite the use of antiretroviral therapy (ART), mild forms of HAND persist. HAND is associated with systemic inflammation and dysregulated metabolism. Although viral proteins have a role in HIV-1 pathogenesis and the development of HAND, the exact role of less common viral proteins such as viral protein R (Vpr), are not known. Little is known about the influence that Vpr amino acid sequence variants have on immune activation, inflammation, and metabolism in people living with HIV. Here, we investigated the relationship between Vpr amino acid sequence variants, inflammation, and metabolism in a cohort of South African people living with HIV.

Methods

A systematic review was conducted to determine the association of Vpr amino acid variants with clinical outcomes in people living with HIV by creating a search criterion and selecting articles based on this selection criterion. Plasma samples from n = 48 South African, subtype C people living with HIV were used to investigate the association between Vpr amino acid sequence variation and immune activation, inflammation, and metabolism in an antiretroviral (ART)-naïve cohort. Viral protein sequence analysis was done to determine the presence of amino acid variants at positions 22, 41, 45, and 55 which are associated with neurological outcomes in people living with HIV. Enzyme-Linked Immunosorbent Assay (ELISA) analysis was performed to measure sCD163, neutrophil gelatinase-associated lipocalin (NGAL), high-sensitivity C-reactive protein (hsCRP), soluble urokinase plasminogen activator receptor (suPAR), and interleukin-6 (IL-6) concentrations in people living with HIV. For liquid chromatography–tandem mass spectrometry (LC-MS/MS), plasma samples from n = 32 people living with HIV were used to investigate the levels of tryptophan, kynurenine, kynurenic acid, quinolinic acid (QUIN), and the tryptophan/kynurenine ratio. Various statistical analyses were conducted to determine the association between Vpr amino acid sequence variation and the levels of immune markers and metabolites of the tryptophan-kynurenine pathway.

Results

A systematic review of the existing literature suggested that Vpr amino acid variants are associated with clinical outcomes such as disease progression, neurological outcomes, and treatment status in people living with HIV. In this regard, 77H and 85P Vpr amino acid variants were considered noteworthy. Furthermore, Vpr amino acid variants associated with negative neurological outcomes were identified. Among the many neuropathogenic Vpr amino acid variants and immune markers examined, the ELISA analysis revealed that after applying Bonferroni corrections ($p = .05/3$) and adjusting for sex and locality, suPAR levels were nearing significance for higher levels in participants with the 41G amino acid variant compared to those with the 41S variant ($p = .035$). Further, amino acid variations at position 41 (between 41G and 41S) exhibited a significant association with suPAR (adj $R^2 = .089$, $\beta = .386$; 95% CI .125 to 3.251; $p = .035$). The metabolomics data found that, out of all the Vpr amino acid substitutions and metabolites that were investigated, only the Vpr 41G and 55A groups was nearing significance for higher levels of QUIN compared to the Vpr 41S and 55T groups, respectively (all $p = .023$) after applying Bonferroni corrections ($p = .05/3$) and adjusting for covariates (age and sex). Multiple regression results revealed that Vpr amino acid variations at position 41 (adj $R^2 = .049$, $\beta = .505$; $p = .023$), and 55 (adj $R^2 = .126$, $\beta = .444$; $p = .023$) displayed significant associations with QUIN after adjusting for age and sex. Lastly, the higher QUIN levels observed in the Vpr 41G group were found to be correlated with suPAR ($r = .588$, $p = .005$).

Conclusions

These findings suggest that Vpr amino acid sequence variations might contribute to dysregulated inflammation in people living with HIV, which could explain the observed association between specific Vpr variants and HAND found in prior research. Furthermore, the Vpr amino acid sequence variations could have an influence on metabolism and inflammation, potentially contributing to HIV-1 pathogenesis and the development of HAND. Thus, these Vpr variants merit further investigation to fully understand their roles in HIV-1 pathogenesis and neuropathogenesis.

Keywords: Human immunodeficiency virus, viral protein R, sequence variation, HIV-associated neurocognitive disorders, immune activation, inflammation, Tryptophan-Kynurenine pathway

Key terms

Acquired immunodeficiency syndrome (AIDS)

Antiretroviral therapy (ART)

Antiretroviral (ARV)

Asymptomatic neurocognitive impairment (ANI)

Blood-brain barrier (BBB)

Cardiovascular Disease (CVD)

Central nervous system (CNS)

Combination antiretroviral therapy (cART)

Enzyme-linked Immunosorbent assay (ELISA)

Global deficit score (GDS)

Glycoprotein 120 (gp120)

High-sensitivity C-reactive protein (hsCRP)

Highly active antiretroviral therapy (HAART)

HIV-associated neurocognitive disorders (HAND)

HIV-associated dementia (HAD)

Human immunodeficiency virus (HIV)

Indoleamine-2,3-dioxygenase-1 (IDO-1)

Interferon (IFN)

Interleukin (IL)

Kynurenine (Kyn)

Kynurenic acid (KA)

Kynurenine/Tryptophan (Kyn/Trp)

Liquid chromatography–tandem mass spectrometry (LC-MS/MS)

Long-term non-progressors (LTNP)

Mild neurocognitive disorder (MND)

Negative regulatory factor (Nef)

Neutrophil gelatinase-associated lipocalin (NGAL)

Polymerase chain reaction (PCR)

Quinolinic acid (QUIN)

Regulator of expression of virion proteins (Rev)

Soluble urokinase plasminogen activator receptor (suPAR)

Soluble CD163 (sCD163)

Transactivator of transcription (Tat)

Tryptophan (Trp)

Tryptophan-Kynurenine (Trp-Kyn)

Viral infectivity factor (Vif)

Viral protein R (Vpr)

Virus protein U (Vpu)

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CHAPTER 1: INTRODUCTION

The influence of HIV-1 Vpr sequence variation on immune activation, inflammation, and metabolism: implications for neurocognitive impairment

Context

Human immunodeficiency virus (HIV) infects approximately 37 million people globally to date (Heath *et al.*, 2021) with more than half of people living with HIV exhibiting mild forms of HIV-associated neurocognitive disorders (HAND) (Heaton *et al.*, 2010). Although the introduction of antiretroviral therapy (ART) has aided in managing HIV pathogenesis, the infection persists as the virus is not eliminated but rather suppressed to a point where HIV becomes undetectable (Heaton *et al.*, 2010). Furthermore, a limitation of ART is that it does not cross the blood-brain barrier (BBB), therefore the dysregulated inflammation in the central nervous system (CNS) persists (Osborne *et al.*, 2020). This persisting immune response leads to neuronal damage which becomes clinically defined as HAND (Ances & Ellis, 2007; Spudich & González-Scarano, 2012). The extent to which HAND occurs varies, thus the severity of HAND can be classified into three categories: asymptomatic neurocognitive disorders (ANI), mild neurocognitive disorders (MND), and HIV-associated dementia (HAD) (Antinori *et al.*, 2007). Common clinical features that are exhibited by patients who are suffering from HAND may include impairments in reasoning, problem-solving, planning, impaired executive functioning, mental slowness, and sensory-perpetual impairments (Antinori *et al.*, 2007).

HIV-1 pathogenesis and neuropathogenesis is multifactorial and not clearly understood. Despite the high genetic variability of HIV, particularly in HIV-1 subtypes, most studies are conducting investigations in HIV-1 B subtypes which are more prevalent in American and European regions (Hemelaar *et al.*, 2019). However, the less commonly investigated HIV-1 subtype C accounts for 40-60% of HIV-1 infections worldwide and is more prevalent in Southern African regions (Hemelaar *et al.*, 2019). Considering the differential influences of all HIV-1 subtypes on HIV-1 pathogenesis and neuropathogenesis (e.g. inflammation and metabolism), the development and severity of HAND are affected by subtype differences (e.g. subtype B vs. subtype C) (Tyor *et al.*, 2013). In this study we focus on subtype C as it is the most common globally and is predominant in South Africa (Hemelaar *et al.*, 2019), where our cohort originates from.

The severity of HIV infection varies between different subtypes of HIV-1. This difference may even be associated in the amino acid sequences of HIV-1 viral proteins. This is supported by studies that have found that viral protein sequences of Transactivator of transcription (Tat), viral protein R (Vpr), Pol, negative regulatory factor (Nef), and glycoprotein 120 (gp120) differ across HIV-1 subtypes which have differential effects on the functioning of these viral proteins (Aralaguppe *et al.*, 2016; Asia *et al.*, 2022; Campbell *et al.*, 2011; Jin *et al.*, 2020; Samikkannu *et al.*, 2011; Samikkannu *et al.*, 2015). Amino acid sequence variation has been associated with HIV-1 pathogenesis, inflammation, and development of HAND (Gandhi *et al.*, 2009; Ruhanya *et al.*, 2023a; Samikkannu *et al.*, 2011; Samikkannu *et al.*, 2015; Santerre *et al.*, 2019). Since inflammation occurs due to HIV infection, a consequence of this inflammation is dysregulated metabolic activity/pathways. However, to our knowledge no study has investigated or reported on an association between viral amino acid sequences and metabolic profile of the tryptophan-kynurenine (Trp-Kyn) pathway. Furthermore, most of the studies focussing on the amino acid sequences of HIV-1 viral proteins and (neuro)pathogenesis are focused on invitro studies/investigation whereas limited studies have focused on clinical studies. Considering the functioning of viral proteins particularly in HIV pathogenesis, HIV-1 infection may contribute to dysregulated neuroinflammation and metabolic profiles.

The occurrence of inflammation in the human body is due to exposure to something harmful. In the context of this dissertation, inflammation occurs due to the exposure to a virus, HIV-1. Thus, an inflammatory response aids as a defence mechanism to harmful pathogens by minimizing injury and/or infection (Chen *et al.*, 2018). During HIV-1 infection, this inflammatory response in the body may be dysregulated and hinder the body's ability to fight the infection. Inflammation in the CNS and brain tissue types have been associated with HIV-associated encephalopathy (Fields *et al.*, 2018). However, the invasiveness of acquiring these tissue types makes the periphery a more accessible target to determine whether the periphery can be correlated or even mirror what happens in the cerebrospinal fluid and/or brain tissue sample types. Previous studies in this regard have found that systematic inflammation may provide insight into HIV-associated neuroinflammation and/or neuropathology, as: 1) plasma-soluble CD163 is associated with postmortem brain pathology in HIV-1 infection (Bryant *et al.*, 2017) and 2) neurofilament light chain protein, a sensitive marker for neuronal injury, concentration in plasma vs cerebrospinal fluid were highly correlated ($r = 0.89$, $p < .0001$) (Gisslén *et al.*, 2016). This evidence suggests that systematic inflammation may 1) reflect on the activity of the CNS e.g., inflammation in CNS and/or neuronal activity to a certain extent, 2) correspond to inflammation levels observed in the

CNS, and 3) potentially serve as an alternative to investigating CNS pathology states related to inflammation. Considering the potential information that systematic inflammation may provide regarding the CNS, it may potentially provide insight into the dysregulated metabolism exhibited in patients suffering from HAND.

Metabolism, which is the chemical reaction in cells produces energy to sustain life (Judge & Dodd, 2020). However, inflammation in the human body may result in a cascade of events. One of such which is changes in metabolic activity in the body. In the context of this dissertation, dysregulated inflammation resulting from HIV infection may consequently cause irregularities in metabolic activity in the body. Trp-Kyn metabolism and its association with HAND has spiked interest in recent years (Lovelace *et al.*, 2017). Trp can be metabolised via the Kyn pathway which occurs in the infiltrating macrophages as well as in resident microglial cells (Guillemin *et al.*, 2003a; Guillemin *et al.*, 2005). Considering the mechanism of HIV infection of the brain via monocytes which differentiate into macrophages, there may be a potential link between the Trp-Kyn pathway and HIV neuropathogenesis. Also, considering the dysregulated inflammation caused by HIV neuropathogenesis, inflammation is associated with metabolic changes observed in people living with HIV, particularly the Trp-Kyn metabolism (Keegan *et al.*, 2016; Lovelace *et al.*, 2017). This is supported by inflammatory cytokines which upregulate the enzyme IDO-1 to degrade tryptophan and subsequently produce more Kyn in the Trp-Kyn pathway (Werner-Felmayer *et al.*, 1989; Yasui *et al.*, 1986). This Trp-Kyn pathway consists of metabolites which may have neuroexcitatory and neuroprotectant properties which may have either negative or positive implications for neurocognitive impairments (Baran *et al.*, 2000; Braidy *et al.*, 2009; Heyes *et al.*, 1991; Lovelace *et al.*, 2017; Martin *et al.*, 1992).

There is an association between HIV-1 with both dysregulated inflammation and dysregulated metabolism. However, most of our understanding of this comes from subtype B which is predominant in regions such as Europe and America (Hemelaar *et al.*, 2019). However, subtype C, more prevalent in southern Africa, accounts for most HIV-1 infections but is a less commonly investigated subtype (Hemelaar *et al.*, 2019). In particular, approximately 71% of clinical studies focusing on HIV-1 Vpr sequence variation are performed on subtype B populations compared to 14% of studies focusing on subtype C populations (Asia *et al.*, 2022). In the context of HIV neuropathogenesis, these subtypes, may have differential severities on HIV infection and comorbidities (Santerre *et al.*, 2019). At an amino acid level, Vpr has shown to be associated with

negative neurological outcomes (Dampier *et al.*, 2017; Ruhanya *et al.*, 2023a). Vpr may impair the function and viability of neurons and astrocytes. Therefore, there is a crucial need to investigate the potential influences that Vpr amino acid sequence variation in HIV-1 subtype C populations may have on inflammation and metabolism to determine if there are any differences to our current understanding of these mechanisms in HIV-1 subtype B infections.

Although there is an association between HIV-1 pathogenesis and both inflammation and Trp-Kyn metabolism, the role of HIV-1 viral proteins in these processes is unclear. Particularly the role of HIV-1 Vpr sequence variation and its effect on both inflammation and Trp-Kyn metabolism is not very clear despite Vpr being associated with clinical outcomes in people living with HIV (Asia *et al.*, 2022; Dampier *et al.*, 2017; Soares *et al.*, 2014; Womersley *et al.*, 2019; Zhang *et al.*, 1997). Therefore, this dissertation will attempt to address the influence that HIV-1 Vpr amino acid sequence variation (with specific focus on neurologically-associated amino acids at position 22, 41, 45, and 55) may have on inflammation and Trp-Kyn metabolism.

Research aims

The overall aim of this dissertation was to determine the influence that HIV Vpr amino acid variants, particularly at positions that have shown to be associated with neurological outcomes, have on immune activation, inflammation, and Trp-Kyn metabolism in clinical sample types. In particular, we aimed to 1) perform a systematic review of the existing literature to identify Vpr amino acid substitutions that are associated with neurological outcomes in people living with HIV (Chapter 2), 2) determine the association of neurologically-associated immune activation and inflammation markers with neurologically-associated Vpr amino acid variants (Chapter 3), and 3) determine the association of neurologically-associated metabolic markers of the Trp-Kyn pathway with neurologically-associated Vpr amino acid variants (Chapter 4).

Coherence of the dissertation

This dissertation has a common underlying theme, which includes the investigation of Vpr amino acid variants for clinical outcomes, particularly neurological clinical outcomes, in a South African context. This dissertation resulted in a series of manuscripts of which one has been published to date.

For this dissertation, I have included a review paper (Chapter 2). This is a published systematic review which summarised findings from clinical studies and identified that 1) Vpr amino acid substitutions were associated with specific clinical outcomes including disease progressions, neurological outcomes, treatment status, and other clinical manifestations, 2) Studies consistently showed that the Vpr substitution 63T was associated with slower disease progression, whereas 77H and 85P were associated with no significant contribution to disease progression, and 3) Although not considered a significant finding from the criteria created in the systematic review, we have found that Vpr amino acid variants generally have positive and negative clinical outcomes of which we selected the ones which were associated with neurological outcomes for further investigations.

This dissertation also includes two original empirical papers (Chapter 3 and Chapter 4). The first which is currently under review for publication (Chapter 3), shows the associations between neurologically associated Vpr amino acid positions with neurologically associated inflammation markers. The second, which is also under review for publication (Chapter 4), shows the association between neurologically associated Vpr amino acid positions with neurologically associated metabolic markers from Trp-Kyn metabolism. Both papers investigated people living with HIV that were treatment naïve in a South African context. Therefore, this allowed the investigation of the immune response and metabolic profiles without the possible confounding effects of ART. Lastly, the findings here may be relevant for future investigations of treatment-experienced participants as well, as chronic inflammation persists irrespective of treatment status.

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

CHAPTER 2

The influence of Viral protein R amino acid substitutions on clinical outcomes in people living with HIV: a systematic review

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Abstract

Background: The HIV viral protein R (Vpr) is a multifunction protein involved in the pathophysiology of HIV-1. Recent evidence has suggested that Vpr amino acid substitutions influence the pathophysiology of HIV-1 and clinical outcomes in people living with HIV. Several studies have linked Vpr amino acid substitutions to clinical outcomes in people living with HIV, however, there is no clear consensus as to which amino acids or amino acid substitutions are most important in the pathophysiology and clinical outcomes in people living with HIV. We, therefore, conducted a systematic review of studies investigating Vpr amino acid substitutions and clinical outcomes in people living with HIV.

Methods: PubMed, Scopus, and Web of Science databases were searched according to PRISMA guidelines using a search protocol designed specifically for this study.

Results: A total of twenty-two studies were included for data extraction, comprising fourteen cross-sectional and eight longitudinal studies. Results indicated that Vpr amino acid substitutions were associated with specific clinical outcomes including disease progressions, neurological outcomes, treatment status, and other clinical manifestations. Studies consistently showed that the Vpr substitution 63T was associated with slower disease progression, whereas 77H and 85P were associated with no significant contribution to disease progression.

Conclusions: Vpr-specific amino acid substitutions may be contributors to clinical outcomes in people living with HIV, and future studies should consider investigating the Vpr amino acid substitutions highlighted in this review.

2.1. Introduction

Human immunodeficiency virus (HIV), a retrovirus, is the causative agent of acquired immunodeficiency syndrome (AIDS) (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984). According to the Joint United Nations Programme on HIV/AIDS (UNAIDS), it was approximated in 2021 that 37.7 million people were living with HIV, 1.5 million people were newly infected, and 680 000 people had AIDS-related deaths, globally (UNAIDS, 2021). Even with 75% of people living with HIV having access to antiretroviral therapy (ART) globally and despite effective treatment for people living with HIV with ART, other complications, especially those of the central nervous system (CNS) persist (Ances & Letendre, 2019).

HIV is a genetically diverse pathogen due to high replication rates, error-prone reverse transcriptase, and recombination events that could occur during viral replication (Hemelaar *et al.*, 2019; Santoro & Perno, 2013; Sebastião *et al.*, 2020). Two types of HIV exist, HIV-1 and HIV-2, of which HIV-1 is more prevalent (Bhebhe *et al.*, 2021; Campbell-Yesufu & Gandhi, 2011; Rasmussen *et al.*, 2020b) and is categorized into groups M, N, O, and P (Ayoub *et al.*, 2001; Plantier *et al.*, 2009; Roques *et al.*, 2004; Simon *et al.*, 1998; Vallari *et al.*, 2011; Yousaf *et al.*, 2011). As reviewed by Sharp and colleagues (1999), HIV-1 group M is the more geographically abundant group compared to other HIV-1 groups (Sharp *et al.*, 1999). Furthermore, HIV-1 group M consists of viral subtypes A, B, C, D, F, G, H, J, and K (Yousaf *et al.*, 2011). Furthermore, subtypes A and F have sub-subtypes, namely A1-4 and F1-2, respectively (Gao *et al.*, 2001; Meloni *et al.*, 2004; Triques *et al.*, 1999; Triques *et al.*, 2000; Vidal *et al.*, 2006). The subtypes predominate in particular regions worldwide: CRF02_AG/Subtype G- West Africa; Subtype A- East Africa, Eastern Europe, and Central Asia; Subtype C- Southern Africa, Ethiopia, and South Asia (India); Subtype B- Western and Central Europe, North America, Caribbean, Latin America, and Oceania (Hemelaar *et al.*, 2019). Amongst all HIV-1 groups, M and O are considered the more genetically diverse groups (Ayoub *et al.*, 2001; Plantier *et al.*, 2009; Roques *et al.*, 2004; Simon *et al.*, 1998; Vallari *et al.*, 2011).

HIV-1 genome consists of various viral proteins which play important roles during the viral lifecycle (Frankel & Young, 1998). HIV encodes for fifteen distinct proteins and these include structural: (matrix, capsid, nucleocapsid, p6, surface and transmembrane), Pol enzymes (protease, reverse transcriptase, integrase), regulatory proteins (Transactivation of transcription (Tat) and regulator

of expression of virion proteins (Rev)) and accessory proteins (Negative regulatory factor (Nef), viral infectivity factor (Vif), viral protein R (Vpr) and virus protein U (Vpu)) (Frankel & Young, 1998).

Traditionally, the HIV-1 accessory Vpr has received less attention despite its relevance to pathophysiological mechanisms and clinical outcomes in people living with HIV. Vpr is a fourteen-kilodalton basic protein comprised of 96 amino acids and is made up of three amphiphilic helices (Pandey *et al.*, 2009). These bundled α -helices extend to the amino acid regions 17–33, 38–50, and 55–77 and are flanked by unstructured flexible N- and C-terminal domains that are negatively or positively charged, respectively (Pandey *et al.*, 2009). HIV Vpr is a multifunctional protein involved in the pathophysiology of HIV, more specifically Vpr (1) counteracts the IFN-stimulated macrophage-specific restriction pathway targeting HIV-1 envelope protein (Env) (Mashiba *et al.*, 2015), (2) targets Tet Methylcytosine Dioxygenase 2 (TET2) for degradation by CRL4^{VprBP} E3 Ligase to sustain pro-inflammatory cytokine interleukin-6 (IL-6) expression and enhance HIV-1 replication (Lv *et al.*, 2018) (3) counteracts the restriction of lysosomal-associated transmembrane protein 5 (LAPTM5) to promote HIV-1 infection in macrophages (Zhao *et al.*, 2021) (4) targets cellular proteins to drive systems-level proteomic remodelling in HIV-1 infection (Greenwood *et al.*, 2019), and (5) counteracts helicase-like transcription factor (HLTF)-mediated restriction of HIV-1 infection in T cells (Yan *et al.*, 2019). The effect that Vpr has on monocytes and infected macrophages is associated with HIV-1-associated neuropathogenesis (Barrero *et al.*, 2013). Even in the absence of active viral replication and with the effective use of ART, Vpr can still be detected in the systemic and CNS of people living with HIV (Ferrucci *et al.*, 2013; Jones *et al.*, 2007; Matsunaga *et al.*, 2019). Therefore, Vpr may have a crucial role in the pathophysiology of HIV-1 in the modern-ART era.

Subtype-specific sequence variation exists between HIV-1 subtypes, and this results in changes in the amino acid sequences of the HIV-1 viral proteins, including Vpr. Multiple research groups have extensively sequenced the HIV-1 genome (Ho *et al.*, 2013; White *et al.*, 2022; Zhou *et al.*, 2016; Zhou & Swanstrom, 2020). HIV-1 mutates rapidly and using a wide range of investigations, the HIV-1 mutation rate is estimated to be 10^{-5} – 10^{-3} errors/bp/cycle (Yeo *et al.*, 2020) with the *Env* gene having the fastest mutation rate amongst all viral genes (Cuevas *et al.*, 2015; Shankarappa *et al.*, 1999; Sun *et al.*, 2022). Even though Vpr is an accessory protein, the Vpr gene is generally well conserved in all human (HIV-1, HIV-2) and simian (SIV) immunodeficiency viruses (González, 2017; Guenzel *et al.*, 2014; Tristem *et al.*, 1992) and has lower mutation rates

compared to other viral genes (Sun *et al.*, 2022) and is therefore indicative of its potential importance in the pathogenesis of HIV.

The sequence variation in the HIV-1 viral proteins influences the pathophysiology of HIV-1 (Chui *et al.*, 2006; Gnanakaran *et al.*, 2007; Jones *et al.*, 2007; Na *et al.*, 2011; Rajan *et al.*, 2006; Rao *et al.*, 2014; Williams *et al.*, 2022; Williams, Zulu, *et al.*, 2020). When comparing the Vpr protein sequences between HIV-1 subtypes, several variances are noted (Figure 1) and certain amino acid substitutions have been associated with the pathophysiology of HIV-1 (Dampier *et al.*, 2017; Rajan *et al.*, 2006). This may be due to a change in the structural characteristics of the Vpr protein (Figure 2). Overall, the structure of Vpr may seem similar between HIV-1 subtypes. It is known that the alternation of specific amino acids or the substitutions of amino acids at specific positions within Vpr may significantly alter its function (Soares *et al.*, 2016). As examples of these, mutations (R36W, I64E, L67A, and I70S) in α -helix residues result in a total inability of Vpr to oligomerize at the nuclear envelope (Fritz *et al.*, 2008). In addition, the deletion of the amino acids SRIG sequence (between amino acids 79 and 82) or mutation of all arginine's in the C-terminal domain prevented G2 arrest by Vpr (Morellet *et al.*, 2009).

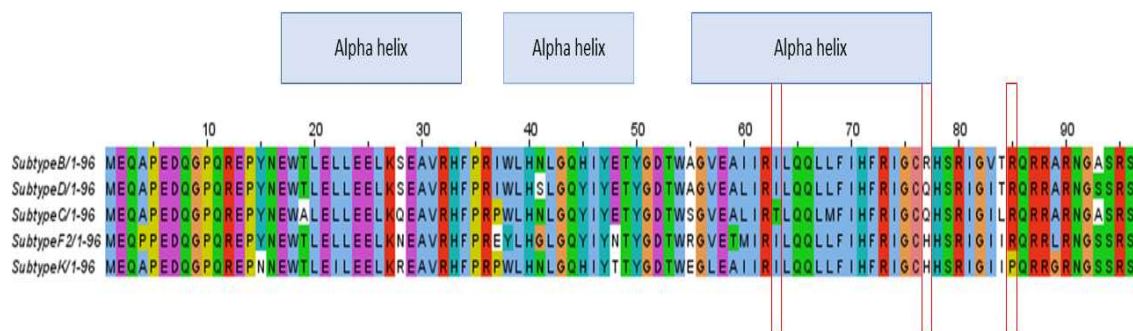


Figure 1: Schematic presentation of HIV-1 Vpr amino acid sequence alignment comparing Subtype B (P12520 VPR_HV1N5), Subtype D (P12519 VPR_HV1Z2), Subtype C (Q75004 VPR_HV1ET), Subtype F2 (F2 P0C1P4 VPR_HV1MP), and Subtype K (P0C1P2 VPR_HV196). Amino acids in boxes indicate noteworthy positions. Alpha helical domains are indicated in blue boxes and are at regions 17–33, 38–50, and 55–77. Each subtype Vpr sequence was extracted from UniProt where available. No Vpr sequence was available for subtype A. Only “pure subtypes” are presented thus circulating recombinant forms (CRFs) are not considered.

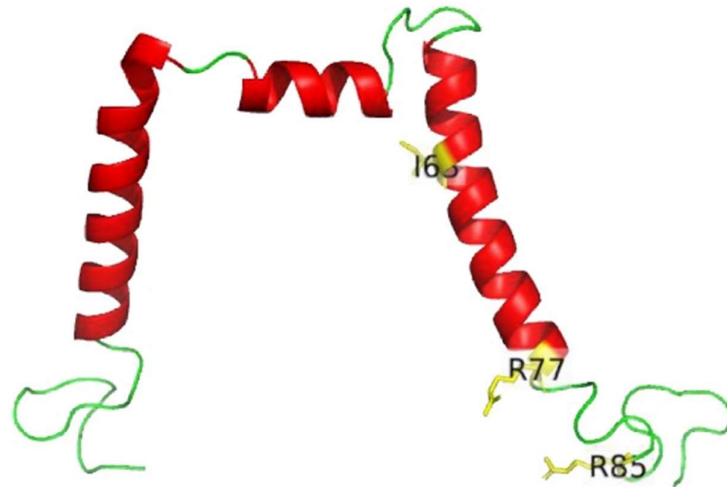


Figure 2: Predicted 3D structures of Vpr (Subtype B (P12520 · VPR_HV1N5) generated by Swiss Model webserver. The Vpr sequence was extracted from UniProt and subjected to 3D structure prediction. Noteworthy amino acid positions are indicated as yellow sticks. Helical structures are indicated in red and unstructured regions are indicated in green.

As reviewed in Fabryova and Strebel (2019), several *in vitro/in vivo* studies have investigated the influence of Vpr sequence variation and their contribution to HIV-1 pathophysiology (Fabryova & Strebel, 2019), however, these amino acid substitutions do not necessarily present the Vpr amino acid substitutions present in people living with HIV. Despite several studies investigating Vpr amino acids' variation in people living with HIV (Fabryova & Strebel, 2019; Soares et al., 2016) there is no clear consensus on which amino acid substitutions are most associated with the pathophysiology (disease progression, neurological outcomes, treatment efficacy, and other clinical manifestation) of HIV-1 in people living with HIV. Therefore, this systematic review aimed to review all literature on this topic to identify the key Vpr amino acid substitutions associated with HIV-1 pathophysiology and clinical outcomes in people living with HIV. Findings from this review may further our understanding of the role of Vpr within various pathophysiological processes.

2.2. Methods

2.2.1. Study design

This systematic review aimed at summarizing the extant literature on the influence of Vpr amino acid substitutions on HIV-1 pathophysiology and clinical outcomes in people living with HIV. This study has been approved by the North-West University Health Research Ethics Committee (NWU-HREC): NWU-00203-22-A 1.

2.2.2. Eligibility criteria

The eligibility criteria were HIV-positive treatment-experienced adults (>18 years old, all medication types included, and no cut-off for treatment duration) with relevant Vpr protein sequence data. All studies had to investigate Vpr protein sequence variations from clinical sample types (blood, cerebrospinal fluid, and post-mortem brain tissue) to investigate HIV-1 proviral DNA and/or viral RNA. For comparability of studies, sequencing had to be done by polymerase chain reaction (PCR) based sequencing methods (e.g., Sanger sequencing and/or Next Generation Sequencing). Studies had to have a control group/comparative arm to be included. Exclusionary criteria were pre-clinical (animal and cell culture models) studies and reviews. Studies investigating Vpr sequence variation without relation to clinical outcomes were also excluded.

2.2.3. Data sources

We electronically searched for publications in PubMed, Scopus, and Web of Science databases based on all studies published until 06/09/2022. Eligible studies included published studies in English only. The search strategy was executed without publication date limitations. The full search criteria for each database are included in Supplementary File 1. 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 (Gene Products, vpr [mh] OR Vpr [tw] or Viral protein R [tw]) AND (mutation [mh] OR protein mutation [tw] OR Polymorphism, Genetic [mh] OR amino acid variation [tw] OR protein signatures [tw]).

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

2.2.4. Data selection

All articles were retrieved and loaded onto a single database using a reference manager (EndNote X9, Clarivate, PA, USA). Two authors 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.

2.2.5. Quality assessment of included studies

The quality of the included studies was assessed, and kappa statistics were calculated. The Joanna Briggs Institute (JBI) critical appraisal tools have been adopted for the quality criterion. Here we have amended the JBI critical appraisal tools by implementing a Likert scale (Likert 1932) to provide a quantitative measure of study quality. For all included studies, we have only considered the JBI quality questions from the Checklist for Analytical Cross-Sectional Studies which may influence the findings of the reviewed studies. These included (1) cohort information, (2) validity and conditions of measurement, and (3) confounding factors and statistical analysis. These were assessed using the following questions (1) Were the criteria for inclusion in the sample clearly defined and were study subjects and the setting described? (2) was the method used to investigate the clinical outcome done in an objective, valid and reliable method (i.e., the inclusion of viral load and CD4⁺ count)? and (3) did they consider confounding factors, and did they employ relevant statistical analysis? This included a total of 3 questions. Each question was rated for 0 = no, 1 = partly and 2 = yes. Studies that addressed all the above questions and had a total rating of ≥ 5 were classified as high quality. Studies with a rating between 3 and 4 were considered intermediate-quality and ≤ 2 as low quality (Supplementary Table 1).

2.2.6. Potential confounders

Although some studies did do additional *in vitro/in vivo* studies, several other factors may influence clinical outcomes in people living with HIV and therefore may influence the findings reported in the reviewed studies investigating Vpr amino acid substitutions and clinical outcomes. Therefore, we wanted to consider certain confounders when analysing the data. In particular, we considered viral load and CD4⁺ count concerning the findings reported in the studies. First, we stratified studies according to viral load, which was defined as <400 copies/millilitre (cps/ml) (viral suppression) versus >400 cps/ml (non-viral suppression) (Biney *et al.*, 2021; Euvrard *et al.*, 2019; Gabagaya *et al.*, 2021) (Supplementary Table 2). Second, studies were also stratified according

to a mean/median CD4⁺ count of <200 cells/μl or >200 cells/μl (Supplementary Table 3). The mean values were primarily considered when stratifying studies, however, where the mean data was not available, the median data was used. We wanted to determine when studies were stratified according to viral load and CD4⁺ counts, whether Vpr amino acid substitutions still influence clinical outcomes in people living with HIV. Lastly, as most studies focused on subtype B we wanted to investigate whether the HIV-1 subtype influenced the prevalence of studies reporting the association of Vpr amino acid substitutions and clinical outcomes.

2.3. Results

2.3.1. Study characteristics

Using the described criterion and search strategy (Section 2.2), 984 abstracts and titles were screened as indicated in Figure 3. Furthermore, n = 3 clinical studies were added from additional sources. Duplicates (n = 121) were removed, resulting in 866 studies. Thereafter, abstracts and titles were screened and a total of 654 studies were excluded which comprised of:

- Review articles/book chapters/conference proceedings (n = 173).
- Studies not investigating HIV-1 in general (n = 48).
- Studies investigating HIV-2 (n = 8).
- Studies not investigating Vpr in general (n = 414)
- Studies only investigating Vpr sequence variation on a genetic level only (n = 4)
- Studies investigating Vpr but not investigating Vpr amino acid substitutions (n = 7)

Full-text articles assessed for eligibility were done for 212 studies, and an additional n = 95 were excluded:

- Review articles/book chapters/conference proceedings (n = 1).
- Studies investigating HIV-2 (n = 6).
- Studies not investigating Vpr in general (n = 1)
- Studies only investigating HIV-1 sequence variation of multiple viral genes on a genetic level only (n = 5)
- Studies investigating Vpr but not investigating Vpr amino acid substitutions (n = 74)

- Paper retraction (n =1)
- Investigated Vpr amino acid substitutions but no correlation with clinical outcomes (n = 5)
- Database sequences (n=1)
- A study that was speculative regarding correlations of Vpr amino acids and clinical outcomes (n = 1)

Using this criterion (section 2.1-2.3), a total of 117 studies resulted which included n= 95 *in vitro/in vivo* studies. The 95 *in vitro/in vivo* studies were excluded as this is part of an ongoing study looking at fundamental Vpr amino acid substitutions. Therefore, a total of 22 studies were included for data extraction, and from these studies, n = 14 (63,64%) were cross-sectional and n = 8 (36,36%) were longitudinal in design. Of all the studies, n = 5 studies were case studies (Cali *et al.*, 2005; Jacquot *et al.*, 2009; Soares *et al.*, 2014; Wang *et al.*, 1996; Zhao *et al.*, 2002). Across all studies, there was a total sample size of n = 2929 people living with HIV and n = 0 HIV-negative controls.

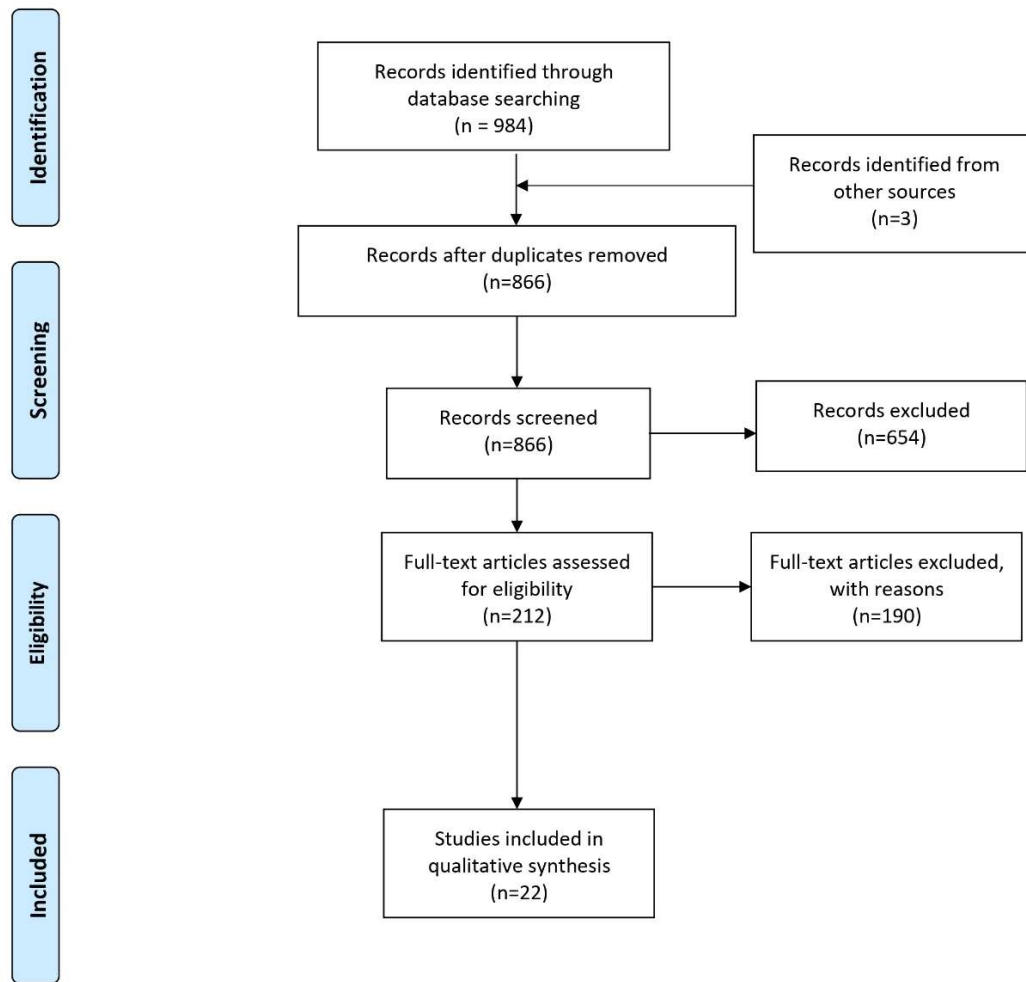


Figure 3: Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram for results of search strategy.

2.3.2. Quality of assessment of included studies

The quality of the studies was rated. The Kappa statistic for interrater agreement and reliability was .757, indicating substantial agreement (McHugh, 2012). Most studies were rated as intermediate (54%- 64%), followed by high (27%- 36%) and low-quality (8%) (Supplementary Table 1). Based on these findings, recommendations are made in the latter part of this review.

2.3.3. Vpr amino acids associated with clinical outcomes

All studies included in this review reported findings for Vpr amino acid substitutions associated with clinical outcomes (Table 1). Positive clinical outcomes were any outcomes that associated with good or improved health whereas negative clinical outcomes were any outcomes related to worsened health in people living with HIV. Associations with negative clinical outcomes were reported for Vpr amino acid substitutions 3R, 21I, 22L, 41N, 41S, 55A, 64P, 75R, 77Q, and 79G. Associations with positive clinical outcomes were reported for Vpr amino acid substitutions 2E, 17A, 18W, 22L, 28N, 29G, 31D, 32K, 33H, 37I, 41S, 41G, 44R, 55A, 63T, 65R, 68M, 70V, 70I, 76C, 77Q, 77R, 78H, 83V, 85P, 86G, 86R, 89G, 89T, 89S, 89A, 93A, 93S, 93R, and 94G (Tables 1). Furthermore, certain Vpr amino acid substitutions had no significant association with clinical outcomes including 17E, 20L, 21E, 22L, 23L, 24E, 25E, 26L, 29E, 30A, 35P, 54W, 73R, 77Q, 77H, 80R, 85P, and 87P (Table 1). Based on the associations of these amino acid substitutions with clinical outcomes, these amino acid substitutions were categorised as (1) disease progression/non-progression, (2) neurological outcomes, and (3) treatment status (Table 1).

The overall findings show that certain Vpr amino acid substitutions were consistently associated with certain clinical outcomes as reported by more than one independent study across different subtypes. However, for this review, we considered an amino acid substitution finding to be “noteworthy” when (1) two or more independent studies investigated a particular Vpr amino acid substitution and (2) when 50% or more of the studies investigating a particular Vpr amino acid substitution found it to be consistently associated with the same clinical outcome (Williams *et al.*, 2021). In other words, if >50% of studies that investigated a particular amino acid substitution found it to be associated with the same positive/negative/no significant contribution clinical outcome, we considered it noteworthy for future investigation (Figures 4-6).

2.3.4. Disease progression/non-progression

Certain Vpr amino acid substitutions were associated with positive (long-term non-progressors, higher CD4⁺, lower viral load) and/or negative clinical outcomes (progressor, lower CD4⁺, higher viral load) or had no significant contribution to clinical outcomes. The amino acid substitutions 2E, 18W, 29G, 31D, 33H, 41G, 44R, 55A, 63T, 65R, 70I, 70V, 76C, 77Q, 78H, 83V, 85P, 86G, 86R, 89G, 89T, 89S, 89A, 93A, 93S, 93R, and 94G were associated with positive clinical outcomes (Table 1 and Figure 4). In particular, these Vpr amino acid substitutions were more prevalent with

long-term non-progressors (LTNP) (Antoni *et al.*, 2007; Jacquot *et al.*, 2009; Lum, Cohen, Nie, Weaver, Gomez, Yao, Lynch, Pilon, Hawley & Kim, 2003; Mologni *et al.*, 2006; Rossenkhan *et al.*, 2016; Zhang *et al.*, 1997; Zhao *et al.*, 2002), lower viral load (Kamori *et al.*, 2017) and higher CD4⁺ count (Chen *et al.*, 2011; Kamori *et al.*, 2017) (Table 1). The amino acid substitutions 3R, 21I, 22L, 41S, 64P, 75R, 77Q, and 79G were associated with negative clinical outcomes (Table 1) and were more prevalent with progressors and/or acute HIV-1 infection (Cali *et al.*, 2005; Jacquot *et al.*, 2009; Rossenkhan *et al.*, 2016), higher viral load (Soares *et al.*, 2014) and lower CD4⁺ count (Soares *et al.*, 2014) (Table 1). The amino acid substitutions 17E, 20L, 21E, 22L, 23L, 24E, 25E, 26L, 29E, 30A, 35P, 54W, 73R, 77Q, 77H, 80R, 85P, and 87P were associated with no significant contribution to disease progression (Cavert *et al.*, 2004; Chui *et al.*, 2006; Fischer *et al.*, 2004; Ge *et al.*, 1996; Mologni *et al.*, 2006; Shen *et al.*, 2008; Wang *et al.*, 1996) (Table 1). The most investigated amino acid substitution across all studies was 77Q (Antoni *et al.*, 2007; Cali *et al.*, 2005; Cavert *et al.*, 2004; Chen *et al.*, 2011; Chui *et al.*, 2006; Fischer *et al.*, 2004; Jacquot *et al.*, 2009; Lum, Cohen, Nie, Weaver, Gomez, Yao, Lynch, Pilon, Hawley & Kim, 2003; Mologni *et al.*, 2006; Shen *et al.*, 2008; Soares *et al.*, 2014). Only 22L, 63T, 77Q, 77H, and 85P were investigated by two or more independent studies related to disease progression (Figure 4), therefore, meeting our first criteria as a noteworthy finding. The second criteria were that for all studies investigating 22L, 63T, 77Q, 77H, and 85P, >50% of these studies had to associate these substitutions with the same positive/negative/no significant contribution clinical outcome. Despite the widespread investigation, the findings for 77Q did not meet our criteria for noteworthy as the findings were mixed with four independent studies linking it to positive clinical outcomes, two independent studies linking it to negative clinical outcomes, and five independent studies linking it to no significant contribution to clinical outcomes. Similarly, the findings for 22L were mixed (Rossenkhan *et al.*, 2016; Shen *et al.*, 2008) (Figure 4). The amino acid substitutions 63T (100%), 77H (100%), and 85P (67%) met our criteria for noteworthy as >50% of studies investigating this substitution linked it to positive (63T), and no significant contribution (77H and 85P) to clinical outcomes (Table 1 and Figure 4).

Table 1 Amino acid substitutions associated with clinical outcomes in people living with HIV.

Amino acid substitutions associated with disease progression						
Amino acid substitution	Positive	Reference	Negative	Reference	No significant contribution	Reference
2E	Well conserved in Long Term non-progressor (LTNP)	(Zhang <i>et al.</i> , 1997)				
3R			Associated with lower CD4 count	(Soares <i>et al.</i> , 2014)		
			Associated with higher viral load	(Soares <i>et al.</i> , 2014)		
17E					No contribution to disease progression	(Shen <i>et al.</i> , 2008)
18W	Well conserved in LTNP	(Zhang <i>et al.</i> , 1997)				
20L					No contribution to disease progression	(Shen <i>et al.</i> , 2008)
21I			Associated with lower CD4 count	(McMullen <i>et al.</i> , 2021)		
21E					No contribution to disease progression	(Shen <i>et al.</i> , 2008)
22L			Significantly enhanced in transmitted/founder (t/f) viruses (single/few viral variants that have a	(Rossenkhani <i>et al.</i> , 2016)	No contribution to disease progression	(Shen <i>et al.</i> , 2008)

			productive infection within a host) compared with chronic sequences			
23L					No contribution to disease progression	(Shen <i>et al.</i> , 2008)
24E					No contribution to disease progression	(Shen <i>et al.</i> , 2008)
25E					No contribution to disease progression	(Shen <i>et al.</i> , 2008)
26L					No contribution to disease progression	(Shen <i>et al.</i> , 2008)
29E					No contribution to disease progression	(Shen <i>et al.</i> , 2008)
29G	Associated with LTNP	(Zhao <i>et al.</i> , 2002)				
30A					No contribution to disease progression	(Shen <i>et al.</i> , 2008)
31D	Associated with LTNP	(Zhao <i>et al.</i> , 2002)				
33H	Well conserved in LTNP	(Zhang <i>et al.</i> , 1997)				
35P					No contribution to disease progression	(Shen <i>et al.</i> , 2008)
41S			Associated with lower CD4 counts	(McMullen <i>et al.</i> , 2021)		
41G	Associated with LTNP	(Zhao <i>et al.</i> , 2002)				
44R	Associated with LTNP	(Zhao <i>et al.</i> , 2002)				

54W					No contribution to disease progression	(Shen <i>et al.</i> , 2008)
55A	Associated with lower viral load	(Kamori <i>et al.</i> , 2017)				
63T	Stable CD4 count	(Chen <i>et al.</i> , 2011)				
	Associated with lower viral load	(Kamori <i>et al.</i> , 2017)				
	Associated with a higher CD4 count	(Kamori <i>et al.</i> , 2017)				
64P			Associated with progressors	(Jacquot <i>et al.</i> , 2009)		
65R	Associated with LTNP	(Jacquot <i>et al.</i> , 2009)				
70V	Stable CD4 count	(Chen <i>et al.</i> , 2011)				
70I	Associated with chronic infection	(Rossenkhan <i>et al.</i> , 2016)				
73R					No contribution to disease progression	(Shen <i>et al.</i> , 2008)
75R			Associated with progressors	(Cali <i>et al.</i> , 2005)		
76C	Well conserved in LTNP	(Zhang <i>et al.</i> , 1997)				

77Q	Associated with LTNP	(Antoni <i>et al.</i> , 2007)	Associated with lower CD4 count	(Soares <i>et al.</i> , 2014)	No contribution to disease progression	(Cavert <i>et al.</i> , 2004)
		(Jacquot <i>et al.</i> , 2009)				(Chen <i>et al.</i> , 2011)
		(Lum, Cohen, Nie, Weaver, Gomez, Yao, Lynch, Pilon, Hawley & Kim, 2003)	Associated with higher viral loads	(Soares <i>et al.</i> , 2014)		(Chui <i>et al.</i> , 2006)
		(Mologni <i>et al.</i> , 2006)				(Fischer <i>et al.</i> , 2004)
						(Shen <i>et al.</i> , 2008)
			Associated with progressors	(Cali <i>et al.</i> , 2005)		
77H					No contribution to disease progression	(Mologni <i>et al.</i> , 2006)
						(Soares <i>et al.</i> , 2014)
78H	Well conserved in LTNP	(Zhang <i>et al.</i> , 1997)				

79G			Associated with progressors	(Jacquot <i>et al.</i> , 2009)		
80R					No contribution to disease progression	(Shen <i>et al.</i> , 2008)
83V	Associated with LTNP	(Zhao <i>et al.</i> , 2002)				
85P	Stable CD4 count	(Chen <i>et al.</i> , 2011)			No contribution to LTNP	(Ge <i>et al.</i> , 1996)
					No contribution to disease progression	(Wang <i>et al.</i> , 1996)
86G	Stable CD4 count	(Chen <i>et al.</i> , 2011)				
86R	Associated with LTNP	(Zhang <i>et al.</i> , 1997)				
87P					No contribution to disease progression	(Wang <i>et al.</i> , 1996)
89G	Stable CD4 count	(Chen <i>et al.</i> , 2011)				
89T	Associated with LTNP	(Zhang <i>et al.</i> , 1997)				
89S	Associated with LTNP	(Zhang <i>et al.</i> , 1997)				
89A	Associated with non-progressors	(Wang <i>et al.</i> , 1996)				
93A	Associated with chronic infection	(Rossenkhan <i>et al.</i> , 2016)				
93S	Associated with LTNP	(Zhang <i>et al.</i> , 1997)				
93R	Associated with LTNP	(Zhang <i>et al.</i> , 1997)				

94G	Stable CD4 count	(Chen <i>et al.</i> , 2011)				
Amino acid substitutions associated with neurological outcomes						
Amino acid substitution	Positive	Reference	Negative	Reference	No significant contribution	Reference
21I			Associated with ischaemic stroke	(McMullen <i>et al.</i> , 2021)		
22L	Associated with the non-stroke control group	(McMullen <i>et al.</i> , 2021)				
37I	Decrease in pronounced neurocognitive deficits (Lower GDS)	(Dampier <i>et al.</i> , 2017)				
41N			Increase in pronounced neurocognitive deficits (Higher GDS)	(Dampier <i>et al.</i> , 2017)		
41S	Decrease in pronounced neurocognitive deficits (Lower GDS)	(Dampier <i>et al.</i> , 2017)	Associated with ischaemic stroke	(McMullen <i>et al.</i> , 2021)		
41G	Associated with the non-stroke control group	(McMullen <i>et al.</i> , 2021)				
55A			Increase in pronounced neurocognitive deficits (higher GDS)	(Dampier <i>et al.</i> , 2017)		

77Q			Predominant in HAD clones of the brain	(Na <i>et al.</i> , 2011)		
77R	Predominant in Non-Dementia (ND) clones of blood	(Na <i>et al.</i> , 2011)				
Clinical outcomes associated with treatment status						
Amino acid substitution	Positive	Reference	Negative	Reference	No significant contribution	Reference
17A	More prevalent in HAART use but not in treatment naïve	(Fourati <i>et al.</i> , 2012)				
28N	More prevalent in HAART use but not in ART-naïve patients	(Cali <i>et al.</i> , 2005)				
32K	More prevalent in treatment patient use but not in treatment naïve	(Fourati <i>et al.</i> , 2012)				
68M	More prevalent in HAART use but not in ART-naïve patients	(Cali <i>et al.</i> , 2005)				

Abbreviations: ART: antiretroviral therapy; GDS: global deficit score; HAART: highly active antiretroviral therapy; HAD: HIV-associated dementia; LTNP: long-term non-progressor; ND: non-dementia; t/f: transmitted/founder.

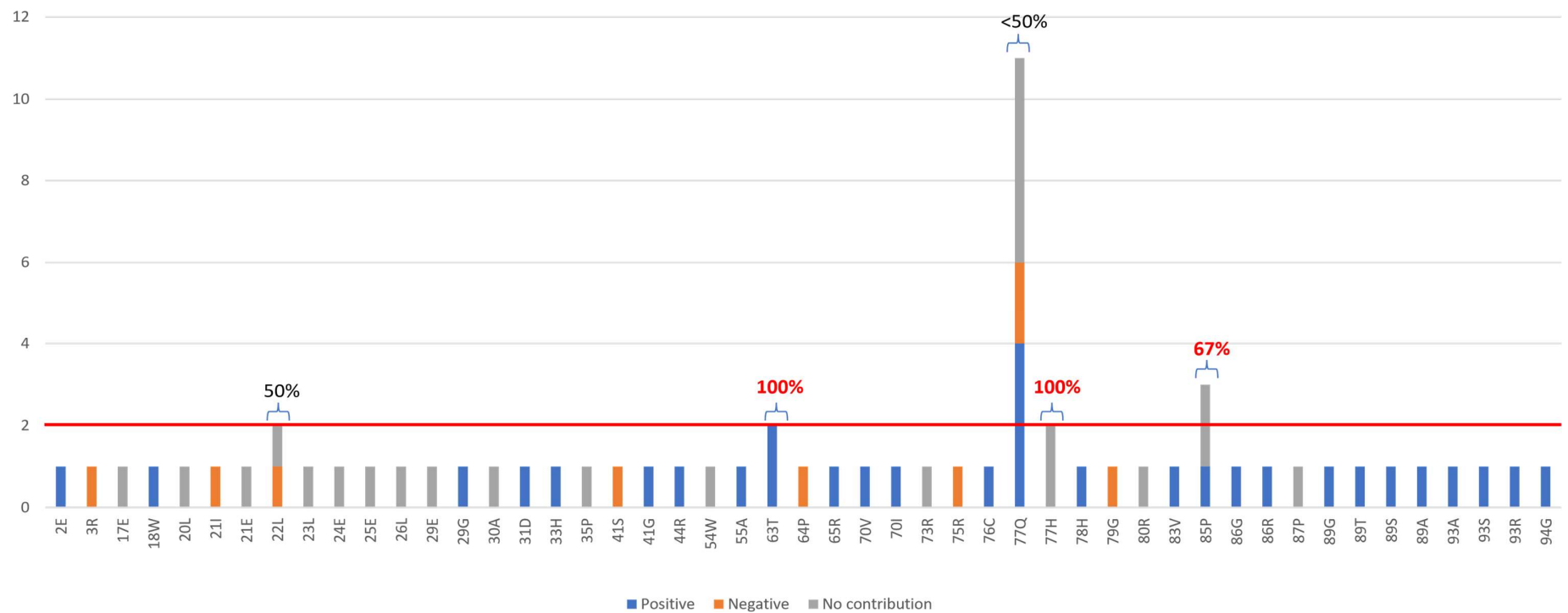


Figure 4: The frequency an amino acid substitution has been investigated in relation to disease progression/non-progression. Blue, Orange and grey bars indicate positive, negative and no contribution to clinical outcomes respectively. Criteria 1 (red line cut-off) was ≥ 2 independent studies that had to investigate a particular substitution. The percentage indicates the number of studies that reported the same clinical outcome for a particular substitution in relation to the number of studies that investigated the substitution (blue). Criteria 2 was that the percentage needed to be $>50\%$ to be considered a noteworthy finding.

2.3.5. Neurological outcomes

Certain amino acid substitutions were associated with positive clinical outcomes (non-stroke and decreased neurocognitive deficits) or negative clinical outcomes (stroke and increased neurocognitive deficits). The Vpr amino acid substitutions 22L, 37I, 41G, 41S, and 77R were associated with positive clinical outcomes and were more prevalent in non-stroke participants (McMullen *et al.*, 2021) and lower neurocognitive deficits (Dampier *et al.*, 2017; Na *et al.*, 2011) in people living with HIV (Table 1). The Vpr amino acid substitutions 21I, 41S, 41N, 55A, and 77Q were associated with negative clinical outcomes and were more prevalent with ischemic stroke (McMullen *et al.*, 2021) and pronounced neurocognitive deficits (Dampier *et al.*, 2017; Na *et al.*, 2011) in people living with HIV (Table 1). The Vpr amino acid substitution 41S was investigated by 2 or more independent studies, therefore, meeting our first criteria as a noteworthy finding (Figure 5). The findings for 41S were mixed as one of the two studies linked it to improved neurological outcomes, whereas the second one linked it to ischaemic stroke. Therefore, none of the Vpr amino acid substitutions investigated met our criteria as noteworthy for neurological outcomes (Table 1 and Figure 5).

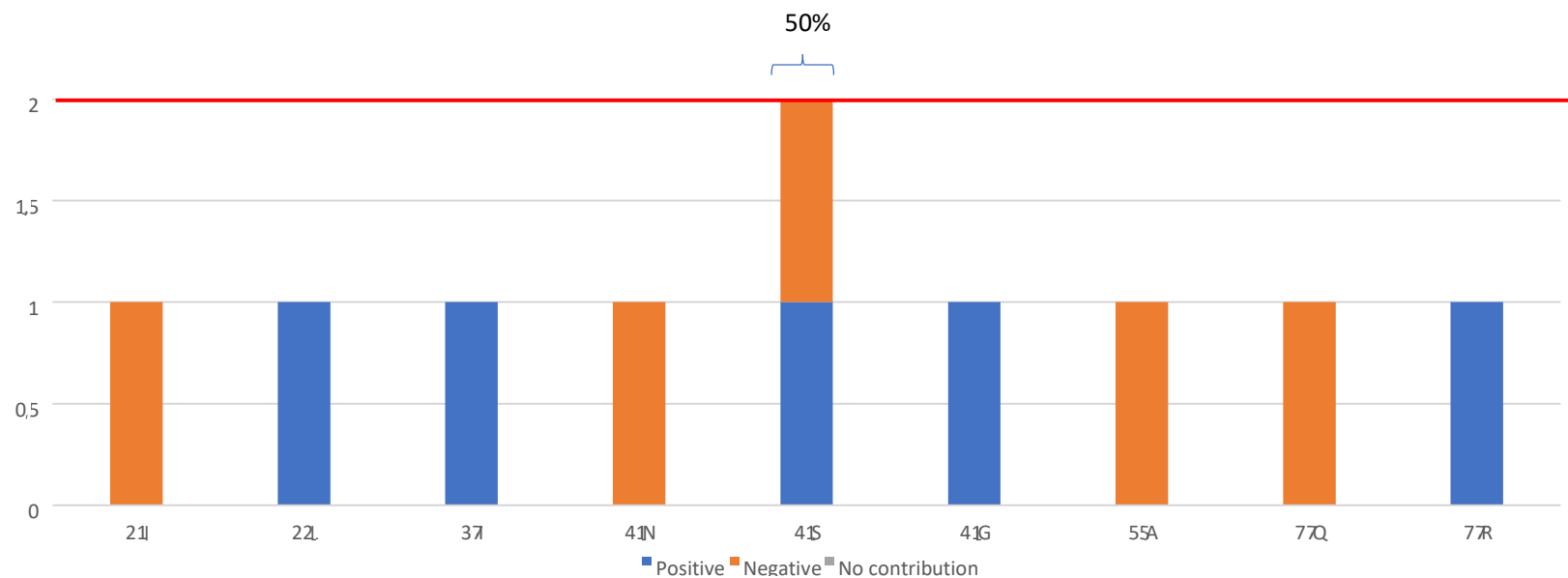


Figure 5: The frequency an amino acid substitution has been investigated in relation to neurological outcomes. Blue, Orange and grey bars indicate positive, negative and no contribution to clinical outcomes respectively. Criteria 1 (red line cut-off) was ≥ 2 independent studies that had to investigate a particular substitution. The percentage indicates the number of studies that reported the same clinical outcome for a particular substitution in relation to the number of studies that investigated the substitution (blue). Criteria 2 was that the percentage needed to be $>50\%$ to be considered a noteworthy finding.

2.3.6. Treatment status

Of all amino acid substitutions investigated; several were associated with treatment status by only occurring in people living with HIV that were using ART (Table 1). The Vpr amino acid substitutions 17A, 28N, 32K, and 68M were associated with positive clinical outcomes and were more prevalent in people living with HIV on HAART (Cali *et al.*, 2005; Fourati *et al.*, 2012) (Table 1). The findings for these amino acid substitutions were only reported by one independent study; therefore, none of the Vpr amino acid substitutions investigated met our criteria as noteworthy for treatment status (Table 1 and Figure 6).

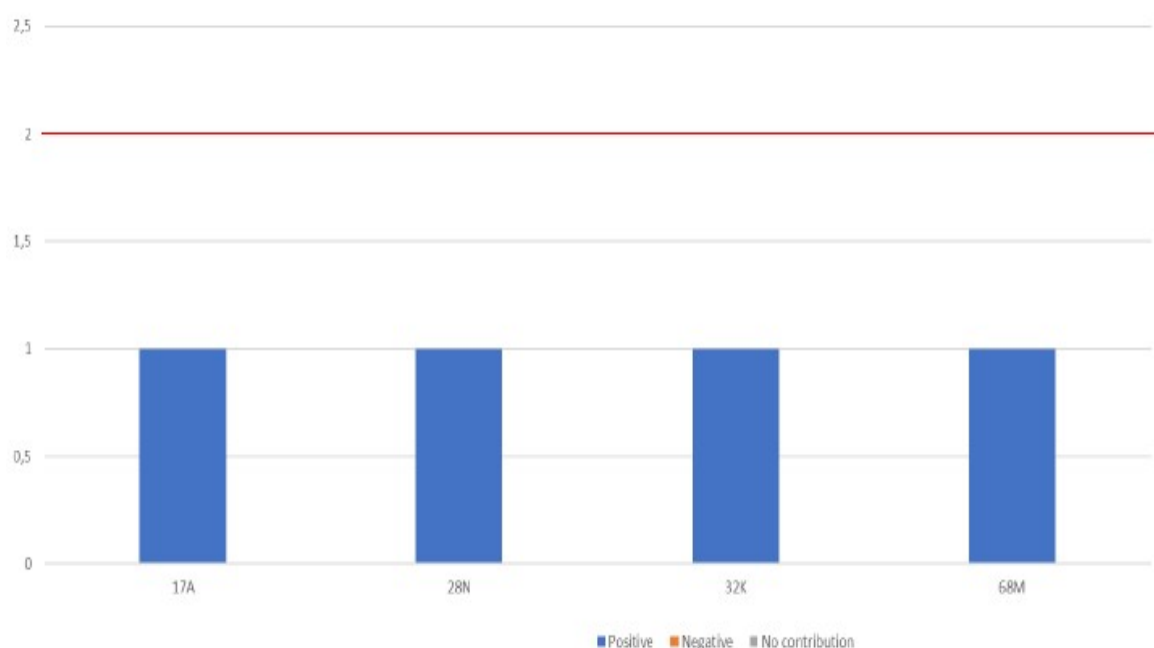


Figure 6: The frequency an amino acid substitution has been investigated in relation to treatment status (17A, 28N, 32K, and 68M). Blue, Orange and grey bars indicate positive, negative and no contribution to clinical outcomes respectively. Criterion 1 (red line cut-off) was >2 independent studies that had to investigate a particular substitution. Criterion 2 was that the percentage needed to be >50% to be considered a noteworthy finding. None of the substitutions met criteria one or two.

2.3.7. Potential confounders: Viral load/CD4⁺ count and HIV-1 subtype

Several factors may influence the reported association of Vpr amino acid substitutions and clinical outcomes. In particular, viral load and CD4⁺ count are well-known determinants of clinical outcomes in people living with HIV (Langford *et al.*, 2007). Therefore, we wanted to determine the potential influence of these confounders on the reported associations between Vpr amino acid substitutions and clinical outcomes in the reviewed studies. Therefore, studies were stratified according to the mean/median viral suppression (<400 cps/ml) or non-viral suppression (>400 cps/ml) (Supplementary Table 2). Studies reported viral load (VL) as a group mean (SD), median [IQR], or percentage distribution. For studies only reporting percentage distribution for virally suppressed participants, we considered viral suppression when more than 50% of participants were virally suppressed (i.e., suppression (<400 cps/ml) within the respective study). We do acknowledge that a fraction of the overall population may have been non-virally suppressed and therefore, findings reported for these studies should be interpreted with caution.

For longitudinal studies that have reported multiple measurements of viral load, the last recordings of viral load were used for stratification. The amino acid substitution investigated was considered at this time-point. If treatment-experienced participants were virally suppressed at baseline/study enrolment, we considered follow-up as virally suppressed due to continued use of ART. For studies that reported non-viral suppression at baseline, we then considered follow-up viral loads where available.

Of all studies reviewed (n = 22), n = 9 studies had not provided sufficient information to stratify participant groups according to viral suppression (Cavert *et al.*, 2004; Chen *et al.*, 2011; Ge *et al.*, 1996; Jacquot *et al.*, 2009; Na *et al.*, 2011; Shen *et al.*, 2008; Srinivasan *et al.*, 2008; Wang *et al.*, 1996; Zhang *et al.*, 1997). Of the remaining 13 studies, the majority (n = 12) investigated cohorts with a group of non-viral suppression (>400 cps/ml), and approximately 38.46% of these studies investigated Vpr amino acid substitutions that had an association with negative clinical outcomes (progressors, low GDS, Ischaemic stroke, t/f viruses, low CD4 count, and high viral load) in people living with HIV (Supplementary Table 2). Stratification showed different associations. One study had participants with a group viral suppression of <400 cps/ml, and this study reported an association between Vpr amino acid substitutions and negative clinical outcomes in people living with HIV. It is also relevant to note that n = 3 studies were case studies and the findings reported were from 3 or fewer participants and these findings may not be representative of larger

populations (Cali *et al.*, 2005; Soares *et al.*, 2014; Zhao *et al.*, 2002). Without stratifying studies according to viral status, a little under half (46.15%) of all studies reported an association between Vpr amino acid substitutions and negative clinical outcomes in people living with HIV.

For the current CD4⁺ count, not all studies used the same measure of CD4⁺ count. Therefore, the studies within this review were stratified into one of two categories depending on which was provided by the studies; group mean/median CD4⁺ count of <200 cells/μl or >200 cells/μl. For longitudinal studies with multiple measures of CD4⁺ count, the CD4⁺ count used for stratification of studies was the timepoint when the correlation between Vpr amino acid substitutions and clinical outcome was done. When study participants are stratified according to CD4⁺ count, most studies included participants with a higher CD4⁺ count (>200 cells/μl), and 26.67% of these studies reported an association between Vpr amino acid substitutions and negative clinical outcomes (progressors, low GDS, ischaemic stroke, t/f viruses, low CD4 count, and high viral load) in people living with HIV. Stratification showed different associations. Certain studies included participants with defined LTNP or non-LTNP status (Jacquot *et al.*, 2009; Shen *et al.*, 2008; Zhao *et al.*, 2002) and therefore for these studies, we also considered the LTNP status when stratifying according to CD4⁺ count (Supplementary Table 3). Of all studies reviewed, n = 4 studies had not provided sufficient information to stratify participant groups according to the current CD4⁺ count (Chen *et al.*, 2011; Ge *et al.*, 1996; Na *et al.*, 2011; Srinivasan *et al.*, 2008). Of the remaining eighteen studies, n = 3 studies could be stratified according to LTNP status. For these studies, LTNP status was linked with CD4⁺ counts of >200 cells/μl and in studies that had LTNP, Vpr amino acid substitutions were not associated with negative clinical outcomes (Jacquot *et al.*, 2009; Shen *et al.*, 2008; Zhao *et al.*, 2002). In studies with participants with progressive disease course of HIV, the findings for the association of Vpr amino acid substitutions with negative clinical outcomes were mixed (Jacquot *et al.*, 2009; Shen *et al.*, 2008; Zhao *et al.*, 2002). Of the remaining n = 15 studies, n = 12 studies investigated cohorts with a mean/median >200 cells/μl, and approximately 33% of these studies investigated Vpr amino acid substitutions with an association with negative clinical outcomes in people living with HIV (Supplementary Table 3). Three studies had participants with a group mean/median of <200 cells/μl, and two studies (66%) reported significant associations between Vpr amino acid substitutions and negative clinical outcomes in people living with HIV. From the studies reporting findings for CD4⁺ count, certain studies were from case studies and/or single participant data (Cali *et al.*, 2005; Jacquot *et al.*, 2009; Soares *et al.*, 2014; Wang *et al.*, 1996; Zhao *et al.*, 2002). Therefore, findings from these studies may not necessarily be representative of larger cohorts.

Most of the studies (64%) reported the HIV-1 subtype of which subtype B was most common (71%), followed by subtype C (14%), subtype A (7%), and circulating recombinant form (CRF)01-AE (7%). For the remaining studies that did not report the HIV-1 subtype, 18% of the total studies reported the geographical region, with one study each conducted in the USA, Canada, Portugal, and a combination of the USA and Australia respectively (Table 2). The remaining studies (17%) did not report the subtype or geographical region. Considering the findings for studies that reported an association of Vpr amino acids with negative clinical outcomes (n = 8) (Table 1) and studies with a confirmed HIV-1 subtype, (Table 2), it was noted that studies with subtypes B and C equally reported findings of Vpr amino acid substitutions with negative clinical outcomes. All key findings are summarised in Table 2.

Table 2: Summary of key findings for all reviewed studies

Reference	Subtype/ Geographical location (percentage of participants)	Cohort	Treatment	Amino acid investigated	Key findings
(Antoni <i>et al.</i> , 2007)	Subtype B: 93.33% Subtype C: 6.67%	People living with HIV: 15 Long-term non-progressors (LTNP): 8 Control (HIV-1 progressors): Treatment-naïve: 4 Rapid progressors (RP): 3 HIV-negative controls: N/A Age: N/A Male (%): N/A CD4 ⁺ count: LTNP: 747.25 (±314.68) cells/μl Control: 650 (±329.50) cells/μl Viral load: LTNP: 4837.5 (±6627.93) copies (cps)/ml Control: 99598.29 (±259465.20) cps/ml	Antiviral therapy (AVT): 13.33%	77Q	83.33% of LTNPs had a 77Q amino acid substitution. - Additional Los Alamos database scans for Vpr sequences: 77Q was present in: 77.6% subtype A sequences 46.0% subtype B sequences 78.0% subtype C sequences 61.4% subtype D sequences 100% subtype E sequences. 77Q was present in 14.29% of HIV-1 progressor control people living with HIV (rapid progressor).
(Cali <i>et al.</i> , 2005)	Subtype B: 100%	People living with HIV: 3	Highly active antiretroviral therapy (HAART): 66.67%	Full-length Vpr	Sequence data from the participant clones showed that:

		HIV-negative: N/A Age: N/A Male: N/A CD4⁺ count: 485 (±91.92) cells/μl Viral load: <10,000 cps/ml			90R occurred in a very high percentage (98%) of clones. 75R occurred in a low percentage (10%) of clones for both recipients A and B (progressors) 77Q occurred in a high percentage (92.8%) of clones of recipient B (progressor) and a low percentage (7.14%) of clones of recipient C (progressor). 68M occurred in a high percentage (85.71%) of clones of recipient B (progressor). 68M occurred in no clones of donor A (non-progressor) and a low percentage of clones of recipient B (progressor) (at a late stage). 28N occurred only in progressors but not in non-progressors. - Mean Vpr peptide diversity: Donor A (non-progressor): 0-5.5% Recipient B (progressor): 1.8-8.5% Recipient C (progressor): 0-15% - Phylogenetic analyses: Donor A (non-progressor) acquired fewer mutations than recipients B and C (progressors)
(Cavert <i>et al.</i> , 2004)	Subtype B: 100%	People living with HIV: 96 HIV-negative controls: N/A Age: N/A Male: N/A CD4⁺ count: 304 {186– 533} cells/μl Viral load: N/A	Prior zidovudine monotherapy: 76%	77Q	Vpr C-terminal (specifically 77Q) had no contribution to disease progression.
(Chen <i>et al.</i> , 2011)	CRF01-AE (51.63%) Subtype C (24.84%) Subtype B (17.65%)	People living with HIV: 208 (Vpr target gene amplified for 74% of participants)	AVT: 0%	Full-length Vpr, with a focus on C-terminal 77Q 77R	77Q was present in 84.3% of cases. - There were no significant differences in clinical indicators between 77Q and 77R. 77Q was more frequently associated with normal disease progression than LTNP.

	CRF03-AB (3.92%) CRF08-BC (1.31%)	HIV-negative controls: N/A Age: 14.5 – 61.4 Male: 59% CD4 ⁺ count: N/A Viral load: N/A			4. Amino acid substitutions in the C-terminal (51-96) were more apparent than that in the N-terminal (1-50). 5. 63T, 70V, 85P, 86G, 89G and 94G were associated with stabilized CD4 cell count and fewer complications citations. 6. M subtype is predominant in China, mainly subtypes AE, B and C.
(Chui <i>et al.</i> , 2006)	Subtype B: 97% Other Subtypes: 2.34%	People living with HIV: 728 HIV-negative controls: N/A Age: 37.2 (32.2–43.7) Male: 84.3% CD4 ⁺ count: 270 [130–415] cells/μl Viral load: 128824.96 [48977.88–331131.12] cps/ml	ART: 0%	77Q	1. 77Q was not a major determinant of HIV disease progression. 2. 77Q did not affect clinical response or survival over the first six years following initiation of HIV therapy.
(Dampier <i>et al.</i> , 2017)	United States of America (USA)	People living with HIV: 112 HIV-negative controls: N/A Age: 51 (46-56) Male: 64% CD4 ⁺ count: 641 (428-904) (cells/μl)	ART: Large % of the cohort received ART (exact % not provided)	41N 55A 37I 41S	1. 41N and 55A amino acid substitutions were associated with an increase in pronounced neurocognitive deficits (higher Global deficit score (GDS)). 2. 37I and 41S were associated with lower GDS.

		Viral load: 20 (13-193) cps/ml			
(Fischer <i>et al.</i> , 2004)	Subtype A: 53% Subtype B: 47%	People living with HIV: 55 Rwanda (29): LTNP: 14 Progressive disease and AIDS-defining conditions: 15 Luxembourg (26): Slow Progressors: 20 Progressors: 6 HIV-negative controls: N/A Age: N/A Male: N/A CD4⁺ count: Rwanda: LTNP: > 500 cells/μl for more than 12 years. Progressive disease and AIDS-defining conditions: N/A Luxembourg: Slow progressors: > 400 cells/μl Progressors: N/A Viral load: Rwanda: N/A	Rwanda Cohort: N/A Luxembourg Cohort: Slow progressors: Treatment: 0% Progressors: N/A	77Q	1. 77Q occurred in 55.9% of LTNP. 2. 77Q occurred in 57.1% of slow progressors. 3. 77Q occurred significantly more frequently in subtype A than in subtype B viruses. 4. A slow progressor had an unusual deletion of the four amino acids 'EPYN' in the N-terminal region of Vpr at positions 13–16. 5. Another slow progressor participant had a deletion in the C-terminal region, at positions 84 and 85.

		Luxembourg: Slow progressors: < 25 000 cps/ml without treatment Progressors: N/A			
(Fourati <i>et al.</i> , 2012)	Subtype B: 100%	People living with HIV: n = 160 HIV-negative controls: N/A Age: people living with HIV failing HAART: 46 (range: N/A) Treatment naïve: N/A Male: people living with HIV failing HAART: 89% Treatment naïve: N/A CD4⁺ count: people living with HIV failing HAART: 331 {N/A} cells/μl Treatment naïve: N/A Viral load: people living with HIV failing HAART: 4680 {N/A} cps/ml Treatment naïve: N/A	Antiretroviral agents (ARV)-experienced failing HAART: 60.63%	17A 32K	17A was associated with didanosine in the treatment histories of people living with HIV. - No associations were reported between 32K and drug-resistant amino acid substitution in Protease (PR) or Reverse Transcriptase (RT). - In treatment naïve people living with HIV, nearly 80% of amino acids were conserved. - From 10.63% of people living with HIV having Vpr 17A and 43.75% of people living with HIV having Vpr 17E, cluster 1 thymidine analog mutations-1 (TAM-1) (M41L, L210W and T215Y) were associated with 17A amino acid substitution. None of the cluster 2 TAM-2 (D67 N, K70R, T215F, and K219Q/E) were associated with Vpr 17A. 17A and 32K amino acid substitutions were more prevalent in treated people living with HIV.

(Ge <i>et al.</i> , 1996)	Subtype B: 100%	People living with HIV: 10 Intravenous Drug Users (IVDU): 8 LTNP: 2 HIV-negative controls: N/A Age: N/A Gender: N/A CD4⁺ count: N/A Viral load: N/A	N/A	Full-length Vpr	1. There were amino acid substitutions within 100% of HIV-1 infected IVDU. 2. LTNP had the most amino acid substitutions in the amino acid 77-96 region resulting in size polymorphism in the Vpr protein. 3. In-frame insertions or deletions occurred in 86% of the HIV-1 strains. 4. The absence of a stop codon occurred in 30% of HIV-1 strains. 5. Chou-Fasman algorithm: Amino acid residues 83-89: amino acid substitutions that have a profound effect on the secondary structure and molecular mass of the Vpr protein. 6. 85P occurred in 60% of analysed clones. Proline did not fit in the α-helix pattern. Its presence resulted in a stretch of the α-helix and therefore becoming terminated. 7. LW-87-1 sequences from this clone affected the Tat start codon. 8. Amino acid substitutions in both IVDU and LTNP are potentially linked to Vpr survival and functioning in HIV-1 pathogenesis
(Jacquot <i>et al.</i> , 2009)	Subtype B: 100%	People living with HIV: 2 LTNP: 1 Progressor: 1 HIV-negative controls: N/A Age: N/A Male: N/A CD4⁺ count: LTNP: >350 cells/μl Progressor: <200 cells/μl Viral load: N/A	ZDV: 100% of Progressor	Full-length Vpr	1. Samples were sampled from LTNP at three- time points- 1986, 1996, and 2004. 2. Sequence diversity of 100% of Vpr alleles from 2004 was consistent with an increased plasma viral load that paralleled a slight decline of the CD4⁺ count. 3. 77Q was present in 100% of Vpr alleles from 1986 LTNP samples and in 100% of Vpr alleles from 2004 LTNP samples. 4. 100% of Vpr alleles from 1996 LTNP samples had 77R. 5. 65R was present in the leucine-rich domain of 20% of Vpr alleles from 2004 LTNP samples 6. 33.33% of progressors had a substitution of 79G. 7. 33.33% of progressors had a 64P substitution.

(Kamori <i>et al.</i> , 2017)	Subtype B: 100%	People living with HIV: 444 HIV-negative controls: N/A Age (years): 34 (29-41) Male: 97.75% CD4⁺ count: 292.5 [183.5-407.0] cells/μl Viral load: 38904.51 [10964.78-141253.75] cps/ml	Treatment: 0%	55A 63T	55A was significantly associated with lower plasma viral load. 63T was significantly associated with lower plasma viral load and higher CD4 count.
(Lum, Cohen, Nie, Weaver, Gomez, Yao, Lynch, Pilon, Hawley & Kim, 2003)	Canada	People living with HIV: 25 LTNP: 10 Progressors: 15 HIV-negative controls: N/A Age: N/A Male: N/A CD4⁺ count: LTNP: N/A Progressor Controls: <500 cells/μl Viral load: LTNP: N/A Progressor Controls: >1,000 cps/ml	LTNP: N/A ART: 0% of controls	77Q	- LTNP: 77Q was present in 80% of people living with HIV and 20% had wild-type Vpr. - ART-naïve people living with HIV (CD4 count < 500): 77Q was present in 33% of people living with HIV. - Los Alamos database: 77Q occurred in 36% of progressors and 75% LTNPs.
(McMullen <i>et al.</i> , 2021)	Subtype C: 100%	People living with HIV: 134 Strokes: 54	ART Prior combination therapy (cART): Antiretroviral	22L 22I 41G	21I and 41S were associated with ischaemic stroke in HIV and had decreased CD4 counts and CD4 nadirs compared to the controls.

		Non-stroke Controls: 80 HIV-negative controls: N/A Age: 18-45 Strokes Male: N/A Non-stroke Controls Male: N/A CD4⁺ count: Strokes: 206 [276] cells/μl Non-stroke Controls: 329 [291] cells/μl CD4⁺ nadir: Strokes: 120 [236.8] Non-stroke Controls: 183 [179.5] Viral load: Strokes: 47863.01 {0-2511886.43} cps/ml Non-stroke Controls: 13803.84 {0-630957.34} cps/ml	Strokes: 48.10% Non-stroke controls: 53.80% Defaulted ART: Strokes: 26.90% Non-stroke controls: 0%	41S	2. 22L and 41G were associated with the non-stroke control group.
(Mologni <i>et al.</i> , 2006)	Subtype B: 100%	People living with HIV: 76 Progressors (Pr): 19 LTNP: 15 Sub-optimal treated people living with HIV: 23 people living with HIV with viral	Pr: 25% ART-naïve Sub-optimal treated people living with HIV: 30.26% on dual combination of either stavudine + lamivudine, stavudine + didanosine, zidovudine + lamivudine, zidovudine + didanosine, zidovudine + zalcitabine	77Q	1. 77Q was significantly higher in LTNP (86.7%) and sub-optimal treated people living with HIV (73.9%) compared to progressors (42.1%) and people living with HIV with viral rebound after treatment failure (42.1%) 2. Differences among these groups were statistically significant.

		rebound after treatment failure: 19 HIV-negative controls: N/A Age: Pr: 34.25 (± 8.68) LTNP: 40.90 (± 6.95) Sub-optimal treated people living with HIV: 42.13 (± 5.07) people living with HIV with viral rebound after treatment failure: 41.58 (± 5.03) Male: Pr: 58% LTNP: 73% Sub-optimal treated people living with HIV: 52% people living with HIV with viral rebound after treatment failure: 74% CD4⁺ count: Pr: 300 (3–763) cells/μl LTNP: 623 (515–1127) cells/μl Sub-optimal treated people living with HIV: 519 (325–952) cells/μl People living with HIV with viral			3. LTNP compared to progressors, there were significant differences in the frequency of 77Q amino acid substitution. 4. In sub-optimal treated people living with HIV and people living with HIV with viral rebound after treatment failure, there were significant differences in the frequency of 77Q. 5. 77H had no significant difference in LTNP and progressors, and in sub-optimal treated people living with HIV and people living with HIV with viral rebound after treatment failure. 6. In non-progressors, 77Q was significantly higher.
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		rebound after treatment failure: 203 (1–449) cells/μl Viral load: Pr: 37734 {187–620000} cps/ml LTNP: 2567 {59–21954} cps/ml Sub-optimal treated people living with HIV: 597 {< 50–9758} cps/ml People living with HIV with viral rebound after treatment failure: 89480 {1880–500000} cps/ml Years of infection: Pr: 1.5 ± 5.41 LTNP: 14 ± 2.30 Sub-optimal treated people living with HIV: 12 ± 4.05 People living with HIV with viral rebound after treatment failure: 12 ± 4.32			
(Na <i>et al.</i> , 2011)	N/A	People living with HIV: N/A HIV-negative controls: N/A Age: N/A Male: N/A	N/A	Full-length Vpr	1. Brain-derived sequences: 77Q predominated in 94.44% of HIV-associated dementia (HAD) clones. 2. 77R was mostly present in 77.77% of Non-Dementia (ND) clones. 3. Blood-derived sequences: No amino acid changes distinguished HAD and ND in blood were not evident.

		CD4⁺ count: N/A Viral load: N/A			4. Synonymous amino acid substitutions within clinical groups: no difference within the blood- or brain-derived sequences. 5. The frequencies of non-synonymous amino acid substitutions were significantly reduced within the HAD brain-derived sequences compared with the HAD blood-derived sequences. 6. dN/dS rates: No significant difference 7. The amount of amino acid differences were significantly reduced in the HAD brain-derived sequences than in HAD blood-derived sequences. 8. The frequency of detection of Vpr transcripts were significantly higher in the brain among people living with HIV with HAD (59%) than in people living with HIV with ND (31%). It was detected in all blood-derived samples examined.
(Rossenkhan <i>et al.</i> , 2016)	Subtype C: 100%	People living with HIV: 17 Acute infected: 6 Early infected: 11 HIV-negative controls: N/A Age: Acute: 31.50 (26.75–34.25) Early: 25.50 (24.75–27.00) Male: Acute: 17% Early: 27% CD4⁺ count: Acute: 337 {258–426} cells/μl Early: 374 {258–490} cells/μl Viral load:	N/A	4P 4A 22L 70I 93A	1. In Vpr, transmitted/founder (t/f) viruses (single/few viral variants that had a productive infection within a host) had a reduced frequency 4P by almost 25% compared to the chronic infection. However, this was not significant. 2. 4P expressed a different dominant amino acid in t/f compared with chronic viruses. 3. 22L was significantly enhanced in t/f viruses compared with chronic sequences. 4. 70I and 93A were significantly underrepresented compared with chronic sequences. 5. Investigating the predicted epitope binding affinity for Vpr variants predicted median IC₅₀ of putative epitopes for Vpr variants that arose during early infection (IC₅₀ 243 [IQR: 99–601 nM]) compared with those encoded in t/f viruses (192 [IQR 171–318] nM). 6. Amino acid substitutions at anchor residues had significantly lower predicted epitope binding affinity compared with the respective epitope in the t/f virus for Vpr (192 vs. 283 nM). 7. Thus, amino acid substitutions at anchor residues in the t/f virus are more likely to activate Major Histocompatibility Complex (MHC) which aids the immune system to recognize foreign material.

		Acute: 89125.09 {10000–331131.12} cps/ml Early: 25118.86 {6918.31–56234.13} cps/ml			
(Shen <i>et al.</i> , 2008)	Subtype B: 100%	People living with HIV: 15 LTNP: 8 AIDS: 7 HIV-negative controls: N/A Age: N/A Male: N/A CD4 ⁺ count: LTNP: 670.5 (±90.43) cells/μl AIDS patients: 147.43 (±27.34) cells/μl Viral load: N/A	ART: 100%	Full-length Vpr	1. The following are all highly conserved in LTNP and AIDS participants of Plasma/Blood Donors (PBDs): all the proline and serine amino acids in the N-terminal or C-terminal of Vpr, amino acids at positions 17, 21, 24, 25, and 29, four hydrophobic polar leucine's at amino acid positions 20, 22, 23, and 26, and alanine at amino position 30 in the first α-helices are all highly conserved in LTNP and AIDS participants of PBDs. 2. 35P and 54W, lying between the first-second helices and second-third helices, respectively, functional amino acids in second and third helices, as well as the H(S/F)RIG motif in the third helix are very well conserved in all 15 people living with HIV, irrespective of disease stages. 3. 73R and 80R were highly conservative in all people living with HIV. 4. 77Q amino acid substitution was present in all PBD people living with HIV irrespective of disease stages. 5. All the predicted binding motifs were highly conserved with slight changes seen in LTNP and AIDS patients. 6. All Vpr sequences were tightly clustered with, firstly, Chinese B and, secondly, Thailand B and intermingled together regardless of disease stage. 7. There was no difference between the asymptomatic people living with HIV and AIDS participants. 8. Analysis of the dS/dN ratio and potential glycosylation sites: No significant difference in the dS/dN ratio and glycosylation sites of Vpr between patients at different stages of the disease. 9. All Vpr sequences in our study had an intact ORF, with 96 amino acids regardless of disease stages.

(Soares <i>et al.</i> , 2014)	Portugal	People living with HIV: 3 HIV-negative controls: N/A Age: Child: 5 Mother: N/A Father: N/A Male: 66.66% CD4⁺ count: Child: 0 cells/μl Mother: N/A Father: N/A Viral load: Child: 1 073 899 cps/ml Mother: N/A Father: N/A	Upon diagnosis: 33.33% received combined oral therapy of lamivudine 4 mg kg ⁻¹ twice a day, zidovudine 180 mg m ⁻² twice a day, lopinavir/ritonavir 10/2.5 mg ⁻¹ kg ⁻¹ twice a day	77Q 3R 77H	1. Child: amino acid substitutions 77Q and 3R were present, initial CD4 count was 0 cells/μl and initial viral load was 1 073 899 cps/ml. 2. Mother and the father: 77H amino acid substitution was present. The mother was asymptomatic, and the father rapidly progressed to AIDS.
(Srinivasan <i>et al.</i> , 2008)	N/A	People living with HIV: 976 Vpr sequences HIV-negative controls: N/A Age: N/A Male: N/A CD4⁺ count: N/A Viral load: N/A	N/A	Full-length Vpr	• Influence of amino acid substitutions on CTL epitope, which was associated with immune control, with respect to HLA-A2 molecule, half-time of dissociation values were as follows: • Reference peptide of the epitope located at amino acids 18-26: 1213.356 • Amino acid substitutions at positions 1-9: predicted to be lower than reference peptide. • Amino acid substitutions at position 2: affected the half-time value considerably. • R amino acid substitution: 0.233 • F9L amino acid substitution: 4.233 • Amino acid substitutions at positions 39 and 41: drastically lowered half-time • Amino acid substitution at position 46: contrasting values dependent on the nature of the amino acid substitution present

					Thus, lower half-time dissociations require a higher concentration of amino acid substitutions to produce 50% of the CTL epitope binding at equilibrium.
(Wang <i>et al.</i> , 1996)	N/A	People living with HIV: 32, Mother and child plus 30 other people living with HIV HIV-negative controls: N/A Age: N/A Male: child, 30 other people living with HIV (N/A) CD4⁺ count: 725 (±35,36) cells/μl 30 other people living with HIV: N/A Viral load: N/A	N/A	Full-length Vpr	- Mother and child were non-progressors. The 30 other people living with HIV died of AIDS or have AIDS-related illnesses. - Non-progressors had fewer defects at the C-terminus region and had a peptide length of 95 amino acids due to an in-frame deletion of 88R. - Unique changes in non-progressor RNA clones include an 89A in 69.23% of clones. - Vpr clones of people living with HIV that died of AIDS or have AIDS-related illnesses had more genetic diversity at the C-terminus (in-frame insertions/deletions) causing considerable length polymorphism in the Vpr protein. - Signature amino acid changes between people living with HIV that died of AIDS or have AIDS-related illnesses and non-progressor clones are at positions 28, 37, 41, 55, 77, and 83. - Amino acid substitutions mostly affected β-turns, which are important in the structural stabilization of Vpr. 62% of clones from people living with HIV that died of AIDS or have AIDS-related illnesses had a proline at positions 85 and 87, replacing the highly charged arginine at those positions. 69% of non-progressor clones had proline at positions 85 and 87. - Clones without prolines had amino acid substitutions at position 70, which plays an important role in the formation of the leucine zipper motif (LXXXXXXL). Natural amino acid substitutions (70VT) alter the zipper found between amino acids 60 and 81.
(Zhang <i>et al.</i> , 1997)	N/A	People living with HIV: 10 HIV-negative controls: N/A	N/A	Full-length Vpr	- The degrees of inter-subject sequence diversity of the LTNP and AIDS participants were not significant. - Point deletion causing frameshift was found in two Vpr clones. Average defective frequencies were 3.8% in Vpr.

		Age: 42.56 (± 3.61) Male: 88.89% CD4⁺ count: 400-1200 cells/μl Viral load: N/A			- LTNP sequences: 2E, 18W, 33H, 78H and 76C were well conserved. - The region between amino acids 85 and 95 from the LTNP showed some variation. Particularly, 89T in 55.56% of people living with HIV, or 89S in 11.11% of people living with HIV. 93S in 11.11% of people living with HIV and 86R and 93R, respectively in 11.11% of people living with HIV.
(Zhao <i>et al.</i> , 2002)	12 HIV-1 viral strains: N/A 2 FP: USA LW and JW: Australia	People living with HIV: LTNP: Mother and child Fast progressing children (FP): 2 HIV-negative controls: N/A Age: N/A Male: N/A CD4⁺ count LTNP: LW (mother): 608 (± 90) cells/μl JW (child): 674 (± 102) cells/μl CD4⁺ count FP: FP3254: 176 (± 133) cells/μl FP4496: 148 (± 94) cells/μl Viral load LTNP: LW (mother): 3890.45 (± 3.63) cps/ml JW (child): 3801.89 (± 2.57) cps/ml	ART: 0% The mother started therapy in 1997 and the child was treated briefly for 3 months.	Full-length Vpr	- Vpr gene amino acid substitutions isolated from the JW/LW pair of LTNPs were unique as they interrupted a normally highly conserved domain. 5 unique variants were identified for FP. - Two Vpr sequences isolated from the mother in 1994 and 1995 (JW94-1 and JW95-1) were differentiated by Q44R and I83V. - VprLW95-1182 contained numerous amino acid substitutions. All Vpr mutants, carried amino acid substitution(s) within the first conserved block, except Vpr LW94-2. - A few single amino acid substitutions were identified in the first conserved block. 31D and 41G, in VprLW89-1 and VprJW89-3, were within the α-helix-turn-α-helix. 29G in VprLW95-1.

		Viral load FP: FP3254: 446683.59 (± 2.57) cps/ml FP4496: 371535.23 (± 3.47) cps/ml CD4/CD8 ratio LTNP: N/A CD4/CD8 ratio FP: FP3254: 0.17 ± 0.10 FP4496: 0.12 ± 0.05			
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Longitudinal studies: (Cavert *et al.*, 2004), (Chen *et al.*, 2011), (Chui *et al.*, 2006), (Jacquot *et al.*, 2009), (Rossenkhan *et al.*, 2016), (Soares *et al.*, 2014), (Wang *et al.*, 1996), (Zhao *et al.*, 2002). Cross-sectional studies: (Antoni *et al.*, 2007), (Cali *et al.*, 2005), (Dampier *et al.*, 2017), (Fischer *et al.*, 2004), (Fourati *et al.*, 2012), (Ge *et al.*, 1996), (Kamori *et al.*, 2017), (Lum, Cohen, Nie, Weaver, Gomez, Yao, Lynch, Pilon, Hawley & Kim, 2003), (McMullen *et al.*, 2021), (Mologni *et al.*, 2006), (Na *et al.*, 2011), (Shen *et al.*, 2008), (Srinivasan *et al.*, 2008), (Zhang *et al.*, 1997). Case studies: (Cali *et al.*, 2005; Jacquot *et al.*, 2009; Soares *et al.*, 2014; Wang *et al.*, 1996; Zhao *et al.*, 2002). Studies that reported sample types other than blood: (Na *et al.*, 2011), (Zhao *et al.*, 2002). Mean (standard deviation); Range; Median [IQR]; mean (range); median $\pm$ standard deviation; median {range}. Age (years): mean (SD) OR median (range); CD4 count or Viral load: Mean (standard deviation); Range; Median [IQR]; mean (range); median $\pm$ standard deviation; median {range}.

2.4. Discussion

This review summarises the key findings for the association between Vpr substitutions and clinical outcomes in people living with HIV. The studies used to compile this review made use of wild type sequences from sequencing data bases. When these amino acids were different from the wild type sequences, the amino acid substitutions would result in different severity HIV-1 pathogenesis (LTNP, progressors, impaired neurocognitive impairments due to HIV infection). We reported the Vpr amino acid substitutions that were associated with specific clinical outcomes including disease progression, neurological outcomes, and treatment status in people living with HIV. Certain Vpr amino acid substitutions reported consistent associations with clinical outcomes in people living with HIV and were therefore considered noteworthy within this study. (1) The Vpr substitution 63T was associated with characteristics of slower disease progression whereas 77H and 85P were associated with no significant contribution to disease progression, (2) preliminary evidence suggests that Vpr amino acid substitutions may have implications in neurological outcomes and treatment efficacy in people living with HIV, despite limited investigation and (4) regardless of higher viral load and lower CD4⁺ counts, studies still report an association of Vpr amino acid substitutions with negative clinical outcomes in people living with HIV.

First, Vpr amino acid substitutions are associated with clinical outcomes in people living with HIV. Nearly a third (31.82%) of all studies reported an association between Vpr amino acid substitutions and negative clinical outcomes in people living with HIV. Much of our understanding of the role of Vpr amino acid substitutions in HIV-1 pathophysiology has been derived from *in vitro/in vivo* studies. However, these amino acid substitutions do not necessarily represent the Vpr amino acid substitutions present in people living with HIV. To our best knowledge, this is the first study that systematically reviewed the Vpr amino acid substitutions found in people living with HIV and their association with clinical outcomes.

Second, we considered the following findings as noteworthy; (1) 63T was more commonly associated with positive clinical outcomes including higher CD4⁺ counts and lower viral load, and Vpr 77H and 85P were more commonly associated with no significant contribution to disease progression. Limited studies have reported findings for the Vpr 63T substitution in clinical or *in vitro/in vivo* studies, and no findings for 63T have been reported in previous reviews done on this topic (Fabryova & Strebel, 2019; Soares *et al.*, 2016). However, as

reported in this review, 63T should be investigated for its potential influence on positive clinical outcomes in people living with HIV. In particular, the 63T has been associated with characteristics of slower disease progression (Chen *et al.*, 2011; Kamori *et al.*, 2017). When comparing the Vpr sequences between subtypes (Figure 1), subtype C was the only subtype that presented with the 63T substitution. Considering the fact that subtype C has been associated with slower disease progression and replication fitness (Abraha *et al.*, 2009; Ball *et al.*, 2003; Marozsan *et al.*, 2005; Venner *et al.*, 2016), it is plausible to speculate that 63T may have a functional role in this outcome. However, too few studies have investigated this particular mutation to make a conclusion and a need exists to further investigate on both clinical and fundamental levels to elucidate the role of 63T in HIV-1 pathogenesis. Further, Vpr 77H was more commonly associated with no significant contribution to disease progression (Mologni *et al.*, 2006; Soares *et al.*, 2014). In general, 77H is a less investigated substitution in contrast to Vpr 77Q. It is relevant to note that the Vpr 77Q substitution was also the most widely investigated amino acid substitution across all studies. However, as reported here, there is no clear consensus on whether 77Q influences disease progression or non-progression in people living with HIV as the findings were mixed. Therefore, as reported here 77H may have no influence on disease progression, however, further investigation is needed to ascertain the findings summarised in this review. The findings for 85P were also associated with no significant contribution to disease progression. However, fundamental studies have associated 85P with characteristics of LTNP (Wang *et al.*, 2013) and with a reduction in the apoptosis-inducing ability (Zheng *et al.*, 2009). This provides some evidence that the 85P amino acid substitution may be an indication of slower disease progression and be investigated as a marker thereof. However, these findings for 85P were reported in fundamental investigations and similar findings may not be reflected in clinical studies.

Third, studies investigating the association between Vpr amino acid substitutions and neurological outcomes in people living with HIV remain limited and therefore no conclusions could be drawn from these studies. However, preliminary studies reviewed here suggest that Vpr amino acid substitutions may influence neurological outcomes. Here we included two studies that reported findings for Vpr amino acid substitutions and neurological outcomes (Dampier *et al.*, 2017; McMullen *et al.*, 2021). Although the amino acid substitutions investigated within this study were not deemed noteworthy, it does provide some evidence of an association between Vpr and neurological outcomes (ischaemic stroke, non-ischaemic stroke, neuroprotection, non-neuroprotection). In general, limited studies have investigated the neurological implications of Vpr amino acid substitutions in people living with HIV;

however, the findings here have highlighted the potential role of amino acid substitutions 22I, 22L, 37I, 41N, 41S, 41G,55A, 77Q and 77R in neurological outcomes in people living with HIV. A recent study (*in press*) has also highlighted that the presence of signature 45Y in subtype C infection was associated with a 3.66 times higher global deficit score (GDS), 525 times higher plasma viral load, 15.84 times higher proviral load and 60% lower absolute CD4-T cell count compared to those without the signature (Ruhanya *et al.*, 2023b). This highlights the potential value of investigating the implication of Vpr amino acid substitutions in the development of neurocognitive impairments.

Fourth, studies investigating the association between Vpr amino acid substitutions and treatment status in people living with HIV remain limited and therefore no conclusions could be drawn from these studies. However, preliminary studies reviewed here suggest that Vpr amino acid substitutions may influence treatment efficacy. Here we included two studies that reported findings for Vpr amino acid substitutions and treatment efficacy. Even though studies investigating this remain limited for Vpr, previous studies of other viral proteins (*e.g.*, gp120 and gag) have shown that sequence substitutions L116P, A204D, S375H/M/T, M426L, M434I, and M475I in gp120 affect the efficacy of treatment, fostemsavir (Lepore *et al.*, 2020). Despite S375T being the most predominant, it had a less substantial role in the reduction of fostemsavir efficacy compared to other sequence substitutions (Lepore *et al.*, 2020). For Gag, cleavage site amino acid substitutions A431V and I437V, found in the NC-SP2-p6 region, contributed to protease inhibition resistance (Dam *et al.*, 2009) whereas, adding N374S, E428, and A431V to major resistance-associated protease substitutions results in increased phenotypic resistance to RTV (Giandhari *et al.*, 2016). Therefore, if Vpr amino acid substitutions follow a similar trend as reported for other HIV-1 viral proteins, we hypothesize that Vpr amino acid substitutions may also influence treatment efficacy and should be investigated as potential indicators thereof.

Last, certain studies (Antoni *et al.*, 2007; Cali *et al.*, 2005; Cavert *et al.*, 2004; Chui *et al.*, 2006; Dampier *et al.*, 2017; Fischer *et al.*, 2004; Fourati *et al.*, 2012; Jacquot *et al.*, 2009; Kamori *et al.*, 2017; Lum, Cohen, Nie, Weaver, Gomez, Yao, Lynch, Pilon, Hawley, Kim, *et al.*, 2003; McMullen *et al.*, 2021; Mologni *et al.*, 2006; Rossenkhani *et al.*, 2016; Shen *et al.*, 2008; Soares *et al.*, 2014; Wang *et al.*, 1996; Zhang *et al.*, 1997; Zhao *et al.*, 2002) have also shown that despite higher CD4⁺ count and lower viral loads, Vpr amino acid substitutions may still

influence clinical outcomes and therefore these findings are relevant considering the modern ART treatment era.

2.5. Limitations

The studies included in this review presented several limitations which we would briefly like to discuss. The studies presented in this review were diverse and heterogeneous. The heterogeneity of studies can be explained as follow: Studies employed different design (longitudinal and cross-sectional) and different measures of clinical outcomes (e.g., neurological, disease progression, etc). Therefore, a meta-analysis could not be conducted. This is particularly related to key cohort information, including the clear description of inclusion/exclusion criteria as well as the setting or infecting viral subtype of the included participant. Further, studies do not clearly define potential confounding factors nor relevant statistical analysis to control for these variances. There were also other confounding factors above and beyond what we investigated which may have influenced the findings collectively of this systematic review. Subtype variation is associated with specific amino acid substitutions within the HIV-1 viral proteins, including Vpr, therefore the clear reporting of this may assist in contextualising the findings. Limited studies have reported viral load and CD4⁺ count and both factors have clear influences on clinical outcomes in people living with HIV (Langford *et al.*, 2007) and this should be clearly defined in studies investigating subtype-specific protein variation and clinical outcomes. Therefore, it is not clear how these findings will reflect in the modern ART era. Sample/cohort sizes varied significantly across studies. This potentially had an influence on the level and/or associations of genetic diversity observed. Case studies or studies including small cohort size may present genetic variation that may not necessarily be representative of larger cohorts. Therefore, the association of genetic variation with clinical outcomes from such studies should be interpreted with caution. It should also be noted that most of the studies included in this review were more than 10 years old and consideration should be taken when interpreting the reported findings. Considering the significant development of HIV-1 research and genome sequencing over the past 10 years, this highlights the need for newer studies related to this topic. This will aid in the further understanding of the correlation between Vpr amino acid substitutions and clinical outcomes in people living with HIV.

2.6. Conclusions

Studies have collectively reported that Vpr amino acid substitutions may be associated with clinical outcomes including disease progressions, neurological outcomes, treatment status, and other clinical manifestations. The Vpr amino acid substitutions, 63T were more commonly associated with positive clinical outcomes, and 77H and 85P were associated with no contribution to disease progression. Preliminary evidence suggests that Vpr amino acid substitutions may have implications for neurological outcomes and treatment efficacy in people living with HIV. The Vpr amino acid substitutions highlighted in this review should be investigated further to determine their potential role as predictive indicators of disease progression or anchor points to better understand their implications in HIV-1 pathophysiology. Therefore, in the next chapters, I used the insight gained from Chapter 2 to investigate amino acid variants present at positions 22, 41, 45, and 55 to determine their influence on immune activation and inflammation (chapter 3) as well as Tryptophan-Kynurenine metabolism (chapter 4) in a treatment-naïve population. Although this systematic review did identify the amino acid variants and their positions which are associated with clinical outcomes in people living with HIV, we aimed to determine the potential influence that the amino acid variants at these positions may potentially have on the inflammatory and metabolic markers which are associated with HAND. The results from chapter 3 and 4, could potentially provide information on the underlying pathological pathways which are associated with the neurological outcomes observed in people living with HIV. Furthermore, this unique population is treatment-naïve and allows us to investigate the immune response in these individuals without the confounding effects of ART.

2.7. LIST OF ABBREVIATIONS

Acquired immunodeficiency syndrome (AIDS), Antiretroviral therapy (ART), Human immunodeficiency virus-1 (HIV-1), Joanna Briggs Institute (JBI), Long-term non-progressors (LTNP), Viral load (VL), Viral protein R (Vpr).

2.8. References

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2.9. Supplementary: Chapter 2

Supplementary file 1

Date of scan: 06/09/2022

Total: 984

Pubmed: 250

(HIV [mh] OR HIV [tw] OR Acquired Immunodeficiency Syndrome [mh] OR "acquired immunodeficiency syndrome" [tw] OR AIDS [tw]) AND (Gene Products, vpr [mh] OR Vpr [tw] or Viral protein R [tw]) AND (mutation [mh] OR protein mutation [tw] OR Polymorphism, Genetic [mh] OR amino acid variation [tw] OR protein signatures [tw])

Scopus 319:

(HIV OR HIV-1 OR Acquired Immunodeficiency Syndrome OR AIDS) AND ("Vpr" OR "vpr" OR "Viral protein R" OR "Viral protein r" or "viral protein r") AND (mutation OR protein mutation OR polymorphism or amino acid variation OR protein signature)

Web of science 415

TS=(HIV OR Acquired Immunodeficiency Syndrome OR "Acquired Immunodeficiency Syndrome" OR AIDS) AND TS=(Vpr OR Viral protein R) AND TS=(mutation OR protein mutation OR polymorphism or amino acid variation OR protein signature)

Supplementary Table 1: Quality Assessment conducted by LKA and MEW

Reference	Q1	Q2	Q3	Score	Rating
(Antoni <i>et al.</i> , 2007)	2	2	2	6	High
(Cali <i>et al.</i> , 2005)	2	2	0	4	Intermediate
(Cavert <i>et al.</i> , 2004)	2	2	1	5	High
(Chen <i>et al.</i> , 2011)	2	2	0	4	Intermediate
(Chui <i>et al.</i> , 2006)	2	2	2	6	High
(Dampier <i>et al.</i> , 2017)	2	2	2	6	High
(Fischer <i>et al.</i> , 2004)	2	2	0	4	Intermediate
(Fourati <i>et al.</i> , 2012)	2	2	1	5	High
(Ge <i>et al.</i> , 1996)	2	1	0	3	Intermediate
(Jacquot <i>et al.</i> , 2009)	2	2	0	4	Intermediate
(Kamori <i>et al.</i> , 2017)	2	2	1	5	High
(Lum, Cohen, Nie, Weaver, Gomez, Yao, Lynch, Pilon, Hawley & Kim, 2003)	2	2	0	4	Intermediate
(McMullen <i>et al.</i> , 2021)	2	2	2	6	High
(Mologni <i>et al.</i> , 2006)	2	2	0	4	Intermediate
(Na <i>et al.</i> , 2011)	1	1	0	2	Low
(Rossenkhan <i>et al.</i> , 2016)	2	2	2	6	High
(Shen <i>et al.</i> , 2008)	2	2	0	4	Intermediate
(Soares <i>et al.</i> , 2014)	2	2	0	4	Intermediate
(Srinivasan <i>et al.</i> , 2008)	1	1	1	3	Intermediate
(Wang <i>et al.</i> , 1996)	1	1	0	2	Low
(Zhang <i>et al.</i> , 1997)	1	2	0	3	Intermediate
(Zhao <i>et al.</i> , 2002)	2	2	0	4	Intermediate

Reference	Q1	Q2	Q3	Score	Rating
(Antoni <i>et al.</i> , 2007)	1	1	1	3	Intermediate
(Cali <i>et al.</i> , 2005)	1	1	1	2	Intermediate
(Cavert <i>et al.</i> , 2004)	2	2	1	5	High
(Chen <i>et al.</i> , 2011)	1	2	1	4	Intermediate
(Chui <i>et al.</i> , 2006)	2	2	2	6	High
(Dampier <i>et al.</i> , 2017)	2	2	2	6	High
(Fischer <i>et al.</i> , 2004)	1	1	1	3	Intermediate
(Fourati <i>et al.</i> , 2012)	1	1	1	3	Intermediate
(Ge <i>et al.</i> , 1996)	1	1	1	3	Intermediate
(Jacquot <i>et al.</i> , 2009)	2	1	1	4	Intermediate
(Kamori <i>et al.</i> , 2017)	2	2	2	6	High
(Lum, Cohen, Nie, Weaver, Gomez, Yao, Lynch, Pilon, Hawley & Kim, 2003)	1	2	1	4	Intermediate
(McMullen <i>et al.</i> , 2021)	2	2	1	5	High
(Mologni <i>et al.</i> , 2006)	1	2	1	4	Intermediate
(Na <i>et al.</i> , 2011)	1	1	1	3	Intermediate
(Rossenkhan <i>et al.</i> , 2016)	2	1	2	5	High
(Shen <i>et al.</i> , 2008)	2	1	1	4	Intermediate
(Soares <i>et al.</i> , 2014)	2	1	1	4	Intermediate
(Srinivasan <i>et al.</i> , 2008)	0	1	1	2	Low
(Wang <i>et al.</i> , 1996)	1	1	0	0	Low
(Zhang <i>et al.</i> , 1997)	1	1	1	3	Intermediate
(Zhao <i>et al.</i> , 2002)	1	1	1	3	Intermediate

Questions (Q) 1- 3. (1) Were the criteria for inclusion in the sample clearly defined and were study subjects and the setting described? (2) was the method used to investigate the clinical

outcome done in an objective, valid and reliable method (inclusion of viral and CD4 count) and (3) did they consider confounding factors, and did they employ relevant statistical analysis? This included a total of 3 questions. Each question was rated for 0 = no, 1 = partly and 2 = yes. Studies that addressed all the above questions and had a total rating of ≥ 5 were classified as high quality. Studies with a rating between 3 and 4 were considered intermediate-quality and ≤ 2 as low quality.

Supplementary Table 2: studies reporting a relationship between Vpr-specific amino acids and clinical outcomes in people living with HIV when stratified according to viral suppression.

Reference	Virally suppressed <400 copies/ml	Non-virally suppressed >400 copies/ml	Association with negative clinical outcomes	
			Yes	No
(Antoni <i>et al.</i> , 2007)		✓		✓
(Cali <i>et al.</i> , 2005)		✓	✓	✓
(Chui <i>et al.</i> , 2006)*		✓		✓
(Dampier <i>et al.</i> , 2017)	✓		✓	✓
(Fischer <i>et al.</i> , 2004)		✓		✓
(Fourati <i>et al.</i> , 2012)		✓		✓
(Kamori <i>et al.</i> , 2017)		✓		✓
(Lum, Cohen, Nie, Weaver, Gomez, Yao, Lynch, Pilon, Hawley & Kim, 2003)		✓		✓
(McMullen <i>et al.</i> , 2021)		✓	✓	✓
(Mologni <i>et al.</i> , 2006)		✓		✓
(Rossenkhan <i>et al.</i> , 2016)*		✓	✓	✓
(Soares <i>et al.</i> , 2014)*		✓	✓	✓
(Zhao <i>et al.</i> , 2002)*		✓		✓

*Longitudinal study

Case studies: (Cali *et al.*, 2005), (Soares *et al.*, 2014)* and (Zhao *et al.*, 2002)*

Supplementary Table 3: studies reporting a relationship between Vpr-specific amino acids and clinical outcomes in people living with HIV when stratified according to current CD4 count (where available, nadir CD4 count)

Reference		<200 cells/ μ l	>200 cells/ μ l	Association with negative clinical outcomes	
				Yes	No
(Antoni <i>et al.</i> , 2007)			✓		✓
(Cali <i>et al.</i> , 2005)			✓	✓	✓
(Cavert <i>et al.</i> , 2004)			✓		✓
(Chui <i>et al.</i> , 2006)			✓		✓
(Dampier <i>et al.</i> , 2017)			✓	✓	✓
(Fischer <i>et al.</i> , 2004)			✓		✓
(Fourati <i>et al.</i> , 2012)			✓		✓
(Jacquot <i>et al.</i> , 2009)	LTNP		✓		✓
	Progressors (Pr)	✓		✓	
(Kamori <i>et al.</i> , 2017)			✓		✓
(Lum, Cohen, Nie, Weaver, Gomez, Yao, Lynch, Pilon, Hawley & Kim, 2003)		✓			✓
(McMullen <i>et al.</i> , 2021)		✓		✓	✓
(Mologni <i>et al.</i> , 2006)			✓		✓
(Rossen Khan <i>et al.</i> , 2016)			✓	✓	✓
(Shen <i>et al.</i> , 2008)	LTNP		✓		✓
	AIDS-defined patients	✓			✓
(Soares <i>et al.</i> , 2014)		✓		✓	✓
(Wang <i>et al.</i> , 1996)			✓	✓	✓
(Zhang <i>et al.</i> , 1997)			✓		✓
(Zhao <i>et al.</i> , 2002)	LTNP		✓		✓
	Fast Progressors (FP)	✓		No clinical outcome	

Case studies: (Zhao *et al.*, 2002), (Soares *et al.*, 2014), (Wang *et al.*, 1996), (Jacquot *et al.*, 2009), and (Cali *et al.*, 2005).

CHAPTER 3

A pilot investigation of the association between Vpr amino acid substitutions and peripheral immune marker levels in people living with HIV: implications for neurocognitive impairment.

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Under review: Open Forum of Infectious Diseases (OFID-D-23-01181)

Abstract

Background

The activity of HIV-1 in the periphery and central nervous system is responsible for its pathogenesis and neuropathogenesis, respectively. Among the various mechanisms influencing HIV-1 disease progression and prevalence, the subtype and subtype-specific variations of HIV-1, observed in the amino acid sequences of viral proteins, play a significant role. Recent evidence suggests that amino acid sequence variations in Viral protein R (Vpr) are linked to clinical outcomes in people living with HIV including disease progression, treatment efficacy and HIV-associated neurocognitive disorder (HAND). Previous research has also shown that Vpr sequence variation is associated with chronic inflammation, a common feature of HIV disease progression and a central mechanism in HIV-1 neuropathogenesis. However, no clinical study has explored the connection between Vpr sequence variation and peripheral inflammation in people living with HIV. This pilot study aimed to determine if specific Vpr amino acid variants, previously associated with HIV-1 neuropathogenesis, associate with specific immune markers in people living with HIV.

Methods

We included a unique cohort of forty-eight treatment-naïve South African people living with HIV to determine the association between blood-derived Vpr sequence variation and peripheral immune marker levels using Sanger sequencing and ELISA analysis, respectively.

Results

Our findings indicate that among the many neuropathogenic Vpr amino acid variants and immune markers examined, after applying Bonferroni corrections ($p = .05/3$) and adjusting for sex and locality, suPAR levels were nearing significance for higher levels in participants with the 41G amino acid variant compared to those with the 41S variant ($p = .035$), after. Further, amino acid variations at position 41 (between 41G and 41S) exhibited a significant association with suPAR (adj $R^2 = .089$, $\beta = .386$; 95% CI .125 to 3.251; $p = .035$).

Conclusions

These findings suggest that Vpr amino acid sequence variations might contribute to dysregulated inflammation, which could explain the observed association between specific Vpr variants and HIV-1 (neuro)pathogenesis found in prior research. These Vpr variants merit further investigation to fully understand their roles in HIV-1 pathogenesis and neuropathogenesis.

1. Introduction

Human immunodeficiency virus (HIV) remains of global concern, with approximately 37.7 million people living with HIV, of which 1.5 million were newly infected in 2021 (Heath *et al.*, 2021). Dysregulated inflammation is a common hallmark of HIV pathogenesis and an accelerator of non-acquired immunodeficiency syndrome (AIDS)-related events and one of the driving forces of CD4⁺ T cell depletion (Decrion *et al.*, 2005; Lv *et al.*, 2021). Similarly, dysregulated peripheral and central nervous system (CNS) inflammation has been associated with HIV-1 neuropathogenesis (Malik & Eugenin, 2016). HIV can invade the CNS when HIV-infected monocytes cross the blood-brain barrier (BBB), acting as a "Trojan horse" (Spudich & González-Scarano, 2012). After penetrating the BBB, these monocytes transform into macrophages, initiating an immune response in the CNS ultimately leading to neuronal damage and manifests clinically as HIV-associated neurocognitive disorders (HAND) (Spudich & González-Scarano, 2012) (Antinori *et al.*, 2007). Despite advancements in treating HIV infections and HAND with combined antiretroviral therapy (cART), chronic inflammation persists, contributing to the ongoing presence of comorbid conditions, including mild forms of HAND (Antinori *et al.*, 2007).

HIV exhibits high genetic variability, influencing the mechanisms related to both HIV-1 pathogenesis and neuropathogenesis (Hemelaar *et al.*, 2019) (Santerre *et al.*, 2019). HIV is categorized into two main types, HIV-1 and HIV-2, due to its high genetic variability. HIV-1 is more prevalent globally (Hemelaar *et al.*, 2019). HIV-1 further consists of groups M, N, O, and P with group M being the most abundant globally (Hemelaar *et al.*, 2019). Based on differences in the viral genome, group M can be further subdivided into subtypes A, B, C, D, F, G, H, J, and K (Hemelaar *et al.*, 2019). Geographically, these subtypes are linked to specific regions worldwide (Hemelaar *et al.*, 2019). Subtype B is predominantly found in America and Europe, while subtype C is prevalent in Southern Africa (Hemelaar *et al.*, 2019). Subtype C accounts for the majority of HIV-1 cases, representing approximately 40-60% of global infections (Hemelaar *et al.*, 2019). Notably, it is the dominant subtype in South Africa (Hemelaar *et al.*, 2019). Further, the prevalence of HIV and HAND varies across these different HIV-1 subtypes (Hemelaar *et al.*, 2019; Santerre *et al.*, 2019).

The differences among subtypes also manifest in changes in the amino acid sequences of HIV-1 viral proteins with different subtypes exhibiting sequence variations in crucial HIV viral proteins, Tat, Vpr, Pol, Nef, and gp120, resulting in distinct functionalities of the viral proteins

across the subtypes (Asia *et al.*, 2022; Jin *et al.*, 2020; Samikkannu *et al.*, 2015). In particular, amino acid sequence variation in several HIV-1 viral proteins was previously associated with a differential HIV-1 pathogenesis, inflammation, and the development of HAND (Gandhi *et al.*, 2009; Ruhanya *et al.*, 2023a; Samikkannu *et al.*, 2015; Santerre *et al.*, 2019).

Despite the numerous mechanisms contributing to the HIV pathogenesis and neuropathogenesis, it is proposed that in the modern cART era, chronic dysregulated inflammation remains a principal factor driving the continual emergence of milder HAND forms (Harezlak *et al.*, 2011). It is well-established that inflammation within the CNS and brain tissue is linked to HIV-associated encephalopathy and the clinical presentation of neurocognitive impairment in people living with HIV (Fields *et al.*, 2018). Given that the cerebrospinal fluid is in indirect contact with the CNS, it can reflect the biochemical milieu of the CNS during disease states (Pendyala & Fox, 2010). However, the invasiveness of cerebrospinal fluid collection means it is not routinely collected for research purposes. This limitation makes peripheral blood a compelling alternative for exploring potential CNS pathologies. In line with this, evidence suggests that peripheral inflammation can shed light on the neuroinflammatory and/or neuropathological profile. A study by Bryant and colleagues reported that plasma-soluble CD163 is associated with postmortem brain pathology in HIV-1 infection (Bryant *et al.*, 2017). A study by Gisslén and colleagues indicated that concentrations of the neurofilament light chain protein in plasma and cerebrospinal fluid—a sensitive marker of neuronal injury—were highly correlated ($r = 0.89$, $p < .0001$) (Gisslén *et al.*, 2016). This suggests that peripheral inflammation may (1) reflect CNS activity to some extent, (2) correspond with inflammation levels in the CNS, and (3) serve as an alternative approach to investigating CNS pathological states related to inflammation. Notably, the activity of the HIV-1 viral proteins is recognized as significant inducers of the dysregulated inflammatory profiles observed in people living with HIV.

However, amongst all the HIV-1 viral proteins investigated (Engelman & Cherepanov, 2012; Li *et al.*, 2005), fewer studies have investigated Vpr despite its function in clinical outcomes in people living with HIV (Asia *et al.*, 2022; Dampier *et al.*, 2017; Na *et al.*, 2011). Vpr presence and sequence variation are associated with various clinical outcomes related to HIV-1 pathogenesis, disease progression, treatment efficacy, and neurological outcomes (Asia *et al.*, 2022) (Ruhanya *et al.*, 2023a). Supporting this notion, fundamental studies have found that particular amino acids within Vpr can induce higher levels of inflammatory markers (Na *et*

al., 2011). However, no clinical studies to date have demonstrated a link between Vpr sequence variation and immune markers. Thus, this study aimed to investigate how Vpr sequence variations, particularly in amino acids previously linked with HIV neuropathogenesis (at positions 22, 41, 45, 55), associates with inflammation. The insights from this research may shed light on the observed correlations between Vpr amino acid sequence variations and inflammation in people living with HIV.

2. Methods

2.1. Study Participants

The Prospective Urban and Rural Epidemiology (PURE) study is a multinational prospective study designed to examine the development of cardiovascular diseases (CVD) in individuals from 27 high-, middle-, and low-income countries (Teo *et al.*, 2009). In particular, the PURE study investigated potential risk factors of CVD, which included HIV-1 infection and peripheral inflammation (Botha *et al.*, 2015). As part of this larger study, individuals of African descent, both men and women above 30 years, were recruited from two urban and two rural communities in the North-West Province of South Africa. Baseline data collection, encompassing n = 2,010 participants, was voluntarily conducted in 2005. Subsequent follow-up data was gathered in 2010 (n = 1,288) and 2015 (n = 923). Participants were re-diagnosed in 2010 to confirm their HIV status. Therefore, individuals who screened positive for HIV-1 and were treatment-naïve at the time of data collection in 2010 were included in this study (n = 103). HIV-1 sequencing was successful for only n = 48 people living with HIV; thus, only these were included in the subsequent analysis. Despite the high prevalence rate of HIV and cardiovascular disease multimorbidity in South Africa, it was previously shown that the participants involved in this study did not exhibit a worse cardiovascular disease risk profile than those without HIV (CVD)(Botha *et al.*, 2014; Fourie *et al.*, 2010; Louwrens *et al.*, 2022). This study presents a unique cohort of individuals who have not been treated for HIV, allowing us to investigate the inflammatory/immune profile without the confounding influence of ART. The study protocol was approved by the Health Research Ethics Committee of the North-West University (NWU-HREC) in South Africa (NWU-00106-22-A and NWU-00106-22-A1) and sub-study (NWU-00106-22-A1-01).

2.2. Questionnaires

Participant information was obtained using the PURE South Africa Adult Questionnaire. This includes the following information: demographic data (including age and sex), lifestyle variables (including tobacco use and alcohol consumption), socioeconomic status and medical history.

2.3. HIV Status

Participants received counselling by trained counsellor prior to their HIV status determination. The South African Department of Health protocol was used to determine the HIV status using the First Response rapid HIV card test (Premier Medical Corporation Limited, Daman, India). The test was confirmed with the SD BIOLINE HIV 1/2 3.0 card test (Standard Diagnostics, INC, Korea). Upon confirmation of a positive result, participants received post-counselling. They were referred to the nearest clinic or hospital for follow-up and CD4⁺ cell count analysis using the flow cytometric method (Beckman COULTER, EPICS XITM, Fullerton, USA).

2.4. Analysis of immune markers in blood samples

Fasting blood samples were collected and were prepared through centrifugation at a speed of $2000 \times g$ for 15 minutes at 10°C within 2 hours of collection. The samples were then transferred to microfuge tubes, snap-frozen on dry ice and stored at -80°C in the laboratory until analysis. For samples collected in rural areas, samples were snap-frozen on dry ice and stored at -18°C for a maximum of five days until they could be transported to the laboratory and stored at -80°C. For this study, the immune markers soluble urokinase plasminogen activator receptor (suPAR), interleukin (IL) 6, high-sensitivity C-reactive protein (hsCRP), soluble CD163 (sCD163) and neutrophil gelatinase-associated lipocalin (NGAL) were selected for investigation. These markers were selected for analysis based on their potential involvement in Vpr-mediated HIV pathogenesis and neuropathogenesis, as reviewed from the scientific literature (Asia *et al.*, 2022; Burdo *et al.*, 2013; Gianella *et al.*, 2019; Rubin *et al.*, 2018; Williams *et al.*, 2019b). Independent of HIV, sCD163 is an immune marker that has been demonstrated to play a role in chronic liver disease and insulin resistance (Grønbaek *et al.*, 2012; Parkner *et al.*, 2012). NGAL has been shown to contribute to kidney function and the duration of end-stage renal failure, cardiovascular disease, and mortality (Daniels *et al.*, 2012; Magnusson *et al.*, 2012). suPAR has been shown to contribute to acute kidney injury and modulate monocyte function to promote atherosclerosis (Hayek *et al.*, 2020; Hindy *et al.*,

2022). IL-6 is a predictor of long-term cardiovascular mortality (Gager *et al.*, 2020), atherosclerotic cardiovascular disease and cardiovascular disease mortality (Susilo *et al.*, 2022). hsCRP has been shown to contribute to acute ischaemic stroke (Cai *et al.*, 2019). suPAR levels were determined from plasma (EDTA) samples with the suPARnostiC® ELISA kit (ViroGates, Copenhagen, Denmark). hsCRP levels were analysed by particle-enhanced turbidimetric assay, and IL-6 was determined in plasma using an Elecsys 2010 (Roche, Basel, Switzerland) apparatus with the electrochemiluminescence immunoassay method. sCD163 and NGAL were measured using ELISA (R&D systems, DuoSet ELISA) according to the manufacturer's instructions. All samples were assayed in duplicate. All tests' intra- and inter-assay coefficients of variation were within acceptable ranges of < 8% and < 10%, respectively.

2.5. Viral protein analysis

RNA was extracted from 200µl of prepared plasma using the Quick-RNA™ Viral Kit (Zymo Research). Total RNA was reverse transcribed by reverse transcriptase-polymerase chain reaction (PCR) using the ProtoScript® II First Strand cDNA Synthesis Kit (New England Biolabs), and DNA was prepared for PCR analysis. The Tat exon 1/Vpr (HXB2 position 4900–6351) was amplified using the primer pair Vif-1 (5'GGGTTTATTACAGGGACAGCAGAG)/CATH-4R(5'-GTACCCCATAGACTGTGACC) PCR, respectively. Amplification conditions were held at 94°C for 2 min, followed by 40 cycles of denaturing (94°C; 30s), annealing (60°C; 30s), extension (68°C; 2 min) and a final extension step for 10 min at 68°C. All PCR products were purified 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 using the Gene studio™ professional sequence analysis software (Version 2.2). Nucleotide sequences were translated into amino acid sequences using Expasy translate (Gasteiger *et al.* 2003), and the key mutations of Vpr were highlighted. Sequences are available in GenBank under the accession numbers OR621303- OR621349.

2.6. Statistical analysis

All analyses were conducted using SPSS (version 27, IBM, USA). *P*-values were considered statistically significant for all analyses at less than 0.05. All variables were assessed for normality by the visual inspection of QQ plots using descriptive statistics. Data distribution for

age and the immune markers suPAR, hsCRP, IL-6, NGAL and sCD163 were skewed. Therefore, the data was log-transformed before statistical analyses.

As the primary aim, we examined whether peripheral inflammation levels differ between groups divided by different Vpr sequence variations. We employed composite risk scores for Vpr variants, similar to a previous study (Womersley *et al.*, 2019). We classified amino acids based on their potential influence on mechanisms related to the development of HAND, assigning higher scores to indicate a greater risk. The Vpr amino acids designated as high-risk (score = 2) were Isoleucine (I)22, Serine (S)41 or Asparagine (N)41, Tyrosine (Y)45 and Alanine (A)55, while low-risk (score = 0) was assigned to Leucine (L)22, Glycine (G)41, Histidine (H)45 and Threonine (T)55. Any other amino acids at the investigated positions were considered neutral risk (score = 1). These selected amino acids have demonstrated influence on neuropathological mechanisms and neurological outcomes in people living with HIV, thus warranting further analysis. A composite “Vpr risk score” was generated by summing the assigned values for each Vpr amino acid variant. Scores ranging from 0 to 3 were considered low-neutral, while scores from 4 to 8 were regarded as high-neutral. The chi-square test was used to assess differences in sex, smoking status, alcohol use and locality between groups. Independent sample T-tests were conducted to determine differences in study characteristics (age and CD4⁺ counts) and immune marker levels between the high-neutral and low-neutral groups. A Pearson correlation analysis was used to determine covariates by determining correlations between sociodemographic variables (age, sex, smoking status, alcohol use, and locality), and immune markers. Analyses of covariance (ANCOVA) were performed to compare the immune marker levels between Vpr high-neutral and low-neutral groups, while adjusting for sex, and locality. Pearson correlations were used to determine correlations between Vpr risk score and immune marker levels. Multiple regression analysis was used to determine associations between Vpr risk score and immune marker levels after adjusting for potential covariates (locality and sex).

As a secondary aim, we wanted to evaluate whether specific immune marker levels could be related to single amino acid variants. Therefore, we stratified participants into groups: 22I vs. 22L, 41N/S vs. 41G, 45Y vs 45H and 55A vs 55T. At position 41, we compared G vs. N and G vs. S, respectively. Pearson’s chi-squared tests were used to test group differences for sex and locality between Vpr amino acid variants. Independent sample T-tests determined differences in study characteristics (age and CD4⁺ counts) and immune marker levels between Vpr amino acid variants. A Bonferroni correction was accounted for the number of immune markers tested ($\alpha/n = .05/5 = .01$) in the secondary aim. An ANCOVA was performed to

compare the immune marker levels between Vpr variants, while adjusting for sex, and locality. A Bonferroni correction was accounted for the number of tests ($\alpha/n = .05/3 = .017$) within the model. Multiple regression analysis was used to determine associations between Vpr amino acid variance at specific positions and immune marker levels after adjusting for potential covariates (sex, and locality).

3. Results

3.1. Study characteristics

This study encompassed a sample of $n = 48$ treatment-naïve South African subtype C participants, with an average age of 47.46 (± 7.604). Men constituted only 25% of the participants, as depicted in Table 1. While the primary study did not record any viral load data, it should be noted that all participants were treatment-naïve at the time of sample collection. CD4⁺ count data was available for 67% of the participants, showing a mean value of 292.00 (± 128.41) cell/mm³. Stratifying participants based on Vpr amino acid variant risks revealed that the Vpr high-neutral group consisted of $n = 26$ participants, while the Vpr low-neutral group had $n = 22$ participants (Table 1). No significant differences in study characteristics (age, sex, CD4⁺ count and locality) were observed between the Vpr high-neutral and Vpr low-neutral groups as shown in Table 1. A Pearson correlation analysis revealed that among all variables (age, sex, smoking status, alcohol use, and locality), only locality and sex were correlated with specific markers. Consequently, we chose to include only these covariates in the relevant analyses.

3.2. Immune marker levels the in Vpr high-neutral group vs. low Vpr low-neutral group

Independent sample T-tests revealed no significant differences in immune marker levels between the Vpr high-neutral and Vpr low-neutral groups (Table 1). To explore the potential influence of covariates on the reported non-significant findings, we performed an ANCOVA analysis. Upon controlling for locality and sex using ANCOVA, the levels of IL-6 ($p = .911$), hsCRP ($p = .744$), suPAR ($p = .219$), NGAL ($p = .826$) and sCD163 ($p = .481$) remained statistically non-significant between the Vpr high-neutral group and the Vpr low-neutral group.

Table 1: Study characteristics of people living with HIV.

	People living with HIV (n = 48)		
Age in years, mean (SD)	47.46 ($\pm$ 7.604)		
Sex, female N (%)	36 (75)		
CD4 ⁺ , mean (SD)	292. ($\pm$ 128.409)		
Locality, rural N (%)	28 (58.3)		
	Vpr high-neutral (n = 26)	Vpr low-neutral (n = 22)	P-value
Age in years, mean (SD)	46.23 (7.016)	48.91 (7.72)	NS
Sex, female N (%)	18 (69)	18 (82)	NS
CD4 ⁺ , mean (SD)	299.8 (145.43)	285.5 (115.5)	NS
Locality, rural N (%)	13 (50)	15 (68)	NS
IL-6 pg/ml, mean (SD)	8.28 (11.37)	7.84 (6.9)	NS
hsCRP mg/l, mean (SD)	11.39 (28.18)	12.23 (12.23)	NS
suPAR ng/ml, mean (SD)	4.26 (1.8)	4.8 (2.01)	NS
NGAL ng/ml, mean (SD)	51.7 (16.4)	53.8 (26.6)	NS
sCD163 ng/ml, mean (SD)	746. 1 (319.2)	792 (420.2)	NS

logarithmically transformed variables has been back transformed for presentation purposes.

NS – not significant

3.3. Correlation between Vpr risk score and immune marker levels.

We aimed to determine if there was a correlation between Vpr risk score and immune marker levels. Using Pearson correlations, we observed no significant correlations between the Vpr risk score and immune markers, namely hsCRP ($R = -.091$, $p = .538$), IL-6 ($R = -.073$, $p = .621$), suPAR ($R = -.200$, $p = .173$), NGAL ($R = -.021$, $p = .888$), and sCD163 ($R = -.103$, $p = .486$). Furthermore, a multiple regression analysis that adjusted for covariates (locality and sex) revealed no significant association between the Vpr risk score and hsCRP (adj $R^2 = -.057$, $\beta = -.097$, 95% CI = -7.8 to 4.0, $p = .523$), IL-6 (adj $R^2 = -.008$, $\beta = -.098$, 95% CI = -2.2 to 1.1, $p = .510$), suPAR (adj $R^2 = .021$, $\beta = -.223$, 95% CI = -0.6 to 0.08, $p = .133$), NGAL (adj $R^2 = -.047$, $\beta = -.100$, 95% CI = -0.09 to .005, $p = .510$) and sCD163 (adj $R^2 = .066$, $\beta = -.067$, 95% CI = -0.11 to .07, $p = .638$).

3.4. Immune marker levels between Vpr amino acid variants

As an alternative to creating composite risk Vpr groups as described above, our secondary aim was to examine whether we could link immune marker levels to specific amino acid variants (Table 2). We stratified participants according to Vpr amino acid variants at position 22 ($n = 44$), 41 ($n = 40$), 45 ($n = 42$), and 55 ($n = 43$). When stratifying participants according to the Vpr amino acids variants, there were no significant differences between study characteristics (age, sex, CD4⁺ count and locality) (Table 2). After applying Bonferroni correction of $p = .05/5$, only suPAR levels were found to be significantly higher in the 41G group compared to the 41N ($p = .008$) (Table 2). However, after applying Bonferroni correction of $p = .05/2$ and when adjusting for locality and sex through ANCOVA, suPAR was nearing significance for higher levels in the 41G group compared to the 41S group ($p = .035$). However, suPAR was no longer significantly higher in the 41G group compared to the 41N group ($p = .115$).

Table 2: Characteristics of HIV-positive participants and immune marker levels when participants stratified according to specific amino acids variants.

	22 (n = 44)		P value	41 (n = 40)			P value	45 (n = 42)		P value	55 (n = 43)		P value
Amino acid	I	L		G	N	S		Y	H		A	T	
N (%)	23 (52)	21 (48)	-	21 (53)	8 (20)	11 (27.5)	-	25 (60)	17 (40)	-	11 (26)	32 (74)	-
Age	48.22 (8.2)	46.9 (7.4)	.581	47.9 (6.8)	46.13 (6.7)	46.3 (8.7)	.544/ .574	48.32 (8.4)	46.88 (5.8)	.545	47.45 (6.8)	47.28 (7.7)	.948
Sex	16 (70)	17 (81)	.862	15 (71)	7 (87.5)	6 (55)	.296	20 (80)	11 (65)	.268	7 (64)	27 (84)	.145
CD4 ⁺ count	274.80 (119.6)	314.4 (137.9)	.416	292 (133.8)	233.6 (60.2)	357.75 (155.3)	.366/ .380	288.07 (101)	279.17 (143)	.851	258.7 (105.8)	317.9 (128.0)	.307
Locality	10 (43)	14 (67)	.123	13 (62)	5 (63)	7 (64)	.995	13 (52)	11 (65)	.618	7 (64)	17 (53)	.800
suPAR, ng/ml	4.51 (1.7)	4.76 (2.2)	.685	5.3 (2.4)	3.65 (0.7)	3.8 (0.9)	.008/ .018	4.98 (2.4)	3.87 (0.8)	.037	5.41 (2.3)	4.12 (1.5)	.056
IL-6, pg/ml	7.93 (6.5)	9 (12.7)	.717	10.5 (13.4)	5.79 (3.57)	6.1 (4.2)	.346/ .305	10.62 (12.5)	5.04 (3.1)	.041	13.08 (16.7)	6.18 (5.4)	.166
hsCRP, mg/l	16.19 (40.0)	10.44 (28.9)	.591	19.38 (48.2)	13.17 (21.5)	3.12 (3.6)	.731/ .139	20.01 (45.2)	4.68 (5.1)	.104	18.67 (39.4)	11.3 (34.2)	.556
NGAL, ng/ml	57.8 (20.1)	45.5 (21.8)	.027	54.4 (26.8)	55.3 (17)	48.7 (15.3)	.661/ .746	54 (22.6)	50.2 (22.3)	.570	50.9 (27.9)	55 (19)	.333
sCD163, ng/ml	801.4 (366.7)	783 (379.8)	.728	759.8 (376)	654.3 (398.1)	807.4 (249.1)	.305/ .465	775.3 (391.7)	631.8 (274.2)	.293	791.3 (292)	747.1 (383.9)	.462

Age in years and immune markers: mean (SD); Sex, female and Locality, rural: N (%), logarithmically transformed variables has been back transformed for presentation purposes. At position 41, we compared G vs N and G vs S respectively. Statistically significant findings are presented in bold. Adjusted for locality and sex.

Further, we aimed to evaluate the relationships between the Vpr amino acid variance at specific positions and immune marker levels while adjusting for covariates. To do this, we adjusted for covariates, namely locality and sex using multiple regression analyses. Across all Vpr amino acid positions investigated only variance at position 41 (between 41G and 41S) exhibited a significant association with suPAR (adj R^2 = .089, β = .386; 95% CI .125 to 3.251; p = .035).

4. Discussion

In this study, we aimed to investigate whether variations in the HIV-1 Vpr amino acid sequence, especially at amino acid positions previously associated with neurological outcomes (namely positions 22, 41, 45, and 55), would influence the levels of peripheral immune markers sCD163, NGAL, hsCRP, suPAR, and IL-6 in people living with HIV. This was in aim of provide commentary on its role on potential underlying HIV-1 related pathology. Amongst all the Vpr amino acids and immune markers investigated, variation at position 44 associated with suPAR, which was shown to be higher in the 41G group when compared to the 41S group after adjustment for covariates.

The previous study by McMullen and colleagues has shown that the Vpr 41G amino acid was associated with non-ischaemic stroke in people living with HIV (McMullen *et al.*, 2021), also suggesting that Vpr 41G may be a low-risk Vpr variant. In the same cohort of South African participants, the 41S amino acid was associated with ischaemic stroke in people living with HIV (McMullen *et al.*, 2021), suggesting that 41S may be a high-risk Vpr variant. However, another study conducted in the United States of America, found that in people living with HIV, the 41S amino acid is associated with a decreased and less pronounced neurocognitive deficit (lower global deficit scores) (Dampier *et al.*, 2017). The contradiction between these findings may be due to the difference in geographical regions and, most likely, different HIV-1 subtypes (Jiang *et al.*, 2022). The study by Dampier and colleagues reported that the 41N amino acid was associated with more pronounced neurocognitive deficits (higher GDS) (Dampier *et al.*, 2017). We initially considered the Vpr variant 41G as low risk. However, our findings suggest that it may contribute to increased suPAR levels since suPAR was also higher in this group - a characteristic expected from the high-risk Vpr variants. The limited number of studies reveals no clear consensus on whether the amino acid variation at position 41, particularly 41G, may contribute negatively or positively to clinical outcomes in people living with HIV.

Incorporating the existing body of evidence, we introduce the possibility that Vpr 41G might play a role in adverse clinical outcomes among people living with HIV by disrupting the peripheral immune response, particularly involving suPAR. It may also be that mutations at position 41 of Vpr generally may influence the structure-function relationship of Vpr, as amino acid 41 is within the second alpha-helix (38-50), which is responsible for key mechanisms including regulation of apoptosis, subcellular transport (Morellet *et al.*, 2009) and virion incorporation of Vpr (Singh *et al.*, 2000). It is established that hydrophilic amino acids, such as 41N and 41S, are situated on a specific side of the helix (Wecker & Roques, 1999). Altering this configuration to a smaller amino acid like 41G could potentially impact the arrangement of the Vpr alpha helices, therefore influencing its potential functioning.

None of the other investigated Vpr amino acid signatures or inflammatory markers demonstrated significance after applying the Bonferroni correction. It is reasonable to speculate that the small number of participants in the included study may have led to low statistical power. However, future studies should explore these Vpr amino acid signatures in larger cohorts, as these signatures have been previously linked to adverse clinical outcomes in people living with HIV (Asia *et al.*, 2022). Studies of this nature may help illuminate the reasons behind the observed variations in clinical severities when comparing participants with different HIV subtype infections. The amino acid at position 77 is extensively studied in the context of Vpr sequence variation (Asia *et al.*, 2022). However, the majority of participants in this study exhibited a Q at position 77 (83%). Due to the limited variation at this position, we were unable to explore its influence on immune markers.

Furthermore, it is known that HIV infection leads to the production of immune markers, often resulting in adverse clinical outcomes for individuals living with HIV (Decrion *et al.*, 2005). Our study revealed an association between the variance at position 41 and suPAR, a marker implicated in various diseases and disorders. Specifically, elevated plasma suPAR levels have been linked to worsened neurological performance in an HIV-treated population (Gianella *et al.*, 2019). Consistent with this, increased suPAR levels in the cerebrospinal fluid have been associated with lower working memory in people living with HIV (De Almeida *et al.*, 2022). This immune marker was also shown to associate with acute kidney injury and modulation of monocyte function, promoting atherosclerosis (Hayek *et al.*, 2020; Hindy *et al.*, 2022). Additionally, suPAR

was found to be associated with organ failure independent of HIV infection (Wang, Wu, *et al.*, 2021).

Our study's findings suggest that Vpr amino acid variants, specifically those at position 41, may be associated with adverse clinical outcomes in people living with HIV through mechanisms related to dysregulated inflammation, particularly, suPAR (De Almeida *et al.*, 2022; Rasmussen *et al.*, 2016). While many studies that aimed to link HIV Vpr amino acid variants to clinical outcomes have been conducted in *in vitro/in vivo* settings (Na *et al.*, 2011), fewer have explored HIV amino acid variations in clinical research (Dampier *et al.*, 2017; McMullen *et al.*, 2021). Moreover, even fewer studies have delved into the influence of HIV Vpr amino acid variants on inflammation, especially those concerning amino acid variations at positions 22, 41, 45 and 55 (Dampier *et al.*, 2017; McMullen *et al.*, 2021).

Given that our cohort was not undergoing any treatment at the time of sample collection, we were uniquely positioned to delineate the relationship between Vpr amino acid sequence variation and systemic inflammation more clearly. However, a more in-depth investigation of these specific Vpr variants is imperative to comprehensively understand their contributions to HIV-1 pathogenesis and possibly neuropathogenesis. Further, most studies examining Vpr sequence variation have been conducted in regions dominated by subtype B (Asia *et al.*, 2022). Few investigations have delved into Vpr sequence variation in the Southern African context. Thus, our findings enrich the understanding of prevalent sequence variations within this setting. Further research is warranted to corroborate the relationship between Vpr amino acid variants and inflammation in people living with HIV. This could provide deeper insights into the potential impacts of such associations on clinical outcomes in people living with HIV.

5. Limitation

This study has several limitations that warrant emphasis. Firstly, the sample size was limited, which might have influenced the reported potential associations. Therefore, our findings should be interpreted in light of this, and future research is recommended to validate our results in larger cohorts. Though we view this as a pilot study, it offers insights into (1) the South African sequence variation, an area with limited investigation, and (2) the association of Vpr sequence variation in

a treatment-naïve cohort, giving a clearer snapshot of systemic inflammatory profile without the potential confounding effects of cART. An initial objective of the study was to assess the combined impact of various amino acid substitutions on peripheral inflammatory levels, utilizing a composite risk score and categorizing participants into high-neutral and low-neutral Vpr risk groups. Although this approach has been employed previously (Womersley *et al.*, 2019), we recognize a limitation in that certain amino acids may exert a more significant influence than others. Summing amino acids together in this manner might attenuate this effect, potentially contributing to the lack of significance observed in the investigation of Vpr risk categories and risk scores. Additionally, this study focused on sequence variation in a single HIV-1 protein. Other pivotal viral proteins, such as Tat and gp120, might have also exhibited specific variations that could have contributed to the observations reported here. Nonetheless, our approach to examining a single HIV-1 protein and its association with clinical outcomes is consistent with methods used in other studies (Dampier *et al.*, 2017; Rao *et al.*, 2014). In this study, we also evaluated peripheral markers to draw insights into their potential CNS functioning. While it could be argued that peripheral markers might not directly reflect CNS activity, previous studies have documented associations between peripheral markers and clinical HAND. Moreover, a direct correlation exists between the levels of specific markers in the periphery and the CNS, suggesting that our findings may be relevant for investigations into CNS activity (Gisslén *et al.*, 2016). Furthermore, examining the concentration of the marker NFL in blood within this study could have provided insights into the potential neuropathology of individuals living with HIV (Gisslén *et al.*, 2016). Therefore, in cases where cerebrospinal fluid or neuropsychological data is unavailable, future studies conducted in blood should explore NFL as a potential indicator of neuropathology. Even though we have considered locality in our analyses, other psychosocial determinants of health may have played a role in influencing immune marker levels and the observed findings. Previous research has demonstrated that mental health, trauma, and stress can impact inflammation in individuals living with HIV (Berk *et al.*, 2013; Chow *et al.*, 2023; Hussain *et al.*, 2023; Memiah *et al.*, 2021) and therefore, our findings should be interpreted in relation to this. This cohort was initially used to investigate CVD in people living with HIV. Thus, evaluation of HAND was not performed; however, the association of certain neuropathogenic Vpr variants with inflammation and metabolic marker hold some translational value into the underlying mechanism that may contribute to HIV-1 pathogenesis and the development of HAND. Thus, for future studies, it would be ideal to select a cohort that have being evaluated for neuropsychological data. It should also be noted that confounding factors such as HLA and other viral proteins, and potential risks to comorbidities may have influenced the outcomes from this investigation. Patient genetics may influence HIV

infectivity and disease progression as certain genes may be protectant against HIV infectivity and other genes rather than increasing the susceptibility of HIV infection (Carrington & O'Brien, 2003; Li *et al.*, 2017). Furthermore, other pivotal viral proteins, such as Tat and gp120, might have also exhibited specific variations that could have contributed to the observations reported here. Nonetheless, our approach to examining a single HIV-1 protein and its association with clinical outcomes is consistent with methods used in other studies (Dampier *et al.*, 2017; Rao *et al.*, 2014). Despite this, there are many more factors in clinical samples that may have an effect; however *in vitro/in vivo* studies may be replicated in clinical models. This has been shown in major viral proteins such as Tat but it is not clear for other less commonly studied viral proteins such as Vpr. Furthermore, due to unavailable data we were unable to adjust for kidney function as we did not have the data available for it.

6. Conclusions

Variations in the Vpr amino acid sequence have been previously linked to neurocognitive impairment in people living with HIV. Yet, the mechanism driving this association remains unclear. Given the pivotal role of dysregulated inflammation in HAND's development, we hypothesized that neuropathogenic Vpr amino acid variants might correlate with specific immune markers, suggesting a potential role in HAND's onset. In this study, we found that the Vpr variants at position 41 was associated with suPAR, and suPAR was higher in people living with HIV with the 41G variant. Therefore, variations in the Vpr amino acid sequence might be related to dysregulated inflammation and the subsequent disease progression. The Vpr signatures highlighted here warrant further investigation to fully understand their roles in HIV-1 pathogenesis and neuropathogenesis. Thus, we use the findings of this Chapter to further investigate how Vpr amino acid variants may influence tryptophan-kynurenine metabolism in Chapter 4. This metabolic pathway is closely linked to HIV-1 pathogenesis and neuropathogenesis and may potentially provide more insight into the roles of these Vpr amino acid variants in both HIV-1 pathogenesis and neuropathogenesis.

7. References

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CHAPTER 4

A pilot investigation of the association between HIV-1 Vpr amino acid sequence diversity and the tryptophan-kynurenine pathway as a potential mechanism for neurocognitive impairment.

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Abstract

HIV infection compromises both the peripheral and central immune systems due to its pathogenic and neuropathogenic features. The mechanisms driving HIV-1 pathogenesis and neuropathogenesis involve a series of events, including metabolic dysregulation. Furthermore, HIV-subtype-specific variations, particularly alterations in the amino acid sequences of key viral proteins, are known to influence the severity of clinical outcomes in people living with HIV. However, the impact of amino acid sequence variations in specific viral proteins, such as Viral protein R (Vpr), on metabolites within the Tryptophan (Trp)-kynurenine (Kyn) pathway in people living with HIV remains unclear. Our research aimed to explore the relationship between variations in the Vpr amino acid sequence (specifically at positions 22, 41, 45, and 55, as these have been previously linked to neurocognitive function) and peripheral Trp-Kyn metabolites. Additionally, we sought to clarify the systems biology of Vpr sequence variation by examining the link between Trp-Kyn metabolism and peripheral inflammation, as a neuropathogenic mechanism. In this preliminary study, we analyzed a unique cohort of thirty-two ($n = 32$) South African cART naïve people living with HIV. We employed Sanger sequencing to ascertain blood-derived Vpr amino acid sequence variations and a targeted LC-MS/MS metabolomics platform to assess Trp-Kyn metabolites, such as Trp, Kyn, kynurenic acid (KA), and quinolinic acid (QUIN). Enzyme-linked immunosorbent assays were used to measure immune markers, hsCRP, IL-6, suPAR, NGAL and sCD163. After applying Bonferroni corrections ($p = .05/3$) and adjusting for covariates (age and sex), only the Vpr 41G and 55A groups was nearing significance for higher levels of QUIN compared to the Vpr 41S and 55T groups, respectively (all $p = .023$). Multiple regression results revealed that Vpr amino acid variations at position 41 (adj $R^2 = .049$, $\beta = .505$; $p = .023$), and 55 (adj $R^2 = .126$, $\beta = .444$; $p = .023$) displayed significant associations with QUIN after adjusting for age and sex. Lastly, the higher QUIN levels observed in the Vpr 41G group were found to be correlated with suPAR ($r = .588$, $p = .005$). These results collectively underscore the importance of specific amino acid substitutions in influencing QUIN and inflammation (specifically suPAR levels), potentially contributing to our understanding of their roles in the pathogenesis and neuropathogenesis of HIV-1.

Keywords: Kynurenine pathway, metabolism, inflammation, and HIV-associated neurocognitive disorders.

1. Introduction

Human immunodeficiency virus (HIV) affects approximately 39 million people globally of which 37.5 million are adults and 1.5 million are children (0-14 years of age) (Heath *et al.*, 2021). Despite advancements in HIV treatment, twenty million people living with HIV are situated in eastern and southern Africa. HIV-1 impacts immune cells, including CD4⁺ cells, leading to the deterioration of the immune system (Vidya Vijayan *et al.*, 2017). This deterioration creates conditions conducive to the development of comorbidities (Deeks *et al.*, 2015). HIV infection commonly results in the virus spreading into the central nervous system (CNS) of people living with HIV, consequently heightening the risk of developing HIV-associated neurocognitive disorders (HAND) (Ances & Ellis, 2007). However, the severity of HIV pathogenesis and neuropathogenesis may differ due to the genetic diversity of HIV (Hemelaar *et al.*, 2019).

While both HIV-1 and HIV-2 can contribute to HIV pathogenesis, HIV-1 is more virulent and is the predominant type causing HIV infections (Bhebhe *et al.*, 2021; Campbell-Yesufu & Gandhi, 2011; Rasmussen *et al.*, 2020a). Due to the considerable genetic variability of HIV, different subtypes of HIV-1 have emerged globally, (Hemelaar *et al.*, 2019), each exerting distinct influences on HIV pathogenesis, neuropathogenesis, and the severity of clinical outcomes (Santerre *et al.*, 2019). Among the HIV-1 groups, including M, N, O, and P (Ayoub *et al.*, 2001; Plantier *et al.*, 2009; Roques *et al.*, 2004; Simon *et al.*, 1998; Vallari *et al.*, 2011; Yousaf *et al.*, 2011), group M is the most prevalent globally (Sharp *et al.*, 1999). Furthermore, group M consists of subtypes A, B, C, D, F, G, H, J, and K (Yousaf *et al.*, 2011). Subtypes B and C represent most HIV infections, accounting for 12.1% and 46.6%, respectively (Hemelaar *et al.*, 2019). Subtype C is the most common in Southern Africa whereas subtype B is most common in Eastern European and Central Asia regions (Hemelaar *et al.*, 2019).

Clinical outcomes related to HIV progression and neuropathogenesis vary between HIV-1 subtypes (Boivin *et al.*, 2010; Gupta *et al.*, 2007; Sacktor *et al.*, 2009; Santoro & Perno, 2013). Consequently, the functions of HIV viral proteins vary depending on the HIV subtype (Asia *et al.*, 2022; Gotor *et al.*, 2023; Jin *et al.*, 2020; Nagata *et al.*, 2017; Samikkannu *et al.*, 2015; Williams, 2023). Thus, studies have investigated the effects of HIV-1 subtype-specific viral proteins such as transactivator of transcription (Tat) and glycoprotein 120 (gp120) in people living with HIV (Aralaguppe *et al.*, 2016; Campbell *et al.*, 2011; Samikkannu *et al.*, 2011; Samikkannu *et al.*,

2015). While major viral proteins like Tat and gp120 receive significant attention, accessory viral proteins such as viral protein R (Vpr) are less frequently studied. This is in spite of research indicating Vpr's contribution to clinical outcomes (Cali *et al.*, 2005; Jacquot *et al.*, 2009; Lum, Cohen, Nie, Weaver, Gomez, Yao, Lynch, Pilon, Hawley, Kim, *et al.*, 2003; Soares *et al.*, 2014), including its association with HIV neuropathogenesis (Ruhanya *et al.*, 2023a).

On a metabolic level, the tryptophan-kynurenine (Trp-Kyn) metabolism has garnered interest because of its association with HIV-1 pathogenesis and neuropathogenesis (Lovelace *et al.*, 2017; Routy *et al.*, 2015). Once Trp is catabolized, it produces several downstream metabolites via the kynurenine (Kyn) pathway such as Kyn, kynurenic acid (KA), and quinolinic acid (QUIN) (Lovelace *et al.*, 2017). In the brain, Trp-Kyn metabolism occurs mainly in infiltrating macrophages and resident microglial cells (Guillemin *et al.*, 2003a; Guillemin *et al.*, 2005). Given that HIV infects macrophages that traverse the blood-brain barrier, a potential link between the Trp-Kyn metabolism and HIV neuropathogenesis might exist. Furthermore, dysregulated inflammation is a major driver of disease progression (Deeks *et al.*, 2013; Roberts *et al.*, 2010), HIV persistence (Klatt *et al.*, 2013), neuropathogenesis, and the development of milder forms of HAND in the modern-ART era (Heaton *et al.*, 2010; Williams, Ipser, *et al.*, 2020). Dysregulated inflammation is observed in plasma and cerebrospinal fluid (CSF) of people living with HIV (Burdo *et al.*, 2013; Guha *et al.*, 2023; Roberts *et al.*, 2010). This inflammation is closely associated with metabolic changes, especially in Trp metabolism (Keegan *et al.*, 2016). Furthermore, the enzyme, IDO-1, which is responsible for Trp degradation into Kyn is upregulated by inflammatory molecules and cytokines like interferon-gamma (IFN- γ) (Werner-Felmayer *et al.*, 1989; Yasui *et al.*, 1986). Subsequently, Trp degradation via the Trp-Kyn pathway results in metabolites which may have excitatory and inhibitory properties (Baran *et al.*, 2000; Braidy *et al.*, 2009). Thus, these excitatory properties play a role in the HIV infection in the CNS (Heyes *et al.*, 1991; Martin *et al.*, 1992). The HAND population often exhibits lowered Trp and Kynurenine/Tryptophan (Kyn/Trp) ratio levels in plasma (Keegan *et al.*, 2016).

Although there is an association between Trp-Kyn metabolism and HIV neuropathogenesis (Lovelace *et al.*, 2017), the association between Trp-Kyn metabolism and HIV-1 viral proteins are unclear; especially the association between HIV-1 Vpr sequence variation and the Trp-Kyn metabolism. In a previous systematic review conducted by our group, Vpr's association with

clinical outcomes in people living with HIV was evident (Asia *et al.*, 2022). However, to our best knowledge, this is the first study to investigate the influence of Vpr amino acid sequence variation on the Trp-Kyn metabolism. Thus, we aimed to assess whether Vpr sequence variation, especially the neurological-associated amino acids at positions 22, 41, 45, and 55, associates with metabolites of Trp-Kyn metabolism in a South African cohort.

2. Methods

2.1. Study Participants

The Prospective Urban and Rural Epidemiology (PURE) study focuses on cardiovascular diseases in people living with HIV and the underlying mechanism that can contribute to the development of CVD such as inflammation, in individuals from 20 countries with varying income levels, encompassing high, middle, and low-income nations (Teo *et al.*, 2009). All demographic data was collected by questionnaires. As a subset of this extensive research, men and women aged 30 and above of African descent were enlisted from both urban and rural regions in the North-West Province of South Africa. The participants were excluded if they had chronic medication use and existing chronic conditions. Baseline data collection, encompassing $n = 2,010$ participants, was voluntarily conducted in 2005. Subsequent follow-up data was gathered in 2010 ($n = 1,288$) and 2015 ($n = 923$). Participants were re-diagnosed in 2010 to confirm their HIV status. Therefore, individuals who screened positive for HIV-1 and were treatment-naïve at the time of data collection in 2010 were included in this study ($n = 103$). For this chapter, a total of 32 participants were used due to depleted samples upon completion of Chapter 3. This selection allowed us to explore metabolic profiles without the interference of antiretroviral treatment (ART), given that ART is recognized for its impact on metabolic profiles in people living with HIV (Keegan *et al.*, 2019). The study's protocol received approval from the Health Research Ethics Committee of the North-West University in South Africa under the reference numbers NWU-00106-22-A1 and NWU-00106-22-A1-01.

2.2. Questionnaires

Participant information including demographic data (including age and sex), lifestyle variables (including tobacco use and alcohol consumption), socioeconomic status and medical history was obtained using the PURE South Africa Adult Questionnaire.

2.3. HIV Status

Before establishing the HIV status of the participants, they underwent counselling sessions provided by trained counsellors. The HIV status was determined using the First Response rapid HIV card test, manufactured by Premier Medical Corporation Limited in Daman, India, in accordance with the protocol established by the South African Department of Health. As a confirmation step, an SD BIOLINE HIV 1/2 3.0 card test from Standard Diagnostics, INC in Korea was used. Participants who received a positive result were provided with post-counselling and were then referred to the nearest clinic or hospital for further assessment. At the clinic or hospital, their CD4⁺ cell count was analysed using the flow cytometric method with the Beckman COULTER EPICS XITM machine from Fullerton, USA.

2.4. Analysis of metabolites in blood samples

Fasting blood samples were collected and were centrifuged at 2000 × g for 15 minutes at a temperature of 10°C, all within 2 hours of collection. After centrifugation, the samples were transferred into microfuge tubes, promptly frozen by placing them on dry ice, and then stored at -80°C in the laboratory until they were ready for analysis. In cases where samples were collected from rural areas, they were also snap-frozen on dry ice but stored at -18°C for a maximum of five days until they could be transported to the laboratory. Upon arrival, they were then stored at -80°C for subsequent analysis.

We investigated the Trp-Kyn metabolism metabolites, specifically Trp, Kyn, Kyn:Trp ratio (in direct measure of IDO activity), KA, and QUIN, due to their potential involvement in the pathophysiology of Vpr-mediated neuropathogenesis and HAND, as discussed in scientific literature references (Baran *et al.*, 2000; Braidy *et al.*, 2009; Guillemin *et al.*, 2003a; Guillemin *et al.*, 2005; Keegan *et al.*, 2016; Lovelace *et al.*, 2017; Werner-Felmayer *et al.*, 1989; Yasui *et al.*, 1986). These metabolic profiles were determined using high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) as described in the following sections.

2.4.1. Chemicals

Kynurenic acid-d5 (2.5mg), L-Kynurenine-d4 [2-aminophenyl-3,5-d2] (5mg), 2,3-pyridinedicarboxylic acid-d3 (Major) (1mg) were purchased from TRC Research Chemical. D-Tryptophan (Indole-D5, 98%) (50mg) was purchased from Cambridge Isotope Laboratories. D-Kynurenine free base (25mg), 2,3-dicarboxylic acid, 99% (25g), D-tryptophan, =98.0% (HPLC) (1g) was purchased from MERCK. UPLC water and acetonitrile, 99%, was purchased from Honeywell. Formic acid for LC-MS was purchased from Merck.

2.4.2. Sample preparation for targeted metabolomics

Trp, Kyn, Kyn/Trp ratio, QUIN, and KA were quantified by liquid chromatography–tandem mass spectrometry (LC-MS/MS; Agilent 1200 series HPLC system) using a targeted approach. Proteins were precipitated from HIV plasma samples by adding 300 µl ice-cold acetonitrile to 100 µl HIV plasma samples in addition to 100 µl internal standard mixture (10 ppm, Kynurenic acid-d5, L-Kynurenine-d4 [2-aminophenyl-3,5-d2], 2,3-pyridinedicarboxylic acid-d3 (Major), D-Tryptophan (Indole-D5, 98%)) (Lindeque *et al.*, 2013). Matrix-appropriate external calibrators were prepared similarly. External calibrators were spiked with Kyn, QUIN, and Trp, which were serially diluted to create a calibration curve. Samples were vortexed and incubated on ice for 10 minutes to facilitate protein precipitation. Samples were then centrifuged at 12 000x g for 10 min. The supernatant was collected, dried under nitrogen gas then stored at -80°C until analysis. For analysis, samples were removed from the -80°C freezer and left at room temperature to equilibrate. The samples were then re-dissolved in 50% HPLC water: 50% ACN, left for 30 minutes at room temperature and then vortexed. The resuspended sample was transferred to a glass vial with a vial insert. Samples were then analyzed on the LC-MS/MS. Metabolic profiles were measured via multiple reaction monitoring (MRM) using electrospray ionization mass spectrometry in both positive and negative modes. The MRM transitions and chromatographic conditions on the LC-MS/MS were optimized (with the help of commercial standards) to detect and quantify the target metabolites accurately. The ratio of Kyn to Trp was used for the estimation of IDO activity.

2.4.3. LC-MS/MS analyses

A targeted LC-MS/MS analysis was performed on HIV-positive plasma samples using a 1200 series HPLC system coupled to a 6470-triple quadrupole-mass spectrophotometer (Agilent Technologies). The chromatographic separations were achieved by injecting 1µL sample on an

Acquity UPLC CSH C18 1.7 μ m 2.1x100mm column, kept at 80°C. Mobile phases consisted of HPLC water (solvent A) and acetonitrile (solvent B) with both containing 0.1% formic acid.

For the positive polarization, the chromatographic gradient was set up as t=0, 1% mobile phase B; t=1, 2% mobile phase B; t=2, 5% mobile phase B; t=3, 10% mobile phase B; t=4, 40% mobile phase B; t=5, 50% mobile phase B; t=6, 60% mobile phase B; t=7, 80% mobile phase B; t=8, 100% mobile phase B; t=10, 1% mobile phase B; lastly, 100% mobile phase B for 5 minutes (post-run) to ensure that all analytes are washed out of the column for the next sample analysis. The flow rate was always 0.2 mL/min except at t=1 until t=2, 0.1 mL/min. Source parameters were set as capillary voltage of 3500 V; nitrogen gas at a flow rate of 5 L/min at 300°C; and nebulizer pressure of 45 psi. MRM parameters were set up for all compounds (Supplementary Table 1).

For the negative polarization, the chromatographic gradient was set up as t=0, 1% mobile phase B; t=2, 1% mobile phase B; t=3, 37% mobile phase B; t=4, 40% mobile phase B; t=5, 45% mobile phase B; t=6, 50% mobile phase B; t=7, 100% mobile phase B; t=8, 1% mobile phase B; lastly, 100% mobile phase B for 5 minutes (post-run) to ensure that all analytes are washed out of the column for the next sample analysis. The flow rate was 0.1 mL/min at t=0; t=2; t=3 and 0.2 mL/min at t=4; t=5; t=6; t=7; t=8. Source parameters were set as capillary voltage of 4000 V; nitrogen gas at a flow rate of 8 L/min at 250°C; and nebulizer pressure of 20 psi. MRM parameters were set up for all compounds (Supplementary Table 1).

2.5. Analysis of immune markers in blood samples

Samples were prepared as described above (*Section 2.3*). We selected specific immune markers for investigation, including soluble urokinase plasminogen activator receptor (suPAR), interleukin (IL) 6, high-sensitivity C-reactive protein (hsCRP), soluble CD163 (sCD163), and neutrophil gelatinase-associated lipocalin (NGAL). These markers were chosen based on their potential relevance to Vpr-mediated neuropathogenesis and HAND pathophysiology, as indicated in scientific literature (Asia *et al.*, 2022; Gianella *et al.*, 2019; Rubin *et al.*, 2018; Williams *et al.*, 2019a). To measure suPAR levels, plasma samples with EDTA were used, and the suPARnostic® ELISA kit from ViroGates in Copenhagen, Denmark, was employed. Particle-enhanced turbidimetric assay was used to analyze hsCRP levels (Cobas Integra 400 plus (Roche

Diagnostic, Basel, Switzerland)), while IL-6 was determined in plasma through the electrochemiluminescence immunoassay method using an Elecsys 2010 (Roche, Basel, Switzerland) apparatus. For sCD163 and NGAL, ELISA assays (R&D systems DuoSet) were conducted following the manufacturer's instructions, and all samples were analyzed in duplicate. The intra- and inter-assay coefficients of variation for all tests fell within acceptable ranges of < 8% and < 10%, respectively.

2.6. Viral protein analysis

RNA was isolated from 200 µl of prepared plasma using the Quick-RNA™ Viral Kit from Zymo Research. The total RNA was then subjected to reverse transcription-polymerase chain reaction (PCR) using the ProtoScript® II First Strand cDNA Synthesis Kit from New England Biolabs, and the resulting DNA was prepared for subsequent PCR analysis. For the amplification of the Tat exon 1/Vpr region (HXB2 position 4900–6351), a primer pair consisting of Vif-1 (5'GGGTTTATTACAGGGACAGCAGAG) and CATH-4R (5'-GTACCCCATAATAGACTGTGACC) was employed. The PCR amplification process involved an initial denaturation step at 94°C for 2 minutes, followed by 40 cycles of denaturing at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 68°C for 2 minutes, and a final extension step at 68°C for 10 minutes. Following PCR amplification, purification of all PCR products was carried out using the Nucleospin® Gel and PCR clean-up kit in accordance with the manufacturer's instructions (Machery-Nagel GmbH & Co.KG, Germany). Subsequently, all PCR products were subjected to sequencing using the BigDye Terminator v.3.1 Cycle Sequencing Ready Reaction Kit from ThermoFisher Scientific and were analyzed using the ABI Prism 3130xl automated DNA sequencer from Applied Biosystems, located in Foster City, CA. To analyze the obtained sequences, the GeneStudio™ Professional sequence analysis software (Version 2.2) was utilized. The nucleotide sequences were translated into amino acid sequences using the Expasy translate method (Gasteiger *et al.*, 2003), and the key mutations in the Vpr region were identified and highlighted for further examination. Sequences are available in GenBank under the accession numbers OR621303- OR621349.

2.7. Statistical analysis

All analyses were conducted using SPSS (version 27, IBM, USA). *P*-values were considered statistically significant for all analyses at less than 0.05. All variables were assessed for normality

by the visual inspection of QQ plots using descriptive statistics. Data distribution for age, the metabolites Kyn/Trp ratio, QUIN and KA and the immune markers NGAL and sCD163 were skewed. Therefore, the data of variables that were skewed were log-transformed before statistical analyses. After log transformation, all data presented acceptable skewness and kurtosis values within the range of -2 and 2.

As the primary aim, we wanted to evaluate whether specific metabolites levels could be related to single amino acid variants. Therefore, we stratified participants into groups: 22I vs. 22L, 41N/S vs. 41G, 45Y vs 45H and 55A vs 55T. At position 41, we compared G vs. N and G vs. S, respectively. Chi-squared tests were used to test group differences for sex, smoking, alcohol use and locality between Vpr amino acid variants. Independent sample T-tests determined differences in study characteristics (age, CD4⁺ count, BMI) and metabolite levels between Vpr amino acid variants. A Bonferroni correction was accounted for the number of metabolites tested ($\alpha/n = .05/5 = .01$) in the primary aim. A Pearson correlation analysis was used to determine covariates by determining correlations between sociodemographic variables (age, sex, smoking status, alcohol use, and locality), and specific metabolites. Analyses of covariance (ANCOVA) were performed with levels of metabolites as the dependent variables to compare the metabolite levels between Vpr variants, adjusting for covariates. A Bonferroni correction was accounted for the number of tests ($\alpha/n = .05/3 = .017$) within the model. Multiple regression analysis was used to determine associations between Vpr amino acid variants and metabolite levels after adjusting for potential covariates.

As a secondary aim, we wanted to determine the association between peripheral immune markers and metabolites. Immune markers were investigated in Chapter 3. Pearson correlations were used to determine correlations between metabolite and immune marker levels/ CD4⁺ count. A Bonferroni correction was accounted for the number of immune markers tested ($\alpha/n = .05/5 = .01$) in the secondary aim. Thereafter, using the enter method, multiple regression analysis was used to determine associations between immune marker and metabolites levels after adjusting for potential covariates.

3. Results

3.1. Study characteristics

This pilot study encompassed a sample of $n = 32$ treatment-naïve South African subtype C participants, with an average age of 48.08 (± 7.066) years. For this chapter, a total of 32 participants were used due to depleted samples upon completion of Chapter 3. Only 25% of the participants were males. While the primary study did not record any viral load data, it should be noted that all participants were treatment-naïve at the time of sample collection. CD4⁺ count data was available for 53% of the participants, showing a mean value of 282.76 (± 161.024) cell/mm³. Participants had a mean BMI of 26.64 (± 10.2). Data on smoking status and alcohol consumption were available for 94% ($n = 30$) of the participants, with 56% and 60% of these participants being current or former smokers and alcohol consumers, respectively. Approximately half of the participants were recruited from rural regions in South Africa ($n = 18$, 56%). When participants were stratified according to Vpr amino acid variants (Table 1), no significant differences were observed for any demographic variables (age, sex, CD4⁺ count, BMI, smoking status, alcohol use and locality) (all $p > .05$). After determining correlations between sociodemographic variables and specific metabolites, it was indicated that, no correlations existed for any of the considered variables (age, sex, smoking status, alcohol use, and locality). Nevertheless, we opted to incorporate sex and age as covariates in the pertinent analyses as will be described below, given their established influence on HIV pathogenesis (Rodés *et al.*, 2022; Scully, 2018).

3.2. Metabolite levels between Vpr amino acid variants

We aimed to examine whether we could link metabolite levels to specific amino acid variants (Table 1). We stratified participants according to Vpr amino acid variants at position 22 ($n = 29$), 41 ($n = 27$), 45 ($n = 30$), and 55 ($n = 28$). After applying Bonferroni corrections of $p = .05/5$, none of the metabolite levels showed significant differences between the Vpr variants at positions 22, 41 or 45. However, QUIN levels were nearing significance for higher levels in the 55A group compared to the 55T group ($p = .022$) (Table 1). After applying Bonferroni corrections of $p = .05/3$, this result remained unchanged in the Vpr 55 group ($p = .023$) after adjustment for covariates (age and sex). Additionally, following the adjustment for age and sex, QUIN levels were nearing significance for higher levels ($p = .023$) in the 41G group when compared to the 41S group.

Table 1: Characteristics of HIV-positive participants and metabolite levels when participants stratified according to specific amino acids variants.

	Position 22 (n = 29)		P value	Position 41 (n = 27)			P value	Position 45 (n = 30)		P value	Position 55 (n = 28)		P value
Amino acid	I	L		G	N	S		Y	H		A	T	
N (%)	16 (55%)	13 (45%)	-	15 (55%)	6 (22%)	6 (22%)	-	18 (60%)	12 (40%)	-	7 (25%)	21 (75%)	-
*Age in years, mean (SD)	47.31 (6.23)	49.08 (8.43)	.523	47.47 (4.9)	46.83 (7.6)	45.7 (8.7)	.822/.548	48.44 (6.5)	48.1 (8.4)	.895	49.43 (6.6)	47.57 (6.6)	.525
Sex, female N (%)	10 (63%)	11 (85%)	.624	10 (67%)	5 (83.3%)	4 (67.07%)	.733	15 (83%)	9 (75%)	.576	7 (64%)	27 (84%)	.145
CD4 ⁺ , mean (SD)	274.50 (119.21)	313.71 (139.52)	.662	272.2 (191)	213.7 (74.2)	367 (222.6)	.564/.513	287.71 (164.3)	241.8 (125.4)	.535	150.5 (72.8)	330.92 (152)	.132
Body Mass index (BMI)	235.5 (16)	223 (34)	.532	22.3 (3.4)	23 (8.4)	21.5 (2.5)	.747/.640	23 (4.8)	23.5 (5.8)	.811	20.5 (4)	24.2 (5.4)	.118
Smoker	9 (56%)	7 (64%)	.701	7 (47%)	3 (50%)	6 (100%)	.167	9 (56%)	8 (67%)	.576	6 (86%)	11 (55%)	.148
Alcohol use	9 (56%)	5 (45%)	.581	6 (40%)	2 (33%)	5 (83%)	.253	7 (44%)	6 (50%)	.743	3 (42%)	11 (55%)	.580
Locality, rural N (%)	6 (38%)	9 (69%)	.089	10 (67%)	4 (67%)	3 (50%)	.757	10 (56%)	7 (58%)	.880	4 (57%)	11 (52%)	.827
Trp, ug/ml	0.16 (0.06)	0.13 (0.07)	.223	0.17 (0.07)	0.13 (0.08)	.13 (0.04)	.303/.226	0.15 (0.07)	0.15 (0.06)	.710	0.15 (0.07)	0.15 (0.06)	.822
Kyn, ug/ml	0.0087 (0.003)	0.0088 (0.003)	.904	0.01 (0.002)	0.01 (0.003)	0.008 (0.004)	.587/.254	0.009 (0.002)	0.009 (0.001)	.646	0.009 (0.003)	0.008 (0.003)	.589
Kyn/Trp ratio, ug/ml	.062 (.027)	.085 (.042)	.106	.07 (.034)	.089 (.045)	.0586 (.032)	.290/.418	.068 (.036)	.071 (.032)	.739	.074 (.038)	.061 (.026)	.375
QUIN, ug/ml	.0013 (.0009)	.0015 (.0009)	.543	.0017 (.0009)	.0013 (.0004)	.0009 (.0007)	.368/.073	.0016 (.0009)	.0012 (.0007)	.157	.002 (.0012)	.001 (.0005)	.022
KA, ug/ml	.000014 (00002)	.00002 (.00003)	.115	.00003 (.00003)	.000008 (.00003)	.000007 (.00003)	.076/.126	.00002 (.00003)	.00001 (.00001)	.725	.00001 (.00001)	.00002 (.00003)	.666

Data represented as mean (SD) or N (%). Logarithmically transformed variables has been back transformed for presentation purposes. At position 41, we compared G vs N and G vs S indicated by p-value/p-value, respectively. Data were unavailable for 2 participants in the L22s and Y45 groups and 1 participant in the T55 group (alcohol use and smoking). Adjusted for age and sex.

Further, we aimed to evaluate the relationships between Vpr amino acid sequence variation and metabolite levels while adjusting for covariates (age and sex) as shown in Table 2. Here, variation at position 41 (between 41G and 41S) showed significant associations with QUIN levels ($p = .023$). When investigating the Vpr amino acids at position 55, variation at this position (between 55A and 55T) demonstrated significant associations with QUIN levels after ($p = .023$). No significant associations were detected for the other Vpr amino acid signatures or metabolites.

Table 2: Multiple regression analyses of the association between Vpr group and QUIN, including covariates.

Vpr group	Adj R ²	β	CI	p
41	.204	.505	.133 to 1.544	.023
55	.126	.444	.102 to 1.257	.023

Model 1: adjusted for demographics (age and sex)

3.3. Correlation between immune markers and metabolites in the specific Vpr groups

Given the significant associations between QUIN levels and variation at Vpr amino acid position 41 and 55, we sought to investigate potential correlations between QUIN and immune markers (investigated in Chapter 3) in these respective groups. Following the application of a Bonferroni correction ($p = .05/5$), we observed a significant positive correlation between suPAR and QUIN levels in the Vpr 41 group ($p = .005$) (Figure 1C). In the Vpr 55 group, QUIN was not associated with any of the investigated immune markers (all $p > .01$) (Figure 1F- Figure 1J). Subsequently, we aimed to assess the association between QUIN and suPAR levels while adjusting for covariates. Even after adjusting for covariates, including age and sex, QUIN was significantly associated with suPAR within Vpr 41 group (adj R²= .356, $\beta = .664$; $p = .003$). Given the absence of viral load data for the entire cohort, adjustment for this variable was not possible. However, CD4⁺ count data was accessible for 53% of the participants. Consequently, we sought to explore the extent to which these disease characteristics may have contributed to QUIN levels in our study. In the overall cohort with available CD4⁺ data ($n = 17$), QUIN levels were negatively correlated with CD4⁺ count ($r = -.711$, $p = .001$).

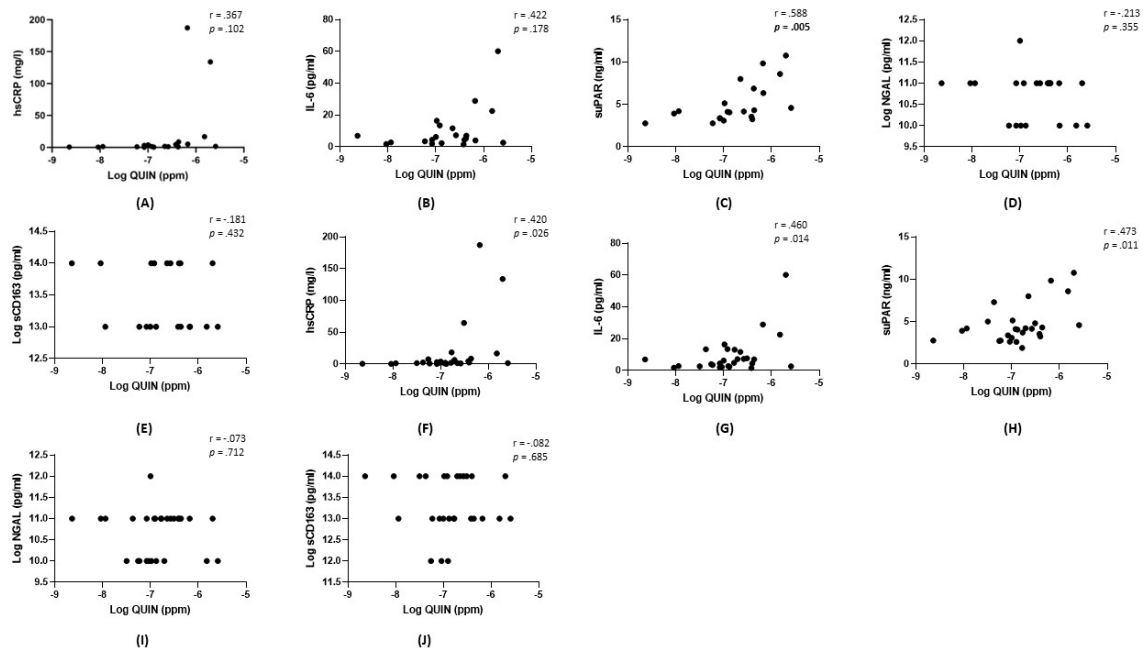


Figure 1: Scatter plot depicting the Pearson correlation between QUIN and immune marker levels. A-E correspond to the Pearson correlation within the Vpr 41 group and F-J correspond to the Pearson correlation within the Vpr 55 group.

4. Discussion

Our study encompassed three main findings. Firstly, among the various Vpr amino acid variants and metabolites examined, only the Vpr 41G and 55A group was nearing significantly higher levels of QUIN when compared to the Vpr 41S and 55T variants respectively. Secondly, amino acid variation at positions 41 and 55 were found to be significantly associated with QUIN levels. Lastly, in the Vpr 41G group, QUIN correlated with higher suPAR levels. These results collectively underscore the importance of specific amino acid substitutions in influencing QUIN and specifically, suPAR levels, potentially contributing to our understanding of their roles in the pathogenesis and neuropathogenesis of HIV-1.

The findings of this study report significant associations between QUIN levels and Vpr variation at positions 41 (between 41G and 41S) and 55 (between 55A and 55T), respectively. These findings may indicate that Vpr variation at these specific positions could increase QUIN levels. Indeed, we showed that the Vpr 41G and 55A variant showed higher levels of QUIN

compared to the 41S ($p = .023$ and 55T groups ($p = .023$), respectively. The higher QUIN levels could potentially be indicative of negative clinical outcomes, as peripheral and CSF QUIN levels have previously been associated with neurological outcomes in psychiatric disorders, including HAND (Anderson *et al.*, 2018; Skorobogatov *et al.*, 2021). Although QUIN cannot freely cross the BBB (Fukui *et al.*, 1991), it is a product of 3-hydroxykynurenine, which can cross the blood-brain barrier freely. Additionally, 3-hydroxykynurenine is a downstream metabolite of tryptophan, which can also readily cross the blood-brain barrier (Schwarcz *et al.*, 2010). As a result, the production of QUIN in the brain may persist, maintaining its neuroexcitatory properties (Braidy *et al.*, 2009). During immune activation in the CNS, over 98% of the Kyn and QUIN present in the brain are produced within the CNS (Kita *et al.*, 2002). However, studies have demonstrated that human monocytes and monocyte-derived macrophages can produce up to 19 times more QUIN than activated microglia (Guillemin *et al.*, 2003b). In line with this, the synthesis of QUIN by microglia in patients with epilepsy was approximately only 15% of the QUIN produced by monocyte-derived macrophages retrieved from brain tissue (Espey *et al.*, 1997). Lastly, it's important to highlight that QUIN concentrations consistently tend to be higher in the bloodstream compared to the CNS across various pathological conditions, with blood-to-cerebrospinal fluid (CSF) ratios of 14:1 in humans, 19:1 in rodents, and even up to 52:1 in nonhuman primates (Morrison *et al.*, 1999). This observation is also pertinent to the CNS because infiltrating activated macrophages could potentially serve as the primary source of QUIN during brain inflammation. Therefore, an investigation into systemic QUIN levels may provide valuable insights into how these levels may be reflected in the CNS. These findings may have translation value to other health conditions. Other studies have also identified associations between plasma Trp-Kyn metabolites and various health conditions, including the presence of depression (Drivsholm *et al.*, 2021), HIV-related gut microbiota alterations, and gut-adipose tissue (Gelpi *et al.*, 2022). Moreover, these metabolites have been shown to predict poor CD4+ T-cell count recovery and increased mortality among individuals with HIV infection. Additionally, they may play a role in discriminating modest detection of active tuberculosis in adults with HIV (Olsson *et al.*, 2022; Wang, Mehra, *et al.*, 2021).

A study investigating treatment experienced people living with HIV found that the 55A amino acid to be linked with adverse neurological outcomes (Dampier *et al.*, 2017). According to Dampier and colleagues, people living with HIV who possess the 55A amino acid variant tend to have higher global deficit scores (Dampier *et al.*, 2017). However, an opposing viewpoint has been proposed, suggesting positive clinical outcomes. A study associated the Vpr 55A

amino acid variant with a lower plasma viral load in treatment naïve people living with (Kamori *et al.*, 2017). Our findings suggest that the 55A variant, by means of the Trp-Kyn metabolism may be associated with negative clinical outcomes by enhancing inflammation as QUIN correlated with suPAR in participants with this variant only. However, further studies are needed to investigate this variant in larger cohorts to validate the findings reported here.

Previous studies have found that the 41G variant is associated with positive clinical outcomes and is observed in treatment experienced long-term non-progressor people living with HIV and non-stroke control groups (McMullen *et al.*, 2021; Zhao *et al.*, 2002). Furthermore, McMullen and colleagues found that the 41G variant is associated with lower CD4 counts and ischemic stroke (McMullen *et al.*, 2021). Conversely, an earlier study by Dampier and colleagues found that the S41 variant in treatment-experienced participants was associated with positive clinical outcomes, as participants exhibited decreased neurocognitive deficits (Dampier *et al.*, 2017), aligning with our findings. Although limited studies have investigated Vpr amino acid variation at position 41, it is evident that there is no clear consensus regarding the influence of 41G/41S amino acids on clinical outcomes in people living with HIV. However, our findings suggest that 41G may be considered a high-risk amino acid in people living with HIV since the 41G group had higher QUIN levels which correlated with suPAR in this group only, which are believed to be characteristic of negative clinical outcomes (Braidy *et al.*, 2009; Smith *et al.*, 2001). It is possible that amino acid 41 is located within the second alpha-helix (38-50), a structurally significant region for key mechanisms such as the regulation of apoptosis, subcellular transport (Morellet *et al.*, 2009) and virion incorporation of Vpr (Singh *et al.*, 2000). Consequently, modifying this configuration and changing the amino acids at this position could potentially disrupt the arrangement of the Vpr alpha helices, thus affecting its functional potential. However, further investigation using modelling and structural techniques is needed to assess the potential functional implications of different amino acids at these positions. While QUIN is known to be neuroexcitatory and linked to worsened neurological outcomes (Braidy *et al.*, 2009), it is important to note that higher suPAR levels have also been associated with decreased neurological performance in people living with HIV (De Almeida *et al.*, 2022; Gianella *et al.*, 2019). Considering the association of suPAR with neurological outcomes, it's worth considering that suPAR may play a role in tryptophan-kynurenine metabolism as an alternate mechanism in the development of HAND. suPAR is known to contribute to an elevated inflammatory environment, and a proinflammatory milieu can stimulate tryptophan-kynurenine metabolism, particularly through the activity of IDO-1. This suggests that suPAR may play a potential role in the breakdown of tryptophan, leading to the subsequent production

of the downstream product QUIN (Werner-Felmayer *et al.*, 1989; Yasui *et al.*, 1986). These findings imply that the inflammatory-associated marker suPAR could further contribute to neurotoxicity due to its significant correlation with the neurotoxic metabolite QUIN. Additionally, suPAR has been shown to be a novel, independent predictive marker of myocardial infarction in people living with HIV (Rasmussen *et al.*, 2016) which may further contribute to the continuous increase in mortality rates in people living with HIV (Lawn *et al.*, 2007; Sidenius *et al.*, 2000).

While our investigation focused on peripheral metabolite levels, we believe that these findings could be relevant to the activity of these metabolites in the CNS. The activation of the Trp-Kyn metabolism in the brain due to cytokine stimulation may have parallels in peripheral tissues (Skorobogatov *et al.*, 2021). In theory, assessments of peripheral metabolites such as QUIN or KA could indirectly offer insights into the conditions within brain tissue (Skorobogatov *et al.*, 2021). Furthermore, in a recent systematic review conducted by our group comprising 22 studies investigating Vpr amino acid substitutions and clinical outcomes, 10 of the studies included treatment-experienced participants, 8 did not report treatment status, and only 4 studies reported on participants who were treatment-naïve (Asia *et al.*, 2022). Therefore, our study provides insight into a relatively scarce population of treatment-naïve individuals within the HIV population.

5. Limitations

This study possesses several noteworthy limitations that deserve attention. First, the sample size was restricted, potentially influencing the reported findings. Therefore, it is essential to interpret our findings considering this limitation. Due to the limited sample size, there is need for future research to confirm our results with larger study cohorts. While we consider this study as preliminary, it provides valuable insights into the link between Vpr sequence variations in a cohort of treatment-naïve individuals, offering a clearer understanding of the metabolic profile without the potential confounding effects of combination cART. Second, we also examined peripheral metabolic and immune markers to gain insights into their potential role in central nervous system (CNS) function (Burdo *et al.*, 2013; Roth *et al.*, 2021). Furthermore, other peripheral markers, including neurofilament light (NFL), which were not investigated in this study, might have offered additional insights into the neuropathology of HIV-1. Previous studies have established a significant correlation between peripheral NFL levels and those within the CNS. Plasma NFL has been shown to detect both severe and

subclinical neuronal injury in HIV (Gisslen *et al.*, 2007). Therefore, incorporating this marker would have been ideal for fully appreciating the translational value of our findings within the CNS. Lastly, studies have identified that plasma Trp-Kyn metabolites predict poor CD4+ T-cell count recovery and increased mortality among individuals with HIV infection. Seeing as CD4+ count and viral load, were unavailable for a substantial subset of participants in our study, we could not consider these variables as covariates. In a subset of participants with CD4+ count data, we observed a significant negative correlation between CD4+ count and QUIN levels. This suggests that CD4+ count may have influenced the reported levels of QUIN in this study. However, further investigation is required, and it is essential to acknowledge that this limitation may have impacted our findings. This cohort was initially used to investigate CVD in people living with HIV. Thus, evaluation of HAND was not performed; however, the association of certain neuropathogenic associated Vpr variants with inflammation and metabolic marker hold some translational value into the underlying mechanism that may contribute to HIV-1 pathogenesis and the development of HAND. Thus, for future studies, it would be ideal to select a cohort that have being evaluated for neuropsychological data. An initial objective of the study was to assess the combined impact of various amino acid substitutions on peripheral inflammatory levels, utilizing a composite risk score and categorizing participants into high-neutral and low-neutral Vpr risk groups. Although this approach has been employed previously³⁶, we recognize a limitation in that certain amino acids may exert a more significant influence than others. Summing amino acids together in this manner might attenuate this effect, potentially contributing to the lack of significance observed in the investigation of Vpr risk categories and risk scores. Another limitation to this study is that we did not adjust for confounding factors such as HLA. Genetics of the participants may play a role in how protective or susceptible participants are to HIV infection and progression of the disease (Carrington & O'Brien, 2003; Li *et al.*, 2017). We only investigated one viral proteins, Vpr, and there are other viral proteins such as Tat and gp120 that may have contribute to the results reported in this study. There are many more factors in clinical samples that may have an effect; however *in vitro/in vivo* studies may be replicated in clinical models. This has been shown in major viral proteins such as Tat but it is not clear for other less commonly studied viral proteins such as Vpr.

6. Conclusion

Here we report evidence that the Vpr amino acid sequence variations may have an influence on metabolic and inflammatory systems, particularly in the Trp-Kyn metabolism. The associations between these sequence variations and Kyn metabolite levels, alongside the significant correlations with immune markers, provide insights into potential mechanisms

contributing to the HIV-1 pathogenesis and potentially neuropathogenesis. This research underscores the importance of understanding the genetic nuances of HIV and their broader systemic implications, emphasizing the need for continued exploration in this area to better understand the pathogenesis and neuropathogenesis in HIV-1 people living with HIV.

7. References

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8. Supplementary

Supplementary Table 1: LC-MS/MS MRM parameter setup

Compound name	Precursor ion (m/z)	Product ion (m/z)	Dwell time	Fragmentor voltage (V)	Collision energy (eV)	Polarization
L-kynurenine-d4	213.1	196	200	80	8	Positive
D-tryptophan-d5	210.1	192	200	75	8	Positive
D-kynurenine	209.1	192	200	75	8	Positive
D-tryptophan	205.1	188	200	70	8	Positive
Kynurenic acid-d5	195.1	149	200	91	24	Positive
Quinolinic acid-d3	171	81.1	200	75	28	Positive
			200			
L-kynurenine-d4	211.1	193	200	75	8	Negative
D-tryptophan-d5	208.1	120.1	200	128	20	Negative
Kynurenic acid-d5	193.1	149.1	200	101	16	Negative
Kynurenic acid	190	144	200	86	20	Negative
Quinolinic acid-d3	169	125	200	70	8	Negative
Quinolinic acid	166	122	200	70	8	Negative

CHAPTER 5

5.1. Summary of findings

This dissertation investigated the influence that HIV-1 Viral protein R (Vpr) sequence variation has on factors such as immune activation, inflammation, and metabolism. Therefore, this investigation was addressed in three manuscripts. The key findings of the dissertation were as follows:

- 1) Based on a systematic review of the current literature, it was concluded that Vpr amino acid variants may contribute to clinical outcomes in people living with HIV. Following the criteria set out, it was found that amino acid variants 63T was associated with positive clinical outcomes and amino acid variants, 77H and 85P had no significant contribution to disease outcome. Although not deemed noteworthy in the criteria set out, we did make use of the findings reported here to select potential neurological-associated amino acid positions that were associated with clinical outcomes for further experiments within this dissertation.
- 2) A pilot study investigating HIV Vpr amino acid variants and their influence on inflammation was conducted on a South African cohort of 48 people living with HIV. After applying Bonferroni corrections ($p = .05/3$) and adjusting for sex and locality, suPAR levels was nearing significance for higher levels in participants with the 41G amino acid variant compared to those with the 41S variant ($p = .035$), after. Further, amino acid variations at position 41 (between 41G and 41S) exhibited a significant association with suPAR ($\text{adj } R^2 = .089$, $\beta = .386$; 95% CI .125 to 3.251; $p = .035$). This is the first study, to our knowledge, to investigate these Vpr amino acid variants and their correlation to inflammation without combination antiretroviral therapy (cART) as a potential confounding factor.
- 3) A pilot study investigating HIV Vpr amino acid variants and their influence on Tryptophan-Kynurenine (Trp-Kyn) pathway was conducted on a South African cohort of 32 people living with HIV. After applying Bonferroni corrections ($p = .05/3$) and adjusting for covariates (age and sex), only the Vpr 41G and 55A groups was nearing significance for higher levels of QUIN compared to the Vpr 41S and 55T groups, respectively (all $p = .023$). Multiple regression results revealed that Vpr amino acid variations at position 41 ($\text{adj } R^2 = .049$, $\beta = .505$; $p = .023$), and 55 ($\text{adj } R^2 = .126$, $\beta = .444$; $p = .023$) displayed significant associations with QUIN after adjusting for age and sex. Lastly, the higher QUIN levels observed in the Vpr 41G group were found to be correlated with suPAR ($r = .588$, $p = .005$). This is the first study to our knowledge

to investigate the Vpr amino acid variants and their correlation to the Trp-Kyn metabolism without cART as a confounding factor.

5.2. Final recommendations

The overall conclusion from this dissertation supports the premise that Vpr amino acid sequence variations may be associated with clinical outcomes in people living with HIV. Vpr amino acid variants may influence neurological-associated inflammatory and metabolic markers in people living with HIV. However, it should be noted that there are confounding factors that may have influenced the findings collectively of Chapters 2-4. Additionally, for Chapter 3 and 4, this cohort was initially used to investigate CVD in people living with HIV. Thus, evaluation of HAND was not performed; however, the association of certain neuropathogenic associated Vpr variants with inflammation and metabolic marker hold some translational value into the underlying mechanism that may contribute to HIV-1 pathogenesis and the development of HAND. Thus, for future studies, it would be ideal to select a cohort that have being evaluated for neuropsychological data. Thus, the major conclusions from this dissertation can be divided into three major components.

Firstly, from a systematic search of literature, Vpr amino acid variants may have implications for disease progression, neurological, and treatments efficacy in people living with HIV. However, the Vpr amino acid variants highlighted in this systematic review warrant further investigation to determine their potential role as predictive indicators of disease progression or anchor points to better understand their implications in HIV-1 pathophysiology.

Second, in an investigation to determine the influence of Vpr amino acid variants on immune activation and inflammation markers in a South African treatment-naïve cohort, sCD136, NGAL, IL-6, suPAR, and hsCRP may be promising targets for future investigation in treatment-naïve participants. Considering that HIV-1 subtype C has lower inflammation levels compared to HIV-1 subtype B, these amino acid variants may potentially have a greater functional role in the HIV-1 subtype B infection. However, more studies should investigate the influence of these amino acid variants on immune activation and inflammation in HIV-1 subtype C as it accounts for the most HIV-1 infections. Also, our study investigated treatment-naïve

participants allowing us to determine the levels of inflammation associated with these amino acid variants without ART as a confounding factor.

Third, in an investigation to determine the influence of Vpr amino acid variants with metabolism in a South African treatment-naïve cohort, Trp, Kyn, KA, and QUIN may be promising targets for future investigation in treatment-naïve participants. Considering the potential role that these amino acid variants may have in the HIV-1 neuro/pathogenesis, more studies are needed to further validate these findings within HIV-1 subtype C as most studies investigate HIV-1 subtype B.

5.3. Strengths

First, this dissertation compiled a systematic review (Chapter 2) of the literature to identify HIV-1 sequence variations which are associated with clinical outcomes in people living with HIV. Thus, Chapter 2 benefits researchers investigating HIV-1 Vpr and clinical outcomes by having all the data in one article, thereby, saving them time from scouring extensive literature. Second, this dissertation further provided new insights into the influence that the specific HIV-1 Vpr sequence variations investigated may have on inflammation, particularly on suPAR and NGAL, which are known to be associated with HIV pathogenesis and HIV-comorbidities. Therefore, Chapter 3 may also provide insights into the contribution of inflammation to HAND through HIV-1 Vpr sequence variation. Third, Chapter 4 provided insights into the influence that the specific HIV-1 Vpr sequence variations investigated here may have on Trp-Kyn pathway metabolites, particularly those metabolites associated with neurological outcomes. Therefore, Chapter 4 suggests a potential mechanism contributing to HIV pathogenesis and co-morbidities e.g., onset of HAND, which is an attractive target for future studies. Fifth, the investigations that were done in this dissertation were performed on a South African HIV-1 subtype C-infected population (urban and rural areas) that was treatment naïve. This not only allowed us to determine the influence of HIV-1 Vpr sequence variation on immune activation, inflammation, and metabolism in a subtype C context but also to determine this influence without cART as a potential confounding factor. Despite most studies investigating HIV-1 subtype B populations, this dissertation provides evidence that HIV-1 subtype C is just as important, particularly in terms of HIV-1 Vpr's influence on immune activation, inflammation, and metabolism. Sixth, this dissertation adapted a standardised method for LC-MS/MS to detect and quantify Trp-Kyn pathway metabolites Trp, Kyn, Kyn/Trp, KA, and QUIN. This

method will be used for further investigations of the Trp-Kyn pathway by other postgraduate students within the facility. Last, this dissertation contributed to literature in the form of three manuscripts which provided insights into Vpr amino acid variants with clinical outcomes, immune activation, inflammation, and metabolism. These manuscripts may anchor a new direction for HIV-1 viral protein investigations including specific variations and clinical outcomes.

5.4. Limitations

This dissertation has several limitations which deserves emphasis. Firstly, in a systematic review of literature, the studies reviewed were diverse and heterogeneous. Thus, a meta-analysis could not be conducted due to this diversity and heterogeneity as the studies reviewed had different study designs (longitudinal and cross-sectional), different measures of clinical outcomes (e.g., neurological and disease progression), and key cohort information. Confounding factors were not clearly defined, nor were relevant statistical analysis to control for these variances. Furthermore, CD4⁺ count and viral load were not reported in all studies even though our review found that they are associated with clinical outcomes. Sample/cohort size varied across the studies compiled in this review, which may potentially result in the associations of genetic diversity observed. Furthermore, most studies that were selected for this review were conducted more than 10 years ago; considering the advancements in HIV-1 research and genome sequencing within this time, newer studies related to this topic are needed. This will aid in the further understanding of Vpr amino acid variants and their associations to clinical outcomes, particularly in a HIV-1 subtype C context.

Secondly, there are several limitations in Chapters 3 and 4. Sample size in Chapters 3 and 4 were limited as they were comprised of a total of 48 and 32 participants, respectively. Although the sample size for these chapters were limited, our study was exploratory in nature and therefore we provide some insight that Vpr amino acid variants may be associated with both inflammation (Chapter 3) and Trp-Kyn metabolism (Chapter 4) in a treatment-naïve subtype C population. Thus, we consider this a preliminary study as it does provide insights into the link between Vpr amino acids with both inflammation and metabolism in a treatment-naïve population, allowing us to understand inflammation and metabolism without the potential confounding effects of cART. However, further studies in different cohorts and/or varying subtypes are needed to validate whether a similar relationship exists between these Vpr amino acid variants with inflammation and metabolism. Furthermore, in chapter 3 and 4 we investigated only one HIV-1 viral protein. It should also be noted that confounding factors such

as HLA and other viral proteins, and potential risks to comorbidities may have influenced the outcomes from this investigation. Patient genetics may influence HIV infectivity and disease progression as certain genes may be protectant against HIV infectivity and other genes rather than increasing the susceptibility of HIV infection (Carrington & O'Brien, 2003; Li *et al.*, 2017). Furthermore, other pivotal viral proteins, such as Tat and gp120, might have also exhibited specific variations that could have contributed to the observations reported here. Nonetheless, our approach to examining a single HIV-1 protein and its association with clinical outcomes is consistent with methods used in other studies (Dampier *et al.*, 2017; Rao *et al.*, 2014). An additional limitation is that peripheral inflammation and metabolic markers were evaluated to draw insights into the CNS as previous studies have shown that peripheral inflammation and metabolism are associated with clinical HAND (Burdo *et al.*, 2013; Roth *et al.*, 2021). Nevertheless, there is a direct correlation between the specific markers in the periphery and CNS which suggest that our finding may be relevant for investigations in the CNS (Gisslén *et al.*, 2016).

5.5. Conclusions

The key findings from this dissertation indicate that Vpr amino acid variants may influence clinical outcomes in people living with HIV, as shown in a systematic search of literature. Further, in a South African context which is primarily HIV-1 subtype C infected, Vpr amino acid variants may influence the levels of neurological-associated inflammatory and metabolic markers in people living with HIV. Taken together, these findings advanced our understanding of how clinical-associated and neurological-associated Vpr amino acid variants relate to inflammation and metabolism in South African HIV-1 subtype C people living with HIV. Further studies should investigate these amino acid variants in larger and varying HIV-1 cohorts to validate if similar findings occur as well as in longitudinal analyses.

ANNEXURES

Annexure A: Published manuscript (<https://doi.org/10.1111/eci.13943>) titled “The influence of viral protein R amino acid substitutions on clinical outcomes in people living with HIV: A systematic review.

Annexure B: Submitted manuscript, under review (OFID-D-23-01181) titled “A pilot investigation of the association between Vpr amino acid substitutions and peripheral immune marker levels in people living with HIV: implications for neurocognitive impairment.”

Annexure C: Submitted manuscript, under review (c713cda8-ebc2-4311-8776-790b6a8867ee) titled “A pilot investigation of the association between HIV-1 Vpr amino acid sequence diversity and the Tryptophan-kynurenine pathway as a potential mechanism for neurocognitive impairment.”

ANNEXURE A:

**THE INFLUENCE OF VIRAL PROTEIN R AMINO ACID SUBSTITUTIONS ON CLINICAL
OUTCOMES IN PEOPLE LIVING WITH HIV: A SYSTEMATIC REVIEW**

The influence of viral protein R amino acid substitutions on clinical outcomes in people living with HIV: A systematic review

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Abstract

Background: The HIV viral protein R (Vpr) is a multifunction protein involved in the pathophysiology of HIV-1. Recent evidence has suggested that Vpr amino acid substitutions influence the pathophysiology of HIV-1 and clinical outcomes in people living with HIV (PLWH). Several studies have linked Vpr amino acid substitutions to clinical outcomes in PLWH; however, there is no clear consensus as to which amino acids or amino acid substitutions are most important in the pathophysiology and clinical outcomes in PLWH. We, therefore, conducted a systematic review of studies investigating Vpr amino acid substitutions and clinical outcomes in PLWH.

Methods: PubMed, Scopus and Web of Science databases were searched according to PRISMA guidelines using a search protocol designed specifically for this study.

Results: A total of 22 studies were included for data extraction, comprising 14 cross-sectional and 8 longitudinal studies. Results indicated that Vpr amino acid substitutions were associated with specific clinical outcomes, including disease progressions, neurological outcomes and treatment status. Studies consistently showed that the Vpr substitution 63T was associated with slower disease progression, whereas 77H and 85P were associated with no significant contribution to disease progression.

Conclusions: Vpr-specific amino acid substitutions may be contributors to clinical outcomes in PLWH, and future studies should consider investigating the Vpr amino acid substitutions highlighted in this review.

KEYWORDS

amino acid substitutions, disease progression, HIV-1 clinical outcomes, neurocognitive deficits, Vpr and sequence variation

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1 | INTRODUCTION

Human immunodeficiency virus (HIV), a retrovirus, is the causative agent of acquired immunodeficiency syndrome (AIDS).^{1,2} According to the Joint United Nations Programme on HIV/AIDS (UNAIDS), it was approximated in 2021 that 37.7 million people were living with HIV (PLWH), 1.5 million people were newly infected and 680,000 people had AIDS-related deaths, globally.³ Even with 75% of PLWH having access to antiretroviral therapy (ART) globally and despite effective treatment for PLWH with ART, other complications, especially those of the central nervous system (CNS), persist.⁴

Human immunodeficiency virus is a genetically diverse pathogen due to high replication rates, error-prone reverse transcriptase and recombination events that could occur during viral replication.⁵⁻⁷ Two types of HIV exist, HIV-1 and HIV-2, of which HIV-1 is more prevalent⁸⁻¹⁰ and is categorised into groups M, N, O and P.¹¹⁻¹⁶ As reviewed by Sharp and colleagues, HIV-1 group M is the more geographically abundant group than other HIV-1 groups.¹⁷ Furthermore, HIV-1 group M consists of viral subtypes A, B, C, D, F, G, H, J and K.¹¹ Furthermore, subtypes A and F have sub-subtypes, namely A1-4 and F1-2, respectively.¹⁸⁻²² The subtypes predominate in particular regions worldwide: CRF02_AG/Subtype G- West Africa; Subtype A- East Africa, Eastern Europe, and Central Asia; Subtype C- Southern Africa, Ethiopia, and South Asia (India); Subtype B- Western and Central Europe, North America, Caribbean, Latin America and Oceania.⁵ Amongst all HIV-1 subtypes, M and O are considered the more genetically diverse subtypes.¹²⁻¹⁶

HIV-1 consists of various viral proteins which play important roles during the viral lifecycle.²³ Human immunodeficiency virus encodes for 15 distinct proteins and these include: structural (matrix, capsid, nucleocapsid, p6, surface and transmembrane), Pol enzymes (protease, reverse transcriptase and integrase), regulatory proteins (transactivation of transcription (Tat) and regulator of expression of virion proteins (Rev)) and accessory proteins (negative regulatory factor (Nef), viral infectivity factor (Vif), viral protein R (Vpr) and virus protein U (Vpu)).²³

Traditionally, the HIV-1 accessory Vpr has received less attention despite its relevance to pathophysiological mechanisms and clinical outcomes in PLWH. Viral protein R is a fourteen-kilodalton basic protein comprised of 96 amino acids and is made up of three amphiphilic helices.²⁴ These bundled α -helices extend to the amino acid regions 17-33, 38-50 and 55-77 and are flanked by unstructured flexible N- and C-terminal domains that are negatively or positively charged, respectively.²⁴ HIV Vpr is a multifunctional protein involved in the pathophysiology of HIV, more specifically Vpr (1) counteracts

the IFN-stimulated macrophage-specific restriction pathway targeting HIV-1 Env,²⁵ (2) targets Tet Methylcytosine Dioxygenase 2 (TET2) for degradation by CRL4^{VprBP} E3 Ligase to sustain pro-inflammatory cytokine interleukin-6 (IL-6) expression and enhance HIV-1 replication,²⁶ (3) counteracts the restriction of lysosomal-associated transmembrane protein 5 (LAPTM5) to promote HIV-1 infection in macrophages,²⁷ (4) targets cellular proteins to drive system-level proteomic remodelling in HIV-1 infection,²⁸ and (5) counteracts helicase-like transcription factor (HLTF)-mediated restriction of HIV-1 infection in T cells.²⁹ The effect that Vpr has on monocytes and infected macrophages is associated with HIV-1-associated neuropathogenesis.³⁰ Even in the absence of active viral replication and with the effective use of ART, Vpr can still be detected in the periphery and CNS of PLWH.³¹⁻³³ Therefore, Vpr may have a crucial role in the pathophysiology of HIV-1 in the modern-ART era.

Subtype-specific sequence variation exists between HIV-1 subtypes, and this results in changes in the amino acid sequences of the HIV-1 viral proteins, including Vpr. Multiple research groups have extensively sequenced the HIV-1 genome.³⁴⁻³⁷ HIV-1 mutates rapidly and using a wide range of investigations, the HIV-1 mutation rate is estimated to be 10^{-5} – 10^{-3} errors/bp/cycle³⁸ with the *Env* gene having the fastest mutation rate amongst all viral genes.³⁹⁻⁴¹ Even though Vpr is an accessory protein, the Vpr gene is generally well-conserved in all human (HIV-1, HIV-2) and simian (SIV) immunodeficiency viruses⁴²⁻⁴⁴ and has lower mutation rates than other viral genes⁴⁰ and therefore indicative of its potential importance in the pathogenesis of HIV.

The sequence variation in the HIV-1 viral proteins influences the pathophysiology of HIV-1.^{31,45-51} When comparing the Vpr protein sequences between HIV-1 subtypes, several variances are noted (Figure 1) and certain amino acid substitutions have been associated with the pathophysiology of HIV-1.^{50,52} This may be due to a change in the structural characteristics of the Vpr protein (Figure 2). Overall, the structure of Vpr may seem similar between HIV-1 subtypes (Figure 2), it is known that the alteration of specific amino acids or the substitutions of amino acids at specific positions within Vpr may significantly alter its function.⁵³ As examples of these, mutations (R36W, I64E, L67A, and I70S) in α -helix residues result in a total inability of Vpr to oligomerize at the nuclear envelope.⁵⁴ In addition, the deletion of the amino acids of the SRIG sequence (between amino acids 79 and 82) or mutation of all arginine's in the C-terminal domain prevented G2 arrest by Vpr.⁵⁵

As reviewed in Fabryova, Strebel,⁵⁶ several preclinical studies have investigated the influence of Vpr sequence variation and their contribution to HIV-1 pathophysiology⁵⁶; however, these amino acid substitutions do not necessarily present the Vpr amino acid substitutions present in PLWH.

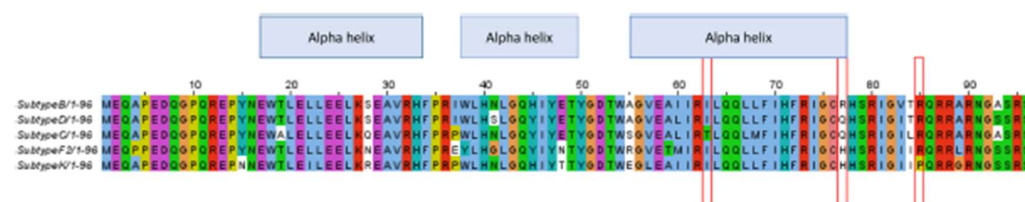


FIGURE 1 Schematic presentation of HIV-1 Vpr amino acid sequence alignment comparing (A) Subtype B (P12520 - VPR_HV1N5) (B) Subtype D (P12519 - VPR_HV1Z2) (C) Subtype C (Q75004 - VPR_HV1ET), (D) Subtype F2 (F2 POC1P4 - VPR_HV1MP), (E) Subtype K (POC1P2 - VPR_HV196). Amino acids in boxes indicate noteworthy positions. Alpha helical domains are indicated in blue boxes and are at regions 17–33, 38–50 and 55–77. Each subtype Vpr sequence was extracted from UniProt where available. No Vpr sequence was available for subtype A. Only the 'pure subtypes' were presented and the sequences from the circulating recombinant forms (CRFs) were not included due to the high number of known CRFs globally.

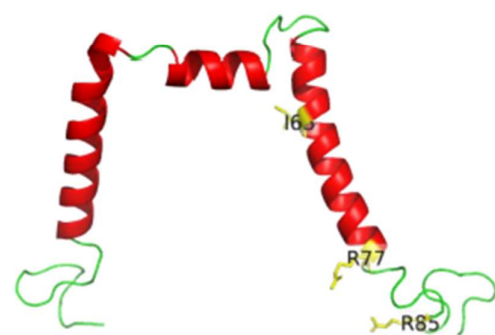


FIGURE 2 Predicted 3D structures of Vpr (Subtype B (P12520 - VPR_HV1N5)) generated by Swiss Model webserver. The Vpr sequence was extracted from UniProt and subjected to 3D structure prediction. Noteworthy amino acid positions are indicated as yellow sticks. Helical structures are indicated in red and unstructured regions are indicated in green.

Despite several studies investigating Vpr amino acids' variation in PLWH,^{53,56} there is no clear consensus on which amino acid substitutions are most commonly associated with the pathophysiology (disease progression, neurological outcomes and treatment efficacy) of HIV-1 in PLWH. Therefore, this systematic review aimed at reviewing all literature on this topic to identify the key Vpr amino acid substitutions associated with HIV-1 pathophysiology in PLWH. Findings from this review may further our understanding of the role of Vpr within various pathophysiological processes.

2 | METHODS

2.1 | Study design

This systematic review aimed at summarising the extant literature on the influence of Vpr amino acid substitutions

on clinical outcomes in PLWH. The study has been carried out according to PRISMA guidelines⁵⁷ and the reporting of the study conforms to broad EQUATOR guidelines.⁵⁸ This study has been approved by the North-West University Health Research Ethics Committee (NWU-HREC): NWU-00203-22-A1.

2.2 | Eligibility criteria

The eligibility criteria were HIV-positive treatment-experienced adults (>18 years old, all medication types included, and no cut-off for treatment duration) with relevant Vpr protein sequence data. All studies had to investigate Vpr protein sequence variations from clinical sample types (blood, cerebrospinal fluid, and post-mortem brain tissue) to investigate HIV-1 proviral DNA and/or viral RNA. For comparability of studies, sequencing had to be carried out by polymerase chain reaction (PCR) based sequencing methods (e.g. Sanger sequencing and/or Next-Generation Sequencing). Studies had to have a control group/comparative arm to be included. Exclusionary criteria were preclinical (animal and cell culture models) studies and reviews. Studies investigating Vpr sequence variation without relation to clinical outcomes were also excluded.

2.3 | Data sources

We electronically searched for publications in PubMed, Scopus and Web of Science databases based on all studies published until 06/09/2022. Eligible studies included published studies in English only. The search strategy was executed without publication date limitations. The full search criteria for each database are included in [File S1](#). 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 (Gene Products, vpr [mh] OR Vpr [tw] or Viral protein R [tw]) AND (mutation [mh] OR protein mutation [tw] OR Polymorphism, Genetic [mh] OR amino acid variation [tw] OR protein signatures [tw]).

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

2.4 | Data selection

All articles were retrieved and loaded onto a single database using a reference manager (EndNote X9, Clarivate, PA, USA). Two authors 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.

2.5 | Quality assessment of included studies

The quality of the included studies was assessed, and kappa statistics were calculated. The Joanna Briggs Institute (JBI) critical appraisal tools have been adopted for the quality criterion. Here, we have amended the JBI critical appraisal tools by implementing a Likert scale⁵⁹ to provide a quantitative measure of study quality. For all included studies, we have only considered the JBI quality questions from the Checklist for Analytical Cross-Sectional Studies which may influence the findings of the reviewed studies. These included (1) cohort information, (2) validity and conditions of measurement and (3) confounding factors and statistical analysis. These were assessed using the following questions (1) Were the criteria for inclusion in the sample clearly defined and were study subjects and the setting described? (2) was the method used to investigate the clinical outcome done in an objective, valid and reliable method (i.e. the inclusion of viral load and CD4⁺ count)? and (3) did they consider confounding factors, and did they employ relevant statistical analysis? This

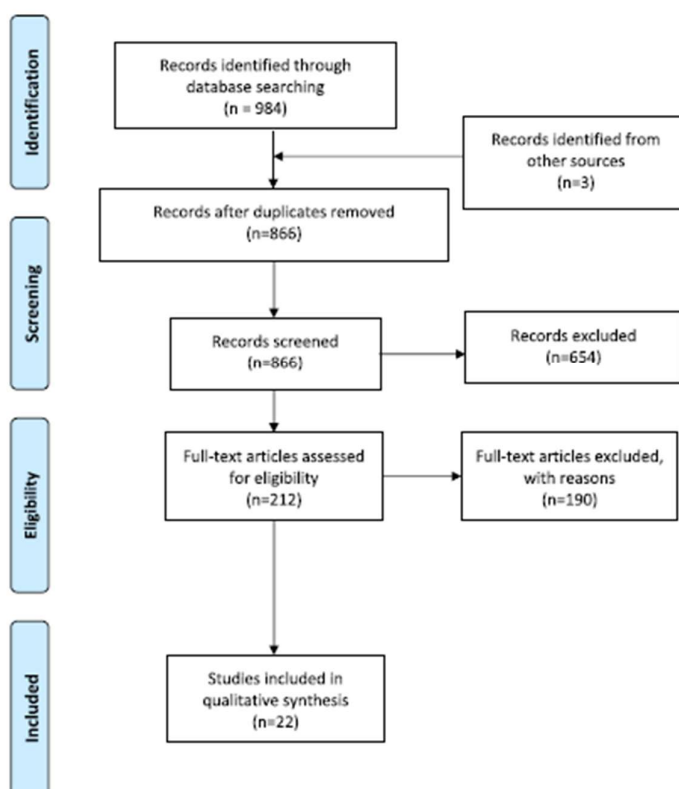


FIGURE 3 Preferred reporting items for systematic reviews and meta-analyses (PRISMA) flow diagram for results of search strategy.

included a total of three questions. Each question was rated 0—no, 1—partly and 2—yes. Studies that addressed all the above questions and had a total rating of ≥ 5 were classified as high quality. Studies with a rating between 3 and 4 were considered intermediate-quality and ≤ 2 as low quality (Table S1).

2.6 | Potential confounders

Several factors may influence clinical outcomes in PLWH and therefore may influence the findings reported in the reviewed studies investigating Vpr amino acid substitutions and clinical outcomes. Therefore, we wanted to consider certain confounders when analysing the data. In particular, we considered viral load and CD4⁺ count concerning the findings reported in the studies. First, we stratified studies according to viral load, which was defined as <400 copies/millilitre (cps/ml) (viral suppression) versus >400 cps/ml (nonviral suppression)^{60–62} (Table S2). Second, studies were also stratified according to a mean/median CD4⁺ count of <200 cells/ μ l or >200 cells/ μ l (Table S3). The mean values were primarily considered when stratifying studies; however, where the mean data were not available, the median data were used. We wanted to determine whether studies were stratified according to viral load and CD4⁺ counts, do Vpr amino acid substitutions still influence clinical outcomes in PLWH. Lastly, we wanted to investigate whether the HIV-1 subtype influenced the prevalence of studies reporting the association of Vpr amino acid substitutions and clinical outcomes.

3 | RESULTS

3.1 | Study characteristics

Using this criterion and search strategy (Section 2.2), 984 abstracts and titles were screened as indicated in Figure 3. Furthermore, $n = 3$ clinical studies were added from additional sources. Duplicates ($n = 121$) were removed, resulting in 866 studies. Thereafter, abstracts and titles were screened and a total of 654 studies were excluded which comprised of:

- Review articles/book chapters/conference proceedings ($n = 173$).
- Studies not investigating HIV-1 in general ($n = 48$).
- Studies investigating HIV-2 ($n = 8$).
- Studies not investigating Vpr in general ($n = 414$).
- Studies only investigating Vpr sequence variation on a genetic level ($n = 4$).

- Studies investigating Vpr but not investigating Vpr amino acid substitutions ($n = 7$).

Full-text articles assessed for eligibility were done for 212 studies, and an additional $n = 95$ were excluded:

- Review articles/book chapters/conference proceedings ($n = 1$).
- Studies investigating HIV-2 ($n = 6$).
- Studies not investigating Vpr in general ($n = 1$).
- Studies only investigating HIV-1 sequence variation of multiple viral genes on a genetic level on ($n = 5$).
- Studies investigating Vpr but not investigating Vpr amino acid substitutions ($n = 74$).
- Paper retraction ($n = 1$).
- Investigated Vpr amino acid substitutions but no correlation with clinical outcomes ($n = 5$).
- Database sequences ($n = 1$).
- A study that was speculative regarding correlations of Vpr amino acids and clinical outcomes ($n = 1$).

Using this criterion (Sections 2.1–2.3), a total of 117 studies resulted which included $n = 95$ preclinical/fundamental studies. The 95 preclinical studies were excluded as this is part of an ongoing study looking at fundamental Vpr amino acid substitutions. Therefore, a total of 22 studies were included for data extraction, and from these studies, $n = 14$ (63.64%) were cross-sectional and $n = 8$ (36.36%) were longitudinal in design. Of all of the studies, $n = 5$ studies were case studies.^{63–67} Across all studies, a total sample size of $n = 2929$ PLWH and $n = 0$ HIV-negative controls.

3.2 | Quality of assessment of included studies

The quality of the studies was rated. The Kappa statistic for interrater agreement and reliability was .757, indicating substantial agreement.⁶⁸ Most studies were rated as intermediate (54%–64%), followed by high (27%–36%) and low-quality (8%) (Table S1). Based on these findings, recommendations are made in the latter part of this review.

3.3 | Vpr amino acids associated with clinical outcomes

All studies included in this review reported findings for Vpr amino acid substitutions associated with clinical outcomes (Table 1). From all the amino acid substitutions investigated in the reviewed studies, associations with negative clinical outcomes were reported for Vpr amino

acid substitutions 3R, 21I, 22L, 41N, 41S, 55A, 64P, 75R, 77Q and 79G. Associations with positive clinical outcomes were reported for Vpr amino acid substitutions 2E, 17A, 18W, 22L, 28N, 29G, 31D, 32K, 33H, 37I, 41S, 41G, 44R, 55A, 63T, 65R, 68M, 70V, 70I, 76C, 77Q, 77R, 78H, 83V, 85P, 86G, 86R, 89G, 89T, 89S, 89A, 93A, 93S, 93R and 94G (Table 1). Furthermore, certain Vpr amino acid substitutions had no significant association with clinical outcomes including 17E, 20L, 21E, 22L, 23L, 24E, 25E, 26L, 29E, 30A, 35P, 54W, 73R, 77Q, 77H, 80R, 85P and 87P (Table 1). Based on the associations of these amino acid substitutions with clinical outcomes, these amino acid substitutions were categorised as (1) disease progression/nonprogression, (2) neurological outcomes, and (3) treatment status (Table 1).

The overall findings show that certain Vpr amino acid substitutions were consistently associated with certain clinical outcomes as reported by more than one independent study. However, for this review, we considered an amino acid substitution finding to be 'noteworthy' when (1) two or more independent studies investigated a particular Vpr amino acid substitution and (2) when 50% or more of the studies investigating a particular Vpr amino acid substitution found it to be consistently associated with the same clinical outcome.⁶⁹ In other words, if >50% of studies that investigated a particular amino acid substitution found it to be associated with the same positive/negative/no significant contribution clinical outcome, we considered it noteworthy for future investigation (Figure 4).

3.3.1 | Disease progression/nonprogression

Certain Vpr amino acid substitutions were associated with positive (long-term nonprogressors, higher CD4⁺, lower viral load) and/or negative clinical outcomes (progressor, lower CD4⁺, higher viral load) or had no significant contribution to clinical outcomes. The amino acid substitutions 2E, 18W, 29G, 31D, 33H, 41G, 44R, 55A, 63T, 65R, 70I, 70V, 76C, 77Q, 78H, 83V, 85P, 86G, 86R, 89G, 89T, 89S, 89A, 93A, 93S, 93R and 94G were associated with positive clinical outcomes (Table 1 and Figure 4A). In particular, these Vpr amino acid substitutions were more prevalent with long-term nonprogressors (LTNP),^{64,67,70-74} lower viral load⁷⁵ and higher CD4⁺ count^{75,76} (Table 1). The amino acid substitutions 3R, 21I, 22L, 41S, 64P, 75R, 77Q and 79G were associated with negative clinical outcomes (Table 1) and were more prevalent with progressors and/or acute HIV-1 infection,^{63,67,74} higher viral load⁶⁶ and lower CD4⁺ count⁶⁶ (Table 1). The amino acid substitutions 17E, 20L, 21E, 22L, 23L, 24E, 25E, 26L, 29E, 30A, 35P, 54W, 73R, 77Q, 77H, 80R, 85P and 87P were associated with no significant contribution to disease progression^{51,65,73,77-80}

(Table 1). The most investigated amino acid substitution across all studies was 77Q.^{51,63,66,67,71-73,76-79} Only 22L, 63T, 77Q, 77H and 85P were investigated by two or more independent studies related to disease progression (Figure 4), therefore, meeting our first criteria as a noteworthy finding. The second criteria were that for all studies investigating 22L, 63T, 77Q, 77H and 85P, >50% of these studies had to associate these substitutions with the same positive/negative/no significant contribution clinical outcome. Despite the widespread investigation, the findings for 77Q did not meet our criteria for noteworthy as the findings were mixed with four independent studies linking it to positive clinical outcomes, two independent studies linking it to negative clinical outcomes, and five independent studies linking it to no significant contribution to clinical outcomes. Similarly, the findings for 22L were mixed^{74,77} (Figure 4A). The amino acid substitutions 63T (100%), 77H (100%) and 85P (67%) met our criteria for noteworthy as >50% of studies investigating this substitution linked it to positive (63T), and no significant contribution (77H and 85P) to clinical outcomes (Table 1 and Figure 4A).

3.3.2 | Neurological outcomes

Certain amino acid substitutions were associated with positive clinical outcomes (nonstroke and decreased neurocognitive deficits) or negative clinical outcomes (stroke and increased neurocognitive deficits). The Vpr amino acid substitutions 22L, 37I, 41G, 41S, and 77R were associated with positive clinical outcomes and were more prevalent in nonstroke participants⁸¹ and lower neurocognitive deficits^{49,52} in PLWH (Table 1). The Vpr amino acid substitutions 21I, 41S, 41N, 55A and 77Q were associated with negative clinical outcomes and were more prevalent with ischaemic stroke⁸¹ and pronounced neurocognitive deficits^{49,52} in PLWH (Table 1). The Vpr amino acid substitution 41S was investigated by two or more independent studies, therefore, meeting our first criteria as a noteworthy finding (Figure 4B). The findings for 41S were mixed as one of the two studies linked it to improved neurological outcomes, whereas the second one linked it to ischaemic stroke. Therefore, none of the Vpr amino acid substitutions investigated met our criteria as noteworthy for neurological outcomes (Table 1 and Figure 4B).

3.3.3 | Treatment status

Of all amino acid substitutions investigated, several were associated with treatment status by higher prevalence in PLWH that were using ART (Table 1). The Vpr amino acid substitutions 17A, 28N, 32K and 68M were associated

TABLE 1 Amino acid substitutions associated with clinical outcomes in people living with HIV

Amino acid substitution	Positive	Reference	Negative	Reference	No significant contribution	Reference
<i>Amino acid substitutions associated with disease progression</i>						
2E	Well-conserved in long-term nonprogressor (LTNP)	[70]				
3R			Associated with lower CD4 count	[66]		
			Associated with higher viral load	[66]		
17E					No contribution to disease progression	[77]
18W	Well-conserved in LTNP	[70]				
20L					No contribution to disease progression	[77]
21I			Associated with lower CD4 count	[81]		
21E					No contribution to disease progression	[77]
22L			Significantly enhanced in transmitted/founder (t/f) viruses (single/few viral variants that have a productive infection within a host) compared with chronic sequences	[74]	No contribution to disease progression	[77]
23L					No contribution to disease progression	[77]
24E					No contribution to disease progression	[77]
25E					No contribution to disease progression	[77]
26L					No contribution to disease progression	[77]
29E					No contribution to disease progression	[77]
29G	Associated with LTNP	[64]				
30A					No contribution to disease progression	[77]
31D	Associated with LTNP	[64]				
33H	Well-conserved in LTNP	[70]				
35P					No contribution to disease progression	[77]
41S			Associated with lower CD4 counts	[81]		
41G	Associated with LTNP	[64]				
44R	Associated with LTNP	[64]				

(Continues)

TABLE 1 (Continued)

Amino acid substitution	Positive	Reference	Negative	Reference	No significant contribution	Reference
54W					No contribution to disease progression	[77]
55A	Associated with lower viral load	[75]				
63T	Stable CD4 count	[76]				
	Associated with lower viral load	[75]				
	Associated with a higher CD4 count	[75]				
64P			Associated with progressors	[67]		
65R	Associated with LTNP	[67]				
70V	Stable CD4 count	[76]				
70I	Associated with chronic infection	[74]				
73R					No contribution to disease progression	[77]
75R			Associated with progressors	[63]		
76C	Well-conserved in LTNP	[70]				
77Q	Associated with LTNP	[71]	Associated with lower CD4 count	[66]	No contribution to disease progression	[78]
		[67]				[76]
		[72]	Associated with higher viral loads	[66]		[51]
		[73]	Associated with progressors	[63]		[79]
						[76]
77H					No contribution to disease progression	[73]
						[66]
78H	Well-conserved in LTNP	[70]				
79G			Associated with progressors	[67]		
80R					No contribution to disease progression	[77]
83V	Associated with LTNP	[64]				
85P	Stable CD4 count	[76]			No contribution to LTNP	[80]
					No contribution to disease progression	[65]
86G	Stable CD4 count	[76]				
86R	Associated with LTNP	[70]				
87P					No contribution to disease progression	[65]
89G	Stable CD4 count	[76]				
89T	Associated with LTNP	[70]				

TABLE 1 (Continued)

Amino acid substitution	Positive	Reference	Negative	Reference	No significant contribution	Reference
89S	Associated with LTNP	[70]				
89A	Associated with nonprogressors	[65]				
93A	Associated with chronic infection	[74]				
93S	Associated with LTNP	[70]				
93R	Associated with LTNP	[70]				
94G	Stable CD4 count	[76]				
<i>Amino acid substitutions associated with neurological outcomes</i>						
21I			Associated with ischaemic stroke	[81]		
22L	Associated with the nonstroke control group	[81]				
37I	Decrease in pronounced neurocognitive deficits (Lower GDS)	[52]				
41N			Increase in pronounced neurocognitive deficits (Higher GDS)	[52]		
41S	Decrease in pronounced neurocognitive deficits (Lower GDS)	[52]	Associated with ischaemic stroke	[81]		
41G	Associated with the nonstroke control group	[81]				
55A			Increase in pronounced neurocognitive deficits (higher GDS)	[52]		
77Q			Predominant in HAD clones of the brain	[49]		
77R	Predominant in Nondementia (ND) clones of blood	[49]				
<i>Amino acid substitutions associated with treatment status</i>						
17A	More prevalent in HAART use but not in treatment naïve	[82]				
28N	More prevalent in HAART use but not in ART-naïve patients	[63]				
32K	More prevalent in treatment patient use but not in treatment naïve	[82]				

(Continues)

TABLE 1 (Continued)

Amino acid substitution	Positive	Reference	Negative	Reference	No significant contribution	Reference
68M	More prevalent in HAART use but not in ART-naïve patients	[63]				

Abbreviations: ART, antiretroviral therapy; GDS, global deficit score; HAART, highly active antiretroviral therapy; HAD, HIV-associated dementia; LTNP, long-term nonprogressor; ND, nondementia; U/I, transmitted/founder.

with positive clinical outcomes and were more prevalent in treated PLWH^{63,82} (Table 1). The findings for these amino acid substitutions were only reported by one independent study; therefore, none of the Vpr amino acid substitutions investigated met our criteria as noteworthy for treatment status (Table 1 and Figure 4C).

3.3.4 | Potential confounders: Viral load/CD4⁺ count and HIV-1 subtype

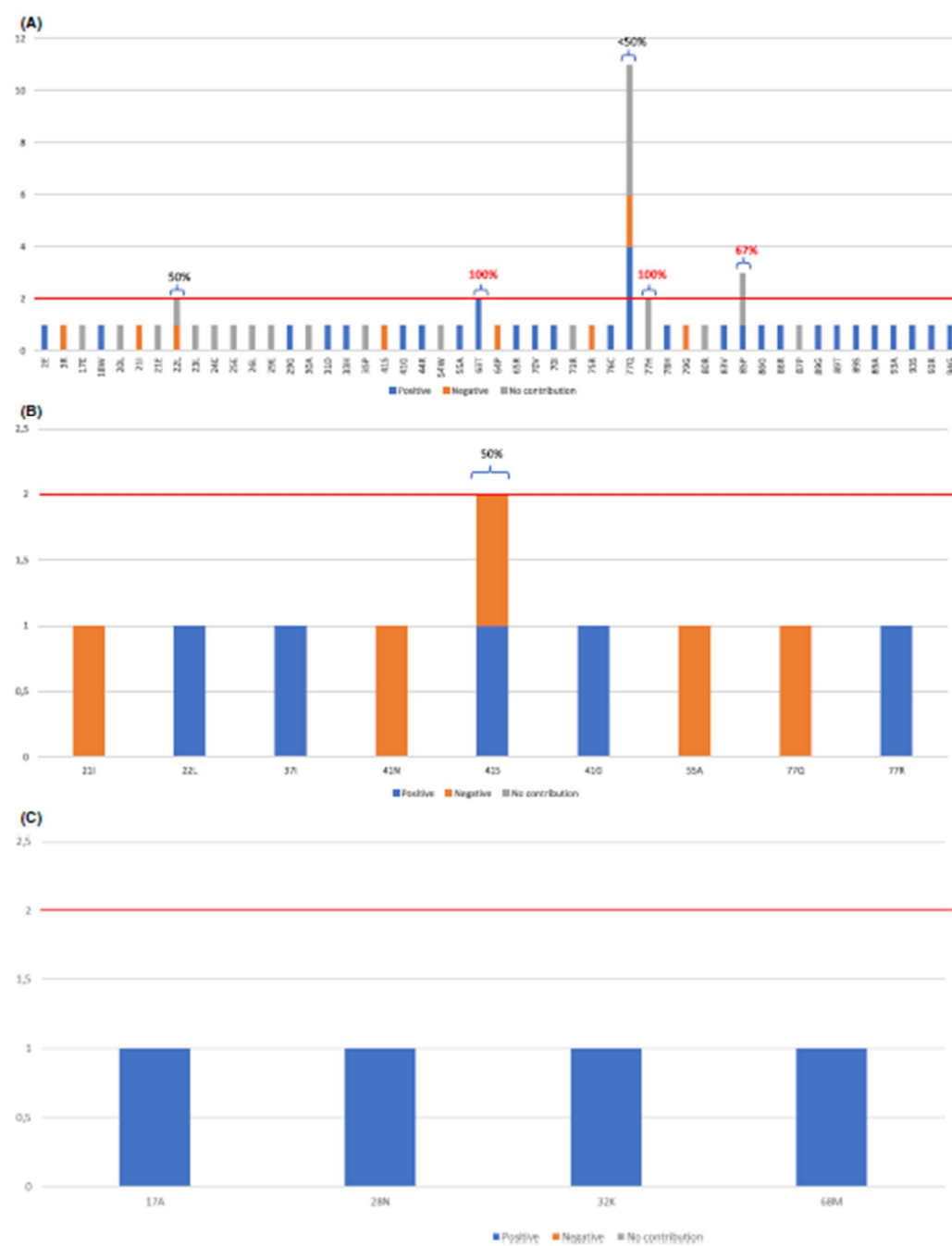
Several findings may influence the reported association of Vpr amino acid substitutions and clinical outcomes. In particular, viral load and CD4⁺ count are well-known determinants of clinical outcomes in PLWH.⁸³ Therefore, we wanted to determine the potential influence of these confounders on the reported associations between Vpr amino acid substitutions and clinical outcomes in the reviewed studies. Therefore, studies were stratified according to the mean/median viral suppression (<400 cps/ml) or nonviral suppression (>400 cps/ml) (Table S2). Studies reported viral load (VL) as a group mean (SD), median [IQR], or percentage distribution. For studies only reporting percentage distribution for virally suppressed participants, we considered viral suppression when more than 50% of participants were virally suppressed (i.e. suppression (<400 cps/ml) within the respective study). We do acknowledge that a fraction of the overall population

may have been nonvirally suppressed and therefore, findings reported for these studies should be interpreted with caution.

For longitudinal studies that have reported multiple measurements of viral load, the last recordings of viral load were used for stratification. If treatment-experienced participants were virally suppressed at baseline, we considered follow-up as virally suppressed due to continued use of ART. For studies that reported nonviral suppression at baseline, we then considered follow-up viral loads where available.

Of all studies reviewed ($n = 22$), $n = 9$ studies had not provided sufficient information to stratify participant groups according to viral suppression.^{49,65,67,70,76–78,80,84} Of the remaining 13 studies, the majority ($n = 12$) investigated cohorts with a group of nonviral suppression (>400 cps/ml), and approximately 38.46% of these studies investigated Vpr amino acid substitutions that had an association with negative clinical outcomes in PLWH (Table S2). One study had participants with a group viral suppression of <400 cps/ml, and this study reported an association between Vpr amino acid substitutions and negative clinical outcomes in PLWH. It is also relevant to note that $n = 3$ studies were case studies and the findings reported were from three or fewer participants and these findings may not be representative of larger populations.^{63,64,66} Without stratifying studies according to viral status, a little under half (46.15%) of all studies reported

FIGURE 4 (A) Frequency an amino acid substitution has been investigated in relation to disease progression/nonprogression. Blue, Orange and grey bars indicate positive, negative and no contribution to clinical outcomes, respectively. Criteria 1 (red line cut-off) was ≥ 2 independent studies that had to investigate a particular substitution. The percentage indicates the number of studies that reported the same clinical outcome for a particular substitution in relation to the number of studies that investigated the substitution. Criteria 2 was that the percentage needed to be >50% to be considered a noteworthy finding. Vpr substitutions 63T, 77H and 85P were considered noteworthy. (B) Frequency an amino acid substitution has been investigated in relation to neurological outcomes. Blue, Orange and grey bars indicate positive, negative and no contribution to clinical outcomes, respectively. Criteria 1 (red line cut-off) was ≥ 2 independent studies that had to investigate a particular substitution. The percentage indicates the number of studies that reported the same clinical outcome for a particular substitution in relation to the number of studies that investigated the substitution. Criteria 2 was that the percentage needed to be >50% to be considered a noteworthy finding. Vpr substitution 41S met criteria one, however, no substitutions were considered noteworthy. (C) Frequency an amino acid substitution has been investigated in relation to treatment status. Blue, Orange and grey bars indicate positive, negative and no contribution to clinical outcomes, respectively. Criteria 1 (red line cut-off) was ≥ 2 independent studies that had to investigate a particular substitution. Criteria 2 was that the percentage needed to be >50% to be considered a noteworthy finding. None of the substitutions met criteria one or two and therefore none were considered noteworthy.



an association between Vpr amino acid substitutions and negative clinical outcomes in PLWH.

For the current CD4⁺ count, not all studies used the same measure of CD4⁺ count. Therefore, the studies within this review were stratified into one of two categories depending on which was provided by the studies; group mean/median CD4⁺ count of <200 cells/μl or >200 cells/μl. For longitudinal studies with multiple measures of CD4⁺ count, the CD4⁺ count used for stratification of studies was the time point when the correlation between Vpr amino acid substitutions and clinical outcome was done. When study participants are stratified according to CD4⁺ count, most studies included participants with a higher CD4⁺ count (>200 cells/μl), and 26.67% of these studies reported an association between Vpr amino acid substitutions and negative clinical outcomes in PLWH. Certain studies included participants with defined LTNP or non-LTNP status^{64,67,77} and therefore for these studies, we also considered the LTNP status when stratifying according to CD4⁺ count (Table S3). Of all studies reviewed, *n* = 4 studies had not provided sufficient information to stratify participant groups according to the current CD4⁺ count.^{49,76,80,84} Of the remaining 18 studies, *n* = 3 studies could be stratified according to LTNP status. For these studies, LTNP status was linked with CD4⁺ counts of >200 cells/μl and in studies that had LTNP, Vpr amino acid substitutions were not associated with negative clinical outcomes.^{64,67,77} In studies with participants with progressive HIV, the findings for the association of Vpr amino acid substitutions with negative clinical outcomes were mixed.^{64,67,77} Of the remaining *n* = 15 studies, *n* = 12 studies investigated cohorts with a mean/median >200 cells/μl, and approximately 33% of these studies investigated Vpr amino acid substitutions with an association with negative clinical outcomes in PLWH (Table S3). Three studies had participants with a group mean/median of <200 cells/μl, and two studies (66%) reported significant associations between Vpr amino acid substitutions and negative clinical outcomes in PLWH. From the studies reporting findings for CD4⁺ count, certain studies were from case studies and/or single-participant data.^{63–67} Therefore, findings from these studies may not necessarily be representative of larger cohorts.

Most of the studies (64%) reported the HIV-1 subtype of which subtype B was most common (71%), followed by subtype C (14%), subtype A (7%), and circulating recombinant form (CRF)01_AE (7%). For the remaining studies that did not report the HIV-1 subtype, 18% of the total studies reported the geographical region, with one study each conducted in the USA, Canada, Portugal and a combination of the USA and Australia, respectively (Table 2). The remaining studies (17%) did not report the subtype or geographical region. Considering the findings for studies

that reported an association of Vpr amino acids with negative clinical outcomes (*n* = 8) (Table 1) and studies with a confirmed HIV-1 subtype, (Table 2), it was noted that studies with subtypes B and C equally reported findings of Vpr amino acid substitutions with negative clinical outcomes. All key findings are summarised in Table 2.

4 | DISCUSSION

This review summarises the key findings for the association between Vpr substitutions and clinical outcomes in PLWH. We reported the Vpr amino acid substitutions that were associated with specific clinical outcomes including disease progressions, neurological outcomes and treatment status in PLWH. Certain Vpr amino acid substitutions reported consistent associations with clinical outcomes in PLWH and were therefore considered noteworthy within this study. (1) The Vpr substitution 63T was associated with characteristics of slower disease progression, whereas 77H and 85P were associated with no significant contribution to disease progression, (2) preliminary evidence suggests that Vpr amino acid substitutions may have implications in neurological outcomes and treatment efficacy in PLWH, despite limited investigation, and (3) regardless of higher viral load and CD4⁺ counts, studies still report an association of Vpr amino acid substitutions with negative clinical outcomes in PLWH.

First, Vpr amino acid substitutions are associated with clinical outcomes in PLWH. Nearly, a third (31.82%) of all studies reported an association between Vpr amino acid substitutions and negative clinical outcomes in PLWH. Much of our understanding of the role of Vpr amino acid substitutions in HIV-1 pathophysiology has been derived from fundamental studies. However, these amino acid substitutions do not necessarily present the Vpr amino acid substitutions present in PLWH. To our best knowledge, this is the first study that systematically reviewed the Vpr amino acid substitutions found in PLWH and their association with clinical outcomes. More specifically, here, we provide a summary of Vpr amino acid substitutions associated with clinical outcomes, particularly, disease progressions, neurological outcomes, and treatment status.

Second, we considered the following findings as noteworthy: (1) 63T was more commonly associated with positive clinical outcomes including higher CD4 counts and lower viral load, and Vpr 77H and 85P were more commonly associated with no significant contribution to disease progression. Limited studies have reported findings for the Vpr 63T substitution in clinical or preclinical studies, and no findings for 63T have been reported in previous reviews performed on this topic.^{33,36} However, as reported

TABLE 2 Summary of key findings for all reviewed studies

Reference	Subtype/Geographical location (percentage of participants)	Cohort	Treatment	Amino acid investigated	Key findings
[71]	Subtype B: 93.33% Subtype C: 6.67%	People living with HIV (PLWH): 15 Long-term nonprogressors (LTNP): 8 Control (HIV-1 progressors): Treatment-naïve: 4 Rapid progressors (RP): 3 HIV- negative controls: N/A Age: N/A Male (%): N/A CD4 ⁺ count: LTNP: 747.25 (±314.68) cells/μl Control: 650 (±328.50) cells/μl Viral load: LTNP: 4837.5 (±6627.93) copies (cps)/ml Control: 99,598.29 (±259,465.20) cps/ml	Antiviral therapy (AVT): 13.33%	77Q	1. 83.33% of LTNP had a 77Q amino acid substitution 2. Additional Los Alamos database scans for Vpr sequences: 77Q was present in: • 77.6% subtype A sequences • 46.0% subtype B sequences • 78.0% subtype C sequences • 61.4% subtype D sequences • 100% subtype E sequences. 77Q was present in 14.29% of HIV-1 progressor control PLWH (rapid progressor).
[63]	Subtype B: 100%	PLWH: 3 HIV-negative: N/A Age: N/A Male: N/A CD4 ⁺ count: 485 (±91.92) cells/μl Viral load: <10,000 cps/ml	Highly active antiretroviral therapy (HAART): 66.67%	Full-length Vpr	1. 90R occurred in a very high percentage (98%) of clones. 2. 75R occurred in a low percentage (10%) of clones for both recipients A and B (progressors) 3. 77Q occurred in a high percentage (92.8%) of clones of recipient B (progressor) and a low percentage (7.14%) of clones of recipient C (progressor). 4. 68M occurred in a high percentage (85.71%) of clones of recipient B (progressor). 5. 68M occurred in no clones of donor A (nonprogressor) and a low percentage of clones of recipient B (progressor) (at a late stage). 6. 28N occurred only in progressors but not in nonprogressors 7. Mean Vpr peptide diversity: Donor A (nonprogressor): 0%–5.5%; Recipient B (progressor): 1.8%–8.5% 8. Recipient C (progressor): 0%–15% 9. Phylogenetic analyses: Donor A (nonprogressor) acquired fewer mutations than recipients B and C (progressors)

TABLE 2 (Continued)

Reference	Subtype/geographical location (percentage of participants)	Cohort	Treatment	Amino acid investigated	Key findings
[78]	Subtype B: 100%	PLWH: 96 HIV-negative controls: N/A Age: N/A Male: N/A CD4 ⁺ count: 304 (186–533) cells/ μ l Viral load: N/A	Prior zidovudine monotherapy; 76%	77Q	1. Vpr C-terminal (specifically 77Q) had no contribution to disease progression.
[76]	CRF01_AE (5.63%) Subtype C (24.84%) Subtype B (17.65%) CRF03_AG (3.92%) CRF08_BC (1.31%)	PLWH: 208 (Vpr target gene amplified for 74% of participants) HIV-negative controls: N/A Age: 14.5–61.4 Male: 59% CD4 ⁺ count: N/A Viral load: N/A	ART: 0%	Full-length Vpr, with a focus on C-terminal 77Q 77R	1. 77Q was present in 84.3% of cases. 2. There were no significant differences in clinical indicators between 77Q and 77R. 3. 77Q was more frequently associated with normal disease progression than 77R. 4. Amino acid substitutions in the C-terminal (51–96) were more apparent than that in the N-terminal (1–50). 5. 63T, 70V, 85P, 86G, 89G and 94G were associated with stabilized CD4 cell count and fewer complications/citations. 6. M subtype is predominant in China, mainly subtypes AE, B and C.
[51]	Subtype B: 97% Other Subtypes: 2.34%	PLWH: 728 HIV-negative controls: N/A Age: 37.2 (32.2–43.7) Male: 84.3% CD4 ⁺ count: 270 (130–415) cells/ μ l Viral load: 128,824.96 [48,977.88–331,131.12] qn/ml	ART: 0%	77Q	1. 77Q was not a major determinant of HIV disease progression. 2. 77Q did not affect clinical response or survival over the first six years following initiation of HIV therapy
[52]	United States of America (USA)	PLWH: 112 HIV-negative controls: N/A Age: 51 (46–56) Male: 64% CD4 ⁺ count: 641 (428–904) cells/ μ l Viral load: 20 (13–193) cpe/ml	ART: Large percentage of the cohort received ART (exact percentage not provided)	41N 55A 37I 41S	1. 41N and 55A amino acid substitutions were associated with an increase in pronounced neurocognitive deficits (higher Global deficit score (GDS)). 2. 37I and 41S were associated with lower GDS.

TABLE 2 (Continued)

Reference	Subtype/Geographical location (percentage of participants)	Cohort	Treatment	Amino acid investigated	Key findings
[79]	Subtype A: 53% Subtype B: 47%	PLWH: 55 Rwanda (29) LTNP: 14 Progressive disease and AIDS-defining conditions: 15 Luxembourg (26): Slow Progressors: 20 Progressors: 6 HIV-negative controls: N/A Age: N/A Male: N/A CD4 ⁺ count: Rwanda: LTNP: >500 cells/μl for more than 12 years. Progressive disease and AIDS-defining conditions: N/A Luxembourg: Slow progressors: >400 cells/μl Progressors: N/A Viral load: Rwanda: N/A Luxembourg: Slow progressors: <25,000 cpm/ml without treatment Progressors: N/A	Rwanda Cohort: N/A Luxembourg Cohort: Slow progressors: Treatment 0% Progressors: N/A	77Q	1. 77Q occurred in 55.9% of LTNP. 2. 77Q occurred in 57.1% of slow progressors. 3. 77Q occurred significantly more frequently in subtype A than in subtype B viruses. 4. A slow progressor had an unusual deletion of the four amino acids "TPVN" in the N-terminal region of Vpr at positions 13–16. 5. Another slow progressor participant had a deletion in the C-terminal region, at positions 84 and 85.
[82]	Subtype B: 100%	PLWH: n = 160 HIV-negative controls: N/A Age: PLWH falling HAAART: 46 (range: N/A) Treatment naïve: N/A Male: PLWH falling HAAART: 89% Treatment naïve: N/A CD4 ⁺ count: PLWH falling HAAART: 331 [N/A] cells/μl Treatment naïve: N/A Viral load: PLWH falling HAAART: 4680 [N/A] cps/ml Treatment naïve: N/A	Antiretroviral agents (ARV)-experienced falling HAAART: 60.63%	17A 32K	1. 17A was associated with didanosine in the treatment histories of PLWH. 2. No associations were reported between 32K and drug-resistant amino acid substitution in Protease (PR) or Reverse Transcriptase (RT). 3. In treatment naïve PLWH, nearly 80% of amino acids were conserved. 4. From 10.63% of PLWH having Vpr 17A and 43.75% of PLWH having Vpr 17E, cluster 1 thymidine analogue mutations-1 (TAM-1) (M41L, L210W and T215V) were associated with 17A amino acid substitution. None of the cluster 2 TAM-2 (D67 N, K70R, T215F, and K219Q/E) were associated with Vpr 17A. 5. 17A and 32K amino acid substitutions were more prevalent in treated PLWH.

(Continues)

TABLE 2 (Continued)

Reference	Subtype/Geographical location (percentage of participants)	Cohort	Treatment	Amino acid investigated	Key findings
[80]	Subtype B: 100%	PLWIE: 10 Intravenous Drug Users (IVDU): 8 LTNP: 2 HIV-negative controls: N/A Age: N/A Gender: N/A CD4 ⁺ count: N/A Viral load: N/A	N/A	Full length Vpr	1. There were amino acid substitutions within 100% of HIV-1 infected IVDU. 2. LTNP had the most amino acid substitutions in the amino acid 77-96 region resulting in size polymorphism in the Vpr protein. 3. In-frame insertions or deletions occurred in 86% of the HIV-1 strains. 4. The absence of a stop codon occurred in 30% of HIV-1 strains. 5. Chou-Fasman algorithm: Amino acid residues 83-89: amino acid substitutions that have a profound effect on the secondary structure and molecular mass of the Vpr protein. 6. RSP occurred in 60% of analysed clones. Proline did not fit in the α-helix pattern. Its presence resulted in a stretch of the α-helix and therefore becoming terminated. 7. LW87-1 sequences from this clone affected the Tat start codon. 8. Amino acid substitutions in both IVIUX and LTNP are potentially linked to Vpr survival and functioning in HIV-1 pathogenesis.
[67]	Subtype B: 100%	PLWIE: 2 LTNP: 1 Progressor: 1 HIV-negative controls: N/A Age: N/A Male: N/A CD4 ⁺ count: N/A LTNP: >350 cells/ μ l Progressor: <200 cells/ μ l Viral load: N/A	ZDV: 100% of Progressor	Full length Vpr	1. Samples were sampled from LTNP at three time points: 1986, 1996, and 2004. 2. Sequence diversity of 100% of Vpr alleles from 2004 was consistent with an increased plasma viral load that paralleled a slight decline of the CD4⁺ count. 3. 77Q was present in 100% of Vpr alleles from 1986 LTNP samples and in 100% of Vpr alleles from 2004 LTNP samples. 4. 100% of Vpr alleles from 1996 LTNP samples had 77R. 5. 68R was present in the leucine-rich domain of 20% of Vpr alleles from 2004 LTNP samples. 6. 33.33% of progressors had a substitution of 79Q. 7. 33.33% of progressors had a 64P substitution.

TABLE 2 (Continued)

Reference	Subtype/Geographical location (percentage of participants)	Cohort	Treatment	Antibody investigated	Key findings
[75]	Subtype B: 100%	PLWH: 444 HIV-negative controls: N/A Age (years): 34 (29–41) Male: 97.75% CD4 ⁺ count: 292.5 [183.5–407.0] cells/μl Viral load: 38,904.51 [10,964.78–141,253.75] cps/ml	Treatment: 0%	55A 63T	1. 55A was significantly associated with lower plasma viral load. 2. 63T was significantly associated with lower plasma viral load and higher CD4 count.
[72]	Canada	PLWH: 25 LTNP: 10 Progressors: 15 HIV-negative controls: N/A Age: N/A Male: N/A CD4 ⁺ count: LTNP: N/A Progressor Controls: <500 cells/μl Viral load: LTNP: N/A Progressor Controls: >1000 cps/ml	LTNP: N/A ART: 0% of controls	77Q	1. LTNP: 77Q was present in 89% of PLWH and 20% had wild-type Vpr. 2. ART-naïve PLWH (CD4 count <500): 77Q was present in 33% of PLWH. 3. Los Alamos database: 77Q occurred in 36% of progressors and 75% LTNP.
[81]	Subtype C: 100%	PLWH: 134 Strokes: 54 Nonstroke Controls: 80 HIV-negative controls: N/A Age: 18–45 Strokes Male: N/A Nonstroke Controls Male: N/A CD4 ⁺ count: Strokes: 206 [276] cells/μl Nonstroke Controls: 329 [291] cells/μl CD4 ⁺ nadir: Strokes: 120 [236.8] Nonstroke Controls: 183 [179.5] Viral load: Strokes: 47,863.01 [0–2,511,886.43] cps/ml Nonstroke Controls: 13,803.84 [0–630,957.34] cps/ml	ART Prior combination Antiretroviral therapy (cART): Strokes: 48.10% Nonstroke controls: 53.80% Deafened ART: Strokes: 26.90% Nonstroke controls: 0%	22L 22L 41G 41S	1. 22L and 41S were associated with ischaemic stroke in HIV and had decreased CD4 counts and CD4 nadirs compared to the controls. 2. 22L and 41G were associated with the nonstroke control group.

(Continues)

TABLE 2 (Continued)

Reference	Subtype/Geographical location (percentage of participants)	Cohort	Treatment	Amino acid investigated	Key findings
[73]	Subtype B: 100%				
		PLWH: 76	Pr: 25% ART-naïve	77Q	
		Progressors (Pr): 19	Sub-optimal treated PLWH:		1. 77Q was significantly higher in LTNP (86.7%) and sub-optimal treated PLWH (73.9%) compared to progressors (42.1%) and PLWH with viral rebound after treatment failure (42.1%)
		LTNP: 15	30.26% on dual combination of either stavudine + lamivudine, didanosine, zidovudine + lamivudine, zidovudine + didanosine, zidovudine + zalcitabine		2. Differences amongst these groups were statistically significant
		Sub-optimal treated PLWH: 23			3. LTNP compared to progressors, there were significant differences in the frequency of 77Q amino acid substitution
		PLWH with viral rebound after treatment failure: 19			4. In sub-optimal treated PLWH and PLWH with viral rebound after treatment failure, there were significant differences in the frequency of 77Q
		HIV-negative controls: N/A			5. 77H had no significant difference in LTNP and progressors, and in sub-optimal treated PLWH and PLWH with viral rebound after treatment failure
		Age: Pr: 34.25 (± 8.68)			6. In nonprogressors, 77Q was significantly higher
		LTNP: 40.90 (± 6.95)			
		Sub-optimal treated PLWH: 42.13 (± 5.07)			
		PLWH with viral rebound after treatment failure: 41.58 (± 5.03)			
		Male: Pr: 58%			
		LTNP: 73%			
		Sub-optimal treated PLWH: 52%			
		PLWH with viral rebound after treatment failure: 74%			
		CD4 ⁺ count:			
		Pr: 300 (3–763) cells/ μ l			
		LTNP: 623 (315–1127) cells/ μ l			
		Sub-optimal treated PLWH: 519 (325–952) cells/ μ l			
		PLWH with viral rebound after treatment failure: 203 (1–449) cells/ μ l			
		Viral load: Pr: 37,734 [187–620,000] cps/ml			
		LTNP: 2567 [99–21,954] cps/ml			
		Sub-optimal treated PLWH: 597 [50–9758] cps/ml			
		PLWH with viral rebound after treatment failure: 89,480 [1880–500,000] cps/ml			
		Years of infection: Pr: 1.5 $\pm$ 5.41			
		LTNP: 14 $\pm$ 2.30			
		Sub-optimal treated PLWH: 12 $\pm$ 4.05			
		PLWH with viral rebound after treatment failure: 12 $\pm$ 4.32			

TABLE 2 (Continued)

Reference	Subtype/Geographical location (percentage of participants)	Cohort	Treatment	Amino acid investigated	Key findings
[49]	N/A	PLWH: N/A HIV-negative controls: N/A Age: N/A Male: N/A CD4 ⁺ count: N/A Viral load: N/A	N/A	Full length Vpr	- Brain-derived sequences: 77Q predominated in 94.44% of HIV-associated dementia (HAD) clones. 77R was mostly present in 77.77% of Non-dementia (ND) clones. - Blood-derived sequences: No amino acid changes distinguished HAD and ND in blood were not evident. - Synonymous amino acid substitutions within clinical groups: no difference within the blood- or brain-derived sequences. - The frequencies of non-synonymous amino acid substitutions were significantly reduced within the HAD brain-derived sequences compared with the HAD blood-derived sequences. - dN/dS ratios: No significant difference. - The amount of amino acid differences were significantly reduced in the HAD brain-derived sequences than in HAD blood-derived sequences. - The frequency of detection of Vpr transcripts were significantly higher in the brain amongst PLWH with HAD (59%) than in PLWH with ND (31%). It was detected in all blood-derived samples examined.

(Continues)

TABLE 2 (Continued)

Reference	Subtype/Geographical location (percentage of participants)	Cohort	Treatment	Amino acid investigated	Key findings
[74]	Subtype C: 100%	PLWH: 17 Acute infected: 6 Early infected: 11 HIV-negative controls: N/A Age: Acute 31.50 (26.75–34.25) Early: 25.50 (24.75–27.00) Male: Acute: 17% Early: 27% CD4 ⁺ count: Acute: 337 (258–426) cells/ μ l Early: 374 (258–490) cells/ μ l Viral load: Acute: 89,125.09 (10,000–331,131.12) cps/ml Early: 25,118.86 (6918.31–56,234.13) cps/ml	N/A	4P 4A 22L 70I 93A	1. In Vpr, transmitted/founder (TF) viruses (single/few viral variants that had a productive infection within a host) had a reduced frequency 4P by almost 25% compared to the chronic infection. However, this was not significant. 2. 4P expressed a different dominant amino acid in TF compared with chronic viruses. 3. 22L was significantly enhanced in TF viruses compared with chronic sequences. 4. 70I and 93A were significantly underrepresented compared with chronic sequences. 5. Investigating the predicted epitope binding affinity for Vpr variants predicted median IC50 of putative epitopes for Vpr variants that arose during early infection (K_{50} 243 [IQR: 99–601 nM]) compared with those encoded in TF viruses (192 [IQR 171–318] nM). 6. Amino acid substitutions at anchor residues had significantly lower predicted epitope binding affinity compared with the respective epitope in the TF virus for Vpr (192 vs. 283 nM). 7. Thus, amino acid substitutions at anchor residues in the TF virus are more likely to activate Major Histocompatibility Complex (MHC) which aids the immune system to recognize foreign material.

TABLE 2 (Continued)

Reference	Subtype/Geographical location (percentage of participants)	Cohort	Treatment	Amino acid investigated	Key findings
[77]	Subtype B: 100%	PLWH: 15 LTNP: 8 AIDS: 7 HIV-negative controls: N/A Age: N/A Male: N/A CD4 ⁺ count LTNP: 670.5 (± 90.43) cells/ μ l AIDS patients: 147.43 (± 27.34) cells/ μ l Viral load: N/A	ART: 100%	Full length Vpr	1. The following are all highly conserved in LTNP and AIDS participants of Plasma/Blood Donors (PBD): all the proline and serine amino acids in the N-terminal or C-terminal of Vpr, amino acids at positions 17, 21, 24, 25, and 29, four hydrophobic polar leucine's at amino acid positions 20, 22, 23, and 26, and alanine at amino position 30 in the first α-helices are all highly conserved in LTNP and AIDS participants of PBDs. 2. 35P and 54W, lying between the first second helices and second third helices, respectively, functional amino acids in second and third helices, as well as the H(S/P)RIG motif in the third helix are very well-conserved in all 15 PLWH, irrespective of disease stages. 3. 73R and 80R were highly conservative in all PLWH. 4. 77Q amino acid substitution was present in all PBD PLWH irrespective of disease stages. 5. All the predicted binding motifs were highly conserved with slight changes seen in LTNP and AIDS patients. 6. All Vpr sequences were tightly clustered with firstly, Chinese B and, secondly, Thailand B and intermingled together regardless of disease stage. 7. There was no difference between the asymptomatic PLWH and AIDS participants. 8. Analysis of the dS/dN ratio and potential glycosylation sites: No significant difference in the dS/dN ratio and glycosylation sites of Vpr between patients at different stages of the disease. 9. All Vpr sequences in our study had an intact ORF, with 96 amino acids regardless of disease stages.

(Continues)

TABLE 2 (Continued)

Reference	Subtype/Geographical location (percentage of participants)	Cohort	Treatment	Amino acid investigated	Key findings
[66]	Portugal	PLWIE 3 HIV-negative controls: N/A Age: Child: 5 Mother: N/A Father: N/A Male: 66.66% CD4 ⁺ count: Child: 0 cells/ μ l Mother: N/A Father: N/A Viral load: Child: 1,073,899 cps/ml Mother: N/A Father: N/A	Upon diagnosis 33.33% received combined oral therapy of lamivudine 4 mg kg ⁻¹ twice a day, zidovudine 180 mg m ⁻² twice a day, lopinavir/ritonavir 102.5 mg ⁻¹ kg ⁻¹ twice a day	77Q 3R 77H	1. Child: amino acid substitutions 77Q and 3R were present. Initial CD4 count was 0 cells/ μ l and initial viral load was 1,073,899 cps/ml 2. Mother and the father: 77H amino acid substitution was present. The mother was asymptomatic, and the father rapidly progressed to AIDS.
[84]	N/A	PLWIE: 976 Vpr sequences HIV-negative controls: N/A Age: N/A Male: N/A CD4 ⁺ count: N/A Viral load: N/A	N/A	Full-length Vpr	1. Influence of amino acid substitutions on CTL epitope, which was associated with immune control, with respect to HLA-A2 molecule, half-time of dissociation values were as follows: 2. Reference peptide of the epitope located at amino acids 18–26: 1213.356 3. Amino acid substitutions at positions 1–9; predicted to be lower than reference peptide. 4. Amino acid substitutions at position 2: affected the half-time value considerably. 5. R amino acid substitution: 233 6. F9L amino acid substitution: 4.233 7. Amino acid substitutions at positions 39 and 41; drastically lowered half-time 8. Amino acid substitution at position 46: contrasting values dependent on the nature of the amino acid substitution present 9. Thus, lower half-time dissociation requires a higher concentration of amino acid substitutions to produce 50% of the CTL-epitope binding at equilibrium.

TABLE 2 (Continued)

Reference	Subtype/Geographical location (percentage of participants)	Cohort	Treatment	Amino acid investigated	Key findings
[65]	N/A	PIWH: 32 Mother and child plus 30 other PIWH HIV-negative controls: N/A Age: N/A Male: child, 30 other PIWH (N/A) CD4 ⁺ count: 725 (± 35.36) cells/ μ l 30 other PIWH: N/A Viral load: N/A	N/A	Full-length Vpr	1. Mother and child were nonprogressors. The 30 other PIWH died of AIDS or have AIDS-related illnesses. 2. Nonprogressors had fewer defects at the C-terminus region and had a peptide length of 95 amino acids due to an in-frame deletion of 88R. 3. Unique changes in nonprogressor RNA clones include an 89A in 69.23% of clones. 4. Vpr clones of PIWH that died of AIDS or have AIDS-related illnesses had more genetic diversity at the C-terminus (in-frame insertions/deletions) causing considerable length polymorphism in the Vpr protein. 5. Signature amino acid changes between PIWH that died of AIDS or have AIDS-related illnesses and nonprogressor clones are at positions 28, 37, 41, 55, 77, and 83. 6. Amino acid substitutions mostly affected β-turns, which are important in the structural stabilization of Vpr. 7. 62% of clones from PIWH that died of AIDS or have AIDS-related illnesses had a proline at positions 85 and 87, replacing the highly charged arginine at those positions. 8. 69% of nonprogressor clones had proline at positions 85 and 87. 9. Clones without prolines had amino acid substitutions at position 70, which plays an important role in the formation of the leucine zipper motif (LXXXXXL). Natural amino acid substitutions (70V77) alter the zipper found between amino acids 60 and 81.

(Continues)

in this review, 63T should be investigated for its potential influence on positive clinical outcomes in PLWH. In particular, the 63T has been associated with characteristics of slower disease progression.^{75,76} When comparing the Vpr sequences between subtypes (Figure 1), subtype C was the only subtype that presented with the 63T substitution. Considering the fact that subtype C has been associated with slower disease progression and replication fitness,^{85–88} it is plausible to speculate that 63T may have a functional role in this outcome. However, too few studies have investigated this particular mutation to make a conclusion and a need exists to further investigate on both clinical and fundamental levels to elucidate the role of 63T in HIV-1 pathogenesis. Furthermore, Vpr 77H was more commonly associated with no significant contribution to disease progression.^{66,73} In general, 77H is a less investigated substitution in contrast to Vpr 77Q. It is relevant to note that the Vpr 77Q substitution was also the most widely investigated amino acid substitution across all studies. However, as reported here, there is no clear consensus on whether 77Q influences disease progression or nonprogression in PLWH as the findings were mixed. Therefore, as reported here, 77H may have no influence on disease progression; however, further investigation is needed to ascertain the findings summarised in this review. The findings for 85P were also associated with no significant contribution to disease progression. However, fundamental studies have associated 85P with characteristics of LTNP⁸⁹ and with a reduction in the apoptosis-inducing ability.⁹⁰ This provides some evidence that the 85P amino acid substitution may be an indication of slower disease progression and be investigated as a marker thereof. However, these findings for 85P were reported in fundamental investigations and similar findings may not be reflected in clinical studies.

Third, studies investigating the association between Vpr amino acid substitutions and neurological outcomes in PLWH remain limited and therefore no conclusions could be drawn from these studies. However, preliminary studies reviewed here suggest that Vpr amino acid substitutions may influence neurological outcomes. Here, we included two studies that reported findings for Vpr amino acid substitutions and neurological outcomes.^{52,81} Although the amino acid substitutions investigated within this study were not deemed noteworthy, it does provide some evidence of an association between Vpr and neurological outcomes (ischaemic stroke, non-ischaemic stroke, neuroprotection and non-neuroprotection). In general, limited studies have investigated the neurological implications of Vpr amino acid substitutions in PLWH; however, the findings here have highlighted the potential role of amino acid substitutions 22I, 22L, 37I, 41N, 41S, 41G, 55A, 77Q and 77R in neurological outcomes in PLWH. A

recent study (*in press*) has also highlighted that the presence of signature 45Y in subtype C infection was associated with a 3.66 times higher global deficit score (GDS), 525 times higher plasma viral load, 15.84 times higher proviral load and 60% lower absolute CD4-T cell count compared those without the signature.⁹¹ This highlights the potential value of investigating the implication of Vpr amino acid substitutions in the development of neurocognitive impairments.

Fourth, studies investigating the association between Vpr amino acid substitutions and treatment status in PLWH remain limited and therefore no conclusions could be drawn from these studies. However, preliminary studies reviewed here suggest that Vpr amino acid substitutions may influence treatment efficacy. Here, we included two studies that reported findings for Vpr amino acid substitutions and treatment efficacy. Even though studies investigating this remain limited for Vpr, previous studies of other viral proteins (e.g. gp120 and group specific antigen; gag) have shown that sequence substitutions L116P, A204D, S375H/M/T, M426L, M434I and M475I in gp120 have shown to affect the efficacy of treatment, fostemsavir.⁹² Despite S375T being the most predominant, it had a less substantial role in the reduction in fostemsavir efficacy than other sequence substitutions.⁹² For gag, cleavage site amino acid substitutions A431V and I437V, found in the NC-SP2-p6 region, contributed to protease inhibition resistance,⁹³ whereas adding N374S, E428 and A431V to major resistance-associated protease substitutions results in increased phenotypic resistance to RTV.⁹⁴ Therefore, if Vpr amino acid substitutions follow a similar trend as reported for other HIV-1 viral proteins, we hypothesise that Vpr amino acid substitutions may also influence treatment efficacy and should be investigated as potential indicators thereof.

Last, certain studies have also shown that despite higher CD4⁺ count and lower viral loads, Vpr amino acid substitutions may still influence clinical outcomes and therefore these findings are relevant considering the modern ART treatment era.

5 | LIMITATIONS

The studies included in this review presented several limitations which we would briefly like to discuss. The studies presented in this review were diverse and heterogeneous. The heterogeneity of studies can be explained as follow: studies employed different design (longitudinal and cross-sectional) and different measures of clinical outcomes (e.g. neurological and disease progression). Therefore, a meta-analysis could not be conducted. This is particularly related to key cohort information, including

the clear description of inclusion/exclusion definition as well as the setting or subtype of the included participant. Furthermore, studies do not clearly define potential confounding factors nor relevant statistical analysis to control for these variances. Subtype variation is associated with specific amino acid substitutions within the HIV-1 viral proteins, including Vpr, therefore the clear reporting of this may assist in contextualising the findings. Limited studies have reported viral load and CD4⁺ count and both factors have clear influences on clinical outcomes in PLWH³³ and this should be clearly defined in studies investigating subtype-specific protein variation and clinical outcomes. Therefore, it is not clear how these findings will reflect in the modern ART era. Sample sizes/cohort sizes varied significantly across studies. This potentially had an influence on the level and/or associations of genetic diversity observed. Case studies or studies including small cohort size present genetic variation that may not necessarily be representative of larger cohorts. Therefore, the association of genetic variation with clinical outcomes from such studies should be interpreted with caution.

It should also be noted that most of the studies included in this review were more than 10 years old and consideration should be taken when interpreting the reported findings. Considering the significant development of HIV-1 research and genome sequencing over the past 10 years, this highlights the need for newer studies related to this topic. This will aid in the further understanding of the correlation between Vpr amino acid substitutions and clinical outcomes in PLWH.

6 | CONCLUSIONS

Studies have collectively reported that Vpr amino acid substitutions are most likely associated with clinical outcomes including disease progressions, neurological outcomes and treatment status. The Vpr amino acid substitutions, 63T were more commonly associated with positive clinical outcomes, and 77H and 85P were associated with no contribution to disease progression. Preliminary evidence suggests that Vpr amino acid substitutions may have implications for neurological outcomes and treatment efficacy in PLWH. The Vpr amino acid substitutions highlighted in this review should be investigated further to determine their potential role as predictive indicators of disease progression or anchor points to better understand their implications in HIV-1 pathophysiology.

AUTHOR CONTRIBUTIONS

Monray E. Williams and Levenco K. Asia conceptualised the study and generated the first draft. Monray E. Williams

and Levenco K. Asia conducted all database scans and articles for inclusion. All tables and figures were generated by Levenco K. Asia. Esmé Jansen Van Vuren provided conceptual input and reviewed all drafts of the manuscript. All authors are aware of and approved of the submission of this manuscript.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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ANNEXURE B:

**A PILOT INVESTIGATION OF THE ASSOCIATION BETWEEN VPR AMINO ACID
SUBSTITUTIONS AND PERIPHERAL IMMUNE MARKER LEVELS IN PEOPLE LIVING
WITH HIV: IMPLICATIONS FOR NEUROCOGNITIVE IMPAIRMENT.**

A pilot investigation of the association between Vpr amino acid substitutions and peripheral immune marker levels in people living with HIV: implications for neurocognitive impairment.

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Running Title: Vpr sequence variation and inflammation

Keywords: HIV neuropathogenesis; Vpr, Polymorphisms; Sequence variations; inflammation

Highlights: In South African people living with HIV, the presence of Vpr amino acids G41 reported significant associations with higher levels of suPAR compared to other amino acids at these positions.

Abstract

Background

The activity of HIV-1 in the periphery and central nervous system is responsible for its pathogenesis and neuropathogenesis, respectively. Among the various mechanisms influencing HIV-1 disease progression and prevalence, the subtype and subtype-specific variations of HIV-1, observed in the amino acid sequences of viral proteins, play a significant role. Recent evidence suggests that amino acid sequence variations in Viral protein R (Vpr) are linked to clinical outcomes in people living with HIV including disease progression, treatment efficacy and HIV-associated neurocognitive disorder (HAND). Previous research has also shown that Vpr sequence variation is associated with chronic inflammation, a common feature of HIV disease progression and a central mechanism in HIV-1 neuropathogenesis. However, no clinical study has explored the connection between Vpr sequence variation and peripheral inflammation in people living with HIV. This pilot study aimed to determine if specific Vpr amino acid variants, previously associated with HIV-1 neuropathogenesis, associate with specific immune markers in people living with HIV.

Methods

We included a unique cohort of forty-eight treatment-naïve South African people living with HIV to determine the association between blood-derived Vpr sequence variation and peripheral immune marker levels using Sanger sequencing and ELISA analysis, respectively.

Results

Our findings indicate that among the many neuropathogenic Vpr amino acid variants and immune markers examined, after applying Bonferroni corrections ($p = .05/3$) and adjusting for sex and locality, suPAR levels was nearing significance for higher levels in participants with the G41 amino acid variant compared to those with the S41 variant ($p = .035$), after. Further, amino acid variations at position 41 (between G41 and S41) exhibited a significant association with suPAR (adj $R^2 = .089$, $\beta = .386$; 95% CI .125 to 3.251; $p = .035$).

Conclusions

These findings suggest that Vpr amino acid sequence variations might contribute to dysregulated inflammation, which could explain the observed association between specific Vpr variants and HIV-1 (neuro)pathogenesis found in prior research. These Vpr variants merit

further investigation to fully understand their roles in HIV-1 pathogenesis and neuropathogenesis.

1. Introduction

Human immunodeficiency virus (HIV) remains of global concern, with approximately 37.7 million people living with HIV, of which 1.5 million were newly infected in 2021 (Heath *et al.*, 2021). Dysregulated inflammation is a common hallmark of HIV pathogenesis and an accelerator of non-acquired immunodeficiency syndrome (AIDS)-related events and one of the driving forces of CD4⁺ T cell depletion (Decrion *et al.*, 2005; Lv *et al.*, 2021). Similarly, dysregulated peripheral and central nervous system (CNS) inflammation has been associated with HIV-1 neuropathogenesis (Malik & Eugenin, 2016). HIV can invade the CNS when HIV-infected monocytes cross the blood-brain barrier (BBB), acting as a "Trojan horse" (Spudich & González-Scarano, 2012). After penetrating the BBB, these monocytes transform into macrophages, initiating an immune response in the CNS ultimately leading to neuronal damage and manifests clinically as HIV-associated neurocognitive disorders (HAND) (Spudich & González-Scarano, 2012) (Antinori *et al.*, 2007). Despite advancements in treating HIV infections and HAND with combined antiretroviral therapy (cART), chronic inflammation persists, contributing to the ongoing presence of comorbid conditions, including mild forms of HAND (Antinori *et al.*, 2007).

HIV exhibits high genetic variability, influencing the mechanisms related to both HIV-1 pathogenesis and neuropathogenesis (Hemelaar *et al.*, 2019) (Santerre *et al.*, 2019). HIV is categorized into two main types, HIV-1 and HIV-2, due to its high genetic variability. HIV-1 is more prevalent globally (Hemelaar *et al.*, 2019). HIV-1 further consists of groups M, N, O, and P with group M being the most abundant globally (Hemelaar *et al.*, 2019). Based on differences in the viral genome, group M can be further subdivided into subtypes A, B, C, D, F, G, H, J, and K (Hemelaar *et al.*, 2019). Geographically, these subtypes are linked to specific regions worldwide (Hemelaar *et al.*, 2019). Subtype B is predominantly found in America and Europe, while subtype C is prevalent in Southern Africa (Hemelaar *et al.*, 2019). Subtype C accounts for the majority of HIV-1 cases, representing approximately 40-60% of global infections (Hemelaar *et al.*, 2019). Notably, it is the dominant subtype in South Africa (Hemelaar *et al.*, 2019). Further, the prevalence of HIV and HAND varies across these different HIV-1 subtypes (Hemelaar *et al.*, 2019; Santerre *et al.*, 2019).

The differences among subtypes also manifest in changes in the amino acid sequences of HIV-1 viral proteins with different subtypes exhibiting sequence variations in crucial HIV viral proteins, Tat, Vpr, Pol, Nef, and gp120, resulting in distinct functionalities of the viral proteins across the subtypes (Asia *et al.*, 2022; Jin *et al.*, 2020; Samikkannu *et al.*, 2015). In particular, amino acid sequence variation in several HIV-1 viral proteins was previously associated with a differential HIV-1 pathogenesis, inflammation, and the development of HAND (Gandhi *et al.*, 2009; Ruhanya *et al.*, 2023a; Samikkannu *et al.*, 2015; Santerre *et al.*, 2019).

Despite the numerous mechanisms contributing to the HIV pathogenesis and neuropathogenesis, it is proposed that in the modern cART era, chronic dysregulated inflammation remains a principal factor driving the continual emergence of milder HAND forms (Harezlak *et al.*, 2011). It is well-established that inflammation within the CNS and brain tissue is linked to HIV-associated encephalopathy and the clinical presentation of neurocognitive impairment in people living with HIV (Fields *et al.*, 2018). Given that the cerebrospinal fluid is in indirect contact with the CNS, it can reflect the biochemical milieu of the CNS during disease states (Pendyala & Fox, 2010). However, the invasiveness of cerebrospinal fluid collection means it is not routinely collected for research purposes. This limitation makes peripheral blood a compelling alternative for exploring potential CNS pathologies. In line with this, evidence suggests that peripheral inflammation can shed light on the neuroinflammatory and/or neuropathological profile. A study by Bryant and colleagues reported that plasma-soluble CD163 is associated with postmortem brain pathology in HIV-1 infection (Bryant *et al.*, 2017). A study by Gisslén and colleagues indicated that concentrations of the neurofilament light chain protein in plasma and cerebrospinal fluid—a sensitive marker of neuronal injury—were highly correlated ($r = 0.89$, $p < .0001$) (Gisslén *et al.*, 2016). This suggests that peripheral inflammation may (1) reflect CNS activity to some extent, (2) correspond with inflammation levels in the CNS, and (3) serve as an alternative approach to investigating CNS pathological states related to inflammation. Notably, the activity of the HIV-1 viral proteins is recognized as significant inducers of the dysregulated inflammatory profiles observed in people living with HIV.

However, amongst all the HIV-1 viral proteins investigated (Engelman & Cherepanov, 2012; Li *et al.*, 2005), fewer studies have investigated Vpr despite its function in clinical outcomes in people living with HIV (Asia *et al.*, 2022; Dampier *et al.*, 2017; Na *et al.*, 2011). Vpr presence

and sequence variation are associated with various clinical outcomes related to HIV-1 pathogenesis, disease progression, treatment efficacy, and neurological outcomes (Asia *et al.*, 2022) (Ruhanya *et al.*, 2023a). Supporting this notion, fundamental studies have found that particular amino acids within Vpr can induce higher levels of inflammatory markers (Na *et al.*, 2011). However, no clinical studies to date have demonstrated a link between Vpr sequence variation and immune markers. Thus, this study aimed to investigate how Vpr sequence variations, particularly in amino acids previously linked with HIV neuropathogenesis (at positions 22, 41, 45, 55), associates with inflammation. The insights from this research may shed light on the observed correlations between Vpr amino acid sequence variations and inflammation in people living with HIV.

2. Methods

2.1. Study Participants

The Prospective Urban and Rural Epidemiology (PURE) study is a multinational prospective study designed to examine the development of cardiovascular diseases (CVD) in individuals from 27 high-, middle-, and low-income countries (Teo *et al.*, 2009). In particular, the PURE study investigated potential risk factors of CVD, which included HIV-1 infection and peripheral inflammation (Botha *et al.*, 2015). As part of this larger study, individuals of African descent, both men and women above 30 years, were recruited from two urban and two rural communities in the North-West Province of South Africa. Baseline data collection, encompassing $n = 2,010$ participants, was voluntarily conducted in 2005. Subsequent follow-up data was gathered in 2010 ($n = 1,288$) and 2015 ($n = 923$). Participants were re-diagnosed in 2010 to confirm their HIV status. Therefore, individuals who screened positive for HIV-1 and were treatment-naïve at the time of data collection in 2010 were included in this study ($n = 103$). HIV-1 sequencing was successful for only $n = 48$ people living with HIV; thus, only these were included in the subsequent analysis. Despite the high prevalence rate of HIV and cardiovascular disease multimorbidity in South Africa, it was previously shown that the participants involved in this study did not exhibit a worse cardiovascular disease risk profile than those without HIV (CVD)(Botha *et al.*, 2014; Fourie *et al.*, 2010; Louwrens *et al.*, 2022). This study presents a unique cohort of individuals who have not been treated for HIV, allowing us to investigate the inflammatory/immune profile without the confounding influence of ART. The study protocol was approved by the Health Research Ethics Committee of the North-West University (NWU-HREC) in South Africa (NWU-00106-22-A and NWU-00106-22-A1) and sub-study (NWU-00106-22-A1-01).

2.2. HIV Status

Participants received counselling by trained councillor prior to their HIV status determination. The South African Department of Health protocol was used to determine the HIV status using the First Response rapid HIV card test (Premier Medical Corporation Limited, Daman, India). The test was confirmed with the SD BIOLINE HIV 1/2 3.0 card test (Standard Diagnostics, INC, Korea). Upon confirmation of a positive result, participants received post-counselling. They were referred to the nearest clinic or hospital for follow-up and CD4⁺ cell count analysis using the flow cytometric method (Beckman COULTER, EPICS XITM, Fullerton, USA).

2.3. Analysis of immune markers in blood samples

Fasting blood samples were collected and were prepared through centrifugation at a speed of 2000 × g for 15 minutes at 10°C within 2 hours of collection. The samples were then transferred to microfuge tubes, snap-frozen on dry ice and stored at -80°C in the laboratory until analysis. For samples collected in rural areas, samples were snap-frozen on dry ice and stored at -18°C for a maximum of five days until they could be transported to the laboratory and stored at -80°C. For this study, the immune markers soluble urokinase plasminogen activator receptor (suPAR), interleukin (IL) 6, high-sensitivity C-reactive protein (hsCRP), soluble CD163 (sCD163) and neutrophil gelatinase-associated lipocalin (NGAL) were selected for investigation. These markers were selected for analysis based on their potential involvement in Vpr-mediated HIV pathogenesis and neuropathogenesis, as reviewed from the scientific literature (Asia *et al.*, 2022; Burdo *et al.*, 2013; Gianella *et al.*, 2019; Rubin *et al.*, 2018; Williams *et al.*, 2019b). Independent of HIV, sCD163 is an immune marker that has been demonstrated to play a role in chronic liver disease and insulin resistance (Grønbaek *et al.*, 2012; Parkner *et al.*, 2012). NGAL has been shown to contribute to kidney function and the duration of end-stage renal failure, cardiovascular disease, and mortality (Daniels *et al.*, 2012; Magnusson *et al.*, 2012). suPAR has been shown to contribute to acute kidney injury and modulate monocyte function to promote atherosclerosis (Hayek *et al.*, 2020; Hindy *et al.*, 2022). IL-6 is a predictor of long-term cardiovascular mortality (Gager *et al.*, 2020), atherosclerotic cardiovascular disease and cardiovascular disease mortality (Susilo *et al.*, 2022). hsCRP has been shown to contribute to acute ischaemic stroke (Cai *et al.*, 2019). suPAR levels were determined from plasma (EDTA) samples with the suPARnostiC® ELISA kit (ViroGates, Copenhagen, Denmark). hsCRP levels were analysed by particle-enhanced turbidimetric assay, and IL-6 was determined in plasma using an Elecsys 2010 (Roche, Basel, Switzerland) apparatus with the electrochemiluminescence immunoassay method. sCD163 and NGAL were measured using ELISA (R&D systems, DuoSet ELISA) according to the

manufacturer's instructions. All samples were assayed in duplicate. All tests' intra- and inter-assay coefficients of variation were within acceptable ranges of < 8% and < 10%, respectively.

2.4. Viral protein analysis

RNA was extracted from 200µl of prepared plasma using the Quick-RNA™ Viral Kit (Zymo Research). Total RNA was reverse transcribed by reverse transcriptase-polymerase chain reaction (PCR) using the ProtoScript® II First Strand cDNA Synthesis Kit (New England Biolabs), and DNA was prepared for PCR analysis. The Tat exon 1/Vpr (HXB2 position 4900–6351) was amplified using the primer pair Vif-1 (5'GGGTTTATTACAGGGACAGCAGAG)/CATH-4R(5'-GTACCCCATAATAGACTGTGACC) PCR, respectively. Amplification conditions were held at 94°C for 2 min, followed by 40 cycles of denaturing (94°C; 30s), annealing (60°C; 30s), extension (68°C; 2 min) and a final extension step for 10 min at 68°C. All PCR products were purified 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 using the Gene studio™ professional sequence analysis software (Version 2.2). Nucleotide sequences were translated into amino acid sequences using Expasy translate (Gasteiger et al. 2003), and the key mutations of Vpr were highlighted. Sequences are available in GenBank under the accession numbers OR621303- OR621349.

2.5. Statistical analysis

All analyses were conducted using SPSS (version 27, IBM, USA). *P*-values were considered statistically significant for all analyses at less than 0.05. All variables were assessed for normality by the visual inspection of QQ plots using descriptive statistics. Data distribution for age and the immune markers suPAR, hsCRP, IL-6, NGAL and sCD163 were skewed. Therefore, the data was log-transformed before statistical analyses.

As the primary aim, we examined whether peripheral inflammation levels differ between groups divided by different Vpr sequence variations. We employed composite risk scores for Vpr variants, similar to a previous study (Womersley *et al.*, 2019). We classified amino acids based on their potential influence on mechanisms related to the development of HAND, assigning higher scores to indicate a greater risk. The Vpr amino acids designated as high-risk (score = 2) were Isoleucine (I)22, Serine (S)41 or Asparagine (N)41, Tyrosine (Y)45 and

Alanine (A)A55, while low-risk (score = 0) was assigned to Leucine (L)22, Glycine (G)41, Histidine (H)45 and Threonine (T)55. Any other amino acids at the investigated positions were considered neutral risk (score = 1). These selected amino acids have demonstrated influence on neuropathological mechanisms and neurological outcomes in people living with HIV, thus warranting further analysis. A composite “Vpr risk score” was generated by summing the assigned values for each Vpr amino acid variant. Scores ranging from 0 to 3 were considered low-neutral, while scores from 4 to 8 were regarded as high-neutral. The chi-square test was used to assess differences in sex, smoking status, alcohol use and locality between groups. Independent sample T-tests were conducted to determine differences in study characteristics (age and CD4⁺ counts) and immune marker levels between the high-neutral and low-neutral groups. A Pearson correlation analysis was used to determine covariates by determining correlations between sociodemographic variables (age, sex, smoking status, alcohol use, and locality), and immune markers. Analyses of covariance (ANCOVA) were performed to compare the immune marker levels between Vpr high-neutral and low-neutral groups, while adjusting for sex, and locality. Pearson correlations were used to determine correlations between Vpr risk score and immune marker levels. Multiple regression analysis was used to determine associations between Vpr risk score and immune marker levels after adjusting for potential covariates (locality and sex).

As a secondary aim, we wanted to evaluate whether specific immune marker levels could be related to single amino acid variants. Therefore, we stratified participants into groups: I22 vs. L22, N/S41 vs. G41, Y45 vs H45 and A55 vs T55. At position 41, we compared G vs. N and G vs. S, respectively. Pearson’s chi-squared tests were used to test group differences for sex and locality between Vpr amino acid variants. Independent sample T-tests determined differences in study characteristics (age and CD4⁺ counts) and immune marker levels between Vpr amino acid variants. A Bonferroni correction was accounted for the number of immune markers tested ($\alpha/n = .05/5 = .01$) in the secondary aim. An ANCOVA was performed to compare the immune marker levels between Vpr variants, while adjusting for sex, and locality. A Bonferroni correction was accounted for the number of tests ($\alpha/n = .05/3 = .017$) within the model. Multiple regression analysis was used to determine associations between Vpr amino acid variance at specific positions and immune marker levels after adjusting for potential covariates (sex, and locality).

3. Results

3.1. Study characteristics

This study encompassed a sample of $n = 48$ treatment-naïve South African subtype C participants, with an average age of 47.46 (± 7.604). Men constituted only 25% of the participants, as depicted in Table 1. While the primary study did not record any viral load data, it should be noted that all participants were treatment-naïve at the time of sample collection. CD4⁺ count data was available for 67% of the participants, showing a mean value of 292.00 (± 128.41) cell/mm³. Stratifying participants based on Vpr amino acid variant risks revealed that the Vpr high-neutral group consisted of $n = 26$ participants, while the Vpr low-neutral group had $n = 22$ participants (Table 1). No significant differences in study characteristics (age, sex, CD4⁺ count and locality) were observed between the Vpr high-neutral and Vpr low-neutral groups as shown in Table 1. A Pearson correlation analysis revealed that among all variables (age, sex, smoking status, alcohol use, and locality), only locality and sex were correlated with specific markers. Consequently, we chose to include only these covariates in the relevant analyses.

3.2. Immune marker levels the in Vpr high-neutral group vs. low Vpr low-neutral group

Independent sample T-tests revealed no significant differences in immune marker levels between the Vpr high-neutral and Vpr low-neutral groups (Table 1). To explore the potential influence of covariates on the reported non-significant findings, we performed an ANCOVA analysis. Upon controlling for locality and sex using ANCOVA, the levels of IL-6 ($p = .911$), hsCRP ($p = .744$), suPAR ($p = .219$), NGAL ($p = .826$) and sCD163 ($p = .481$) remained statistically non-significant between the Vpr high-neutral group and the Vpr low-neutral group.

[Insert Table 1 here]

3.3. Correlation between Vpr risk score and immune marker levels.

We aimed to determine if there was a correlation between Vpr risk score and immune marker levels. Using Pearson correlations, we observed no significant correlations between the Vpr risk score and immune markers, namely hsCRP ($R = -.091$, $p = .538$), IL-6 ($R = -.073$, $p = .621$), suPAR ($R = -.200$, $p = .173$), NGAL ($R = -.021$, $p = .888$), and sCD163 ($R = -.103$, $p = .486$). Furthermore, a multiple regression analysis that adjusted for covariates (locality and sex) revealed no significant association between the Vpr risk score and hsCRP (adj $R^2 = -.057$, $\beta = -.097$, 95% CI = -7.8 to 4.0, $p = .523$), IL-6 (adj $R^2 = -.008$, $\beta = -.098$, 95% CI = -2.2 to 1.1, $p = .510$), suPAR (adj $R^2 = .021$, $\beta = -.223$, 95% CI = -0.6 to 0.08, $p = .133$), NGAL (adj

$R^2 = -.047$, $\beta = -.100$, 95% CI = -0.09 to .005, $p = .510$) and sCD163 (adj $R^2 = .066$, $\beta = -.067$, 95% CI = -0.11 to .07, $p = .638$).

3.4. Immune marker levels between Vpr amino acid variants

As an alternative to creating composite risk Vpr groups as described above, our secondary aim was to examine whether we could link immune marker levels to specific amino acid variants (Table 2). We stratified participants according to Vpr amino acid variants at position 22 ($n = 44$), 41 ($n = 40$), 45 ($n = 42$), and 55 ($n = 43$). When stratifying participants according to the Vpr amino acids variants, there were no significant differences between study characteristics (age, sex, CD4⁺ count and locality) (Table 2). After applying Bonferroni correction of $p = .05/5$, only suPAR levels were found to be significantly higher in the G41 group compared to the N41 ($p = .008$) (Table 2). However, after applying Bonferroni correction of $p = .05/2$ and when adjusting for locality and sex through ANCOVA, suPAR was nearing significance for higher levels in the G41 group compared to the S41 group ($p = .035$). However, suPAR was no longer significantly higher in the G41 group compared to the N41 group ($p = .115$).

[Insert Table 2 here]

Further, we aimed to evaluate the relationships between the Vpr amino acid variance at specific positions and immune marker levels while adjusting for covariates. To do this, we adjusted for covariates, namely locality and sex using multiple regression analyses. Across all Vpr amino acid positions investigated only variance at position 41 (between G41 and S41) exhibited a significant association with suPAR (adj $R^2 = .089$, $\beta = .386$; 95% CI .125 to 3.251; $p = .035$).

4. Discussion

In this study, we aimed to investigate whether variations in the HIV-1 Vpr amino acid sequence, especially at amino acid positions previously associated with neurological outcomes (namely positions 22, 41, 45, and 55), would influence the levels of peripheral immune markers sCD163, NGAL, hsCRP, suPAR, and IL-6 in people living with HIV. This was in aim of provide commentary on its role on potential underlying HIV-1 related pathology. Amongst all the Vpr amino acids and immune markers investigated, variation at position 44 associated with suPAR, which was shown to be higher in the G41 group when compared to the S41 group after adjustment for covariates.

The previous study by McMullen and colleagues has shown that the Vpr G41 amino acid was associated with non-ischaemic stroke in people living with HIV (McMullen *et al.*, 2021), also suggesting that Vpr G41 may be a low-risk Vpr variant. In the same cohort of South African participants, the S41 amino acid was associated with ischaemic stroke in people living with HIV (McMullen *et al.*, 2021), suggesting that S41 may be a high-risk Vpr variant. However, another study conducted in the United States of America, found that in people living with HIV, the S41 amino acid is associated with a decreased and less pronounced neurocognitive deficit (lower global deficit scores) (Dampier *et al.*, 2017). The contradiction between these findings may be due to the difference in geographical regions and, most likely, different HIV-1 subtypes (Jiang *et al.*, 2022). The study by Dampier and colleagues reported that the N41 amino acid was associated with more pronounced neurocognitive deficits (higher GDS) (Dampier *et al.*, 2017). We initially considered the Vpr variant G41 as low risk. However, our findings suggest that it may contribute to increased suPAR levels since suPAR was also higher in this group - a characteristic expected from the high-risk Vpr variants. The limited number of studies reveals no clear consensus on whether the amino acid variation at position 41, particularly G41, may contribute negatively or positively to clinical outcomes in people living with HIV.

Incorporating the existing body of evidence, we introduce the possibility that Vpr G41 might play a role in adverse clinical outcomes among people living with HIV by disrupting the peripheral immune response, particularly involving suPAR. It may also be that mutations at position 41 of Vpr generally may influence the structure-function relationship of Vpr, as amino acid 41 is within the second alpha-helix (38-50), which is responsible for key mechanisms including regulation of apoptosis, subcellular transport (Morellet *et al.*, 2009) and virion incorporation of Vpr (Singh *et al.*, 2000). It is established that hydrophilic amino acids, such as N41 and S41, are situated on a specific side of the helix (Wecker & Roques, 1999). Altering this configuration to a smaller amino acid like G41 could potentially impact the arrangement of the Vpr alpha helices, therefore influencing its potential functioning.

None of the other investigated Vpr amino acid signatures or inflammatory markers demonstrated significance after applying the Bonferroni correction. It is reasonable to speculate that the small number of participants in the included study may have led to low statistical power. However, future studies should explore these Vpr amino acid signatures in larger cohorts, as these signatures have been previously linked to adverse clinical outcomes

in people living with HIV (Asia *et al.*, 2022). Studies of this nature may help illuminate the reasons behind the observed variations in clinical severities when comparing participants with different HIV subtype infections. The amino acid at position 77 is extensively studied in the context of Vpr sequence variation (Asia *et al.*, 2022). However, the majority of participants in this study exhibited a Q at position 77 (83%). Due to the limited variation at this position, we were unable to explore its influence on immune markers.

Furthermore, it is known that HIV infection leads to the production of immune markers, often resulting in adverse clinical outcomes for individuals living with HIV (Decrion *et al.*, 2005). Our study revealed an association between the variance at position 41 and suPAR, a marker implicated in various diseases and disorders. Specifically, elevated plasma suPAR levels have been linked to worsened neurological performance in an HIV-treated population (Gianella *et al.*, 2019). Consistent with this, increased suPAR levels in the cerebrospinal fluid have been associated with lower working memory in people living with HIV (De Almeida *et al.*, 2022). This immune marker was also shown to associate with acute kidney injury and modulation of monocyte function, promoting atherosclerosis (Hayek *et al.*, 2020; Hindy *et al.*, 2022). Additionally, suPAR was found to be associated with organ failure independent of HIV infection (Wang, Wu, *et al.*, 2021).

Our study's findings suggest that Vpr amino acid variants, specifically those at position 41, may be associated with adverse clinical outcomes in people living with HIV through mechanisms related to dysregulated inflammation, particularly, suPAR (De Almeida *et al.*, 2022; Rasmussen *et al.*, 2016). While many studies that aimed to link HIV Vpr amino acid variants to clinical outcomes have been conducted in preclinical settings (Na *et al.*, 2011), fewer have explored HIV amino acid variations in clinical research (Dampier *et al.*, 2017; McMullen *et al.*, 2021). Moreover, even fewer studies have delved into the influence of HIV Vpr amino acid variants on inflammation, especially those concerning amino acid variations at positions 22, 41, 45 and 55 (Dampier *et al.*, 2017; McMullen *et al.*, 2021).

Given that our cohort was not undergoing any treatment at the time of sample collection, we were uniquely positioned to delineate the relationship between Vpr amino acid sequence variation and systemic inflammation more clearly. However, a more in-depth investigation of these specific Vpr variants is imperative to comprehensively understand their contributions to

HIV-1 pathogenesis and possibly neuropathogenesis. Further, most studies examining Vpr sequence variation have been conducted in regions dominated by subtype B (Asia *et al.*, 2022). Few investigations have delved into Vpr sequence variation in the Southern African context. Thus, our findings enrich the understanding of prevalent sequence variations within this setting. Further research is warranted to corroborate the relationship between Vpr amino acid variants and inflammation in people living with HIV. This could provide deeper insights into the potential impacts of such associations on clinical outcomes in people living with HIV.

5. Limitation

This study has several limitations that warrant emphasis. Firstly, the sample size was limited, which might have influenced the reported potential associations. Therefore, our findings should be interpreted in light of this, and future research is recommended to validate our results in larger cohorts. Though we view this as a pilot study, it offers insights into (1) the South African sequence variation, an area with limited investigation, and (2) the association of Vpr sequence variation in a treatment-naïve cohort, giving a clearer snapshot of systemic inflammatory profile without the potential confounding effects of cART. An initial objective of the study was to assess the combined impact of various amino acid substitutions on peripheral inflammatory levels, utilizing a composite risk score and categorizing participants into high-neutral and low-neutral Vpr risk groups. Although this approach has been employed previously (Womersley *et al.*, 2019), we recognize a limitation in that certain amino acids may exert a more significant influence than others. Summing amino acids together in this manner might attenuate this effect, potentially contributing to the lack of significance observed in the investigation of Vpr risk categories and risk scores. Additionally, this study focused on sequence variation in a single HIV-1 protein. Other pivotal viral proteins, such as Tat and gp120, might have also exhibited specific variations that could have contributed to the observations reported here. Nonetheless, our approach to examining a single HIV-1 protein and its association with clinical outcomes is consistent with methods used in other studies (Dampier *et al.*, 2017; Rao *et al.*, 2014). In this study, we also evaluated peripheral markers to draw insights into their potential CNS functioning. While it could be argued that peripheral markers might not directly reflect CNS activity, previous studies have documented associations between peripheral markers and clinical HAND. Moreover, a direct correlation exists between the levels of specific markers in the periphery and the CNS, suggesting that our findings may be relevant for investigations into CNS activity (Gisslén *et al.*, 2016). Furthermore, examining the concentration of the marker NFL in blood within this study could have provided insights into the potential neuropathology of individuals living with HIV (Gisslén

et al., 2016). Therefore, in cases where cerebrospinal fluid or neuropsychological data is unavailable, future studies conducted in blood should explore NFL as a potential indicator of neuropathology. Even though we have considered locality in our analyses, other psychosocial determinants of health may have played a role in influencing immune marker levels and the observed findings. Previous research has demonstrated that mental health, trauma, and stress can impact inflammation in individuals living with HIV (Berk *et al.*, 2013; Chow *et al.*, 2023; Hussain *et al.*, 2023; Memiah *et al.*, 2021) and therefore, our findings should be interpreted in relation to this.

6. Conclusions

Variations in the Vpr amino acid sequence have been previously linked to neurocognitive impairment in people living with HIV. Yet, the mechanism driving this association remains unclear. Given the pivotal role of dysregulated inflammation in HAND's development, we hypothesized that neuropathogenic Vpr amino acid variants might correlate with specific immune markers, suggesting a potential role in HAND's onset. In this study, we found that the Vpr variants at position 41 was associated with suPAR, and suPAR was higher in people living with HIV with the G41 variant. Therefore, variations in the Vpr amino acid sequence might be related to dysregulated inflammation and the subsequent disease progression. The Vpr signatures highlighted here warrant further investigation to fully understand their roles in HIV-1 pathogenesis and neuropathogenesis.

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8. Declarations

Potential Conflict of interests

No conflict

Data availability

All data is attached to this article.

Patient Consent Statement

All participants provided written informed consent, and their information was anonymized to the greatest extent possible. The study protocol received approval from the Health Research Ethics Committee of North-West University (NWU-HREC) in South Africa under the reference numbers NWU-00106-22-A, NWU-00106-22-A1, and the sub-study NWU-00106-22-A1-01.

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Author contributions

LKA and MEW contributed to the conceptualization, investigation, methodology, formal analysis, data curation, and writing. MEW also handled project administration, funding acquisition and supervision. EJVV contributed to formal analysis, writing (both original draft and review & editing), and supervision. LK provided resources and contributed to writing (review & editing).

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List of Tables

Table 1: Study characteristics of people living with HIV.

	People living with HIV (n = 48)
Age in years, mean (SD)	47.46 (±7.604)
Sex, female N (%)	36 (75)
CD4 ⁺ , mean (SD)	292. (±128.409)

Locality, rural N (%)	28 (58.3)		
	Vpr high-neutral (n = 26)	Vpr low-neutral (n = 22)	<i>P</i> -value
Age in years, mean (SD)	46.23 (7.016)	48.91 (7.72)	<i>NS</i>
Sex, female N (%)	18 (69)	18 (82)	<i>NS</i>
CD4 ⁺ , mean (SD)	299.8 (145.43)	285.5 (115.5)	<i>NS</i>
Locality, rural N (%)	13 (50)	15 (68)	<i>NS</i>
IL-6 pg/ml, mean (SD)	8.28 (11.37)	7.84 (6.9)	<i>NS</i>
hsCRP mg/l, mean (SD)	11.39 (28.18)	12.23 (12.23)	<i>NS</i>
suPAR ng/ml, mean (SD)	4.26 (1.8)	4.8 (2.01)	<i>NS</i>
NGAL ng/ml, mean (SD)	51.7 (16.4)	53.8 (26.6)	<i>NS</i>
sCD163 ng/ml, mean (SD)	746.1 (319.2)	792 (420.2)	<i>NS</i>

logarithmically transformed variables has been back transformed for presentation purposes.

NS – not significant

Table 2: Characteristics of HIV-positive participants and immune marker levels when participants stratified according to specific amino acids variants.

	22 (n = 44)		P value	41 (n = 40)			P value	45 (n = 42)		P value	55 (n = 43)		P value
Amino acid	I	L		G	N	S		Y	H		A	T	
N (%)	23 (52)	21 (48)	-	21 (53)	8 (20)	11 (27.5)	-	25 (60)	17 (40)	-	11 (26)	32 (74)	-
Age	48.22 (8.2)	46.9 (7.4)	.581	47.9 (6.8)	46.13 (6.7)	46.3 (8.7)	.544/.574	48.32 (8.4)	46.88 (5.8)	.545	47.45 (6.8)	47.28 (7.7)	.948
Sex	16 (70)	17 (81)	.862	15 (71)	7 (87.5)	6 (55)	.296	20 (80)	11 (65)	.268	7 (64)	27 (84)	.145
CD4 ⁺ count	274.80 (119.6)	314.4 (137.9)	.416	292 (133.8)	233.6 (60.2)	357.75 (155.3)	.366/.380	288.07 (101)	279.17 (143)	.851	258.7 (105.8)	317.9 (128.0)	.307
Locality	10 (43)	14 (67)	.123	13 (62)	5 (63)	7 (64)	.995	13 (52)	11 (65)	.618	7 (64)	17 (53)	.800
suPAR, ng/ml	4.51 (1.7)	4.76 (2.2)	.685	5.3 (2.4)	3.65 (0.7)	3.8 (0.9)	.008/.018	4.98 (2.4)	3.87 (0.8)	.037	5.41 (2.3)	4.12 (1.5)	.056
IL-6, pg/ml	7.93 (6.5)	9 (12.7)	.717	10.5 (13.4)	5.79 (3.57)	6.1 (4.2)	.346/.305	10.62 (12.5)	5.04 (3.1)	.041	13.08 (16.7)	6.18 (5.4)	.166
hsCRP, mg/l	16.19 (40.0)	10.44 (28.9)	.591	19.38 (48.2)	13.17 (21.5)	3.12 (3.6)	.731/.139	20.01 (45.2)	4.68 (5.1)	.104	18.67 (39.4)	11.3 (34.2)	.556
NGAL, ng/ml	57.8 (20.1)	45.5 (21.8)	.027	54.4 (26.8)	55.3 (17)	48.7 (15.3)	.661/.746	54 (22.6)	50.2 (22.3)	.570	50.9 (27.9)	55 (19)	.333

sCD163, ng/ml	801.4 (366.7)	783 (379.8)	.728	759.8 (376)	654.3 (398.1)	807.4 (249.1)	.305/ .465	775.3 (391.7)	631.8 (274.2)	.293	791.3 (292)	747.1 (383.9)	.462
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Age in years and immune markers: mean (SD); Sex, female and Locality, rural: N (%), logarithmically transformed variables has been back transformed for presentation purposes. At position 41, we compared G vs N and G vs S respectively. Statistically significant findings are presented in bold.

ANNEXURE C:

**A PILOT INVESTIGATION OF THE ASSOCIATION BETWEEN HIV-1 VPR AMINO ACID
SEQUENCE DIVERSITY AND THE TRYPTOPHAN-KYNURENINE PATHWAY AS A
POTENTIAL MECHANISM FOR NEUROCOGNITIVE IMPAIRMENT.**

A pilot investigation of the association between HIV-1 Vpr amino acid sequence diversity and the Tryptophan-kynurenine pathway as a potential mechanism for neurocognitive impairment.

HIV-1 Viral protein R sequence diversity

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Abstract

HIV infection compromises both the peripheral and central immune systems due to its pathogenic and neuropathogenic features. The mechanisms driving HIV-1 pathogenesis and neuropathogenesis involve a series of events, including metabolic dysregulation. Furthermore, HIV-subtype-specific variations, particularly alterations in the amino acid sequences of key viral proteins, are known to influence the severity of clinical outcomes in people living with HIV. However, the impact of amino acid sequence variations in specific viral proteins, such as Viral protein R (Vpr), on metabolites within the Tryptophan (Trp)-kynurenine (Kyn) pathway in people living with HIV remains unclear. Our research aimed to explore the relationship between variations in the Vpr amino acid sequence (specifically at positions 22, 41, 45, and 55, as these have been previously linked to neurocognitive function) and peripheral Trp-Kyn metabolites. Additionally, we sought to clarify the systems biology of Vpr sequence variation by examining the link between Trp-Kyn metabolism and peripheral inflammation, as a neuropathogenic mechanism. In this preliminary study, we analyzed a unique cohort of thirty-two ($n = 32$) South African cART naïve people living with HIV. We employed Sanger sequencing to ascertain blood-derived Vpr amino acid sequence variations and a targeted LC-MS/MS metabolomics platform to assess Trp-Kyn metabolites, such as Trp, Kyn, kynurenic acid (KA), and quinolinic acid (QUIN). Enzyme-linked immunosorbent assays were used to measure immune markers, hsCRP, IL-6, suPAR, NGAL and sCD163. After applying Bonferroni corrections ($p = .05/3$) and adjusting for covariates (age and sex), only the Vpr G41 and A55 groups were nearing significance for higher levels of QUIN compared to the Vpr S41 and T55 groups, respectively (all $p = .023$). Multiple regression results revealed that Vpr amino acid variations at position 41 (adj $R^2 = .049$, $\beta = .505$; $p = .023$), and 55 (adj $R^2 = .126$, $\beta = .444$; $p = .023$) displayed significant associations with QUIN after adjusting for age and sex. Lastly, the higher QUIN levels observed in the Vpr G41 group were found to be correlated with suPAR ($r = .588$, $p = .005$). These results collectively underscore the importance of specific amino acid substitutions in influencing QUIN and inflammation (specifically suPAR levels), potentially contributing to our understanding of their roles in the pathogenesis and neuropathogenesis of HIV-1.

Keywords: Kynurenine pathway, metabolism, inflammation, and HIV-associated neurocognitive disorders.

1. Introduction

Human immunodeficiency virus (HIV) affects approximately 39 million people globally of which 37.5 million are adults and 1.5 million are children (0-14 years of age) (Heath *et al.*, 2021). Despite advancements in HIV treatment, twenty million people living with HIV are situated in eastern and southern Africa. HIV-1 impacts immune cells, including CD4⁺ cells, leading to the deterioration of the immune system (Vidya Vijayan *et al.*, 2017). This deterioration creates conditions conducive to the development of comorbidities (Deeks *et al.*, 2015). HIV infection commonly results in the virus spreading into the central nervous system (CNS) of people living with HIV, consequently heightening the risk of developing HIV-associated neurocognitive disorders (HAND) (Ances & Ellis, 2007). However, the severity of HIV pathogenesis and neuropathogenesis may differ due to the genetic diversity of HIV (Hemelaar *et al.*, 2019).

While both HIV-1 and HIV-2 can contribute to HIV pathogenesis, HIV-1 is more virulent and is the predominant type causing HIV infections (Bhebhe *et al.*, 2021; Campbell-Yesufu & Gandhi, 2011; Rasmussen *et al.*, 2020a). Due to the considerable genetic variability of HIV, different subtypes of HIV-1 have emerged globally, (Hemelaar *et al.*, 2019), each exerting distinct influences on HIV pathogenesis, neuropathogenesis, and the severity of clinical outcomes (Santerre *et al.*, 2019). Among the HIV-1 groups, including M, N, O, and P (Ayoub *et al.*, 2001; Plantier *et al.*, 2009; Roques *et al.*, 2004; Simon *et al.*, 1998; Vallari *et al.*, 2011; Yousaf *et al.*, 2011), group M is the most prevalent globally (Sharp *et al.*, 1999). Furthermore, group M consists of subtypes A, B, C, D, F, G, H, J, and K (Yousaf *et al.*, 2011). Subtypes B and C represent most HIV infections, accounting for 12.1% and 46.6%, respectively (Hemelaar *et al.*, 2019). Subtype C is the most common in Southern Africa whereas subtype B is most common in Eastern European and Central Asia regions (Hemelaar *et al.*, 2019).

Clinical outcomes related to HIV progression and neuropathogenesis vary between HIV-1 subtypes (Boivin *et al.*, 2010; Gupta *et al.*, 2007; Sacktor *et al.*, 2009; Santoro & Perno, 2013). Consequently, the functions of HIV viral proteins vary depending on the HIV subtype (Asia *et al.*, 2022; Gotora *et al.*, 2023; Jin *et al.*, 2020; Nagata *et al.*, 2017; Samikkannu *et al.*, 2015; Williams, 2023). Thus, studies have investigated the effects of HIV-1 subtype-specific viral proteins such as transactivator of transcription (Tat) and glycoprotein 120 (gp120) in people living with HIV (Aralaguppe *et al.*, 2016; Campbell *et al.*, 2011; Samikkannu *et al.*, 2011; Samikkannu *et al.*, 2015). While major viral proteins like Tat and gp120 receive significant attention, accessory viral proteins such as viral protein R (Vpr) are less frequently studied. This is in spite of research

indicating Vpr's contribution to clinical outcomes (Cali *et al.*, 2005; Jacquot *et al.*, 2009; Lum, Cohen, Nie, Weaver, Gomez, Yao, Lynch, Pilon, Hawley, Kim, *et al.*, 2003; Soares *et al.*, 2014), including its association with HIV neuropathogenesis (Ruhanya *et al.*, 2023a).

On a metabolic level, the tryptophan-kynurenine (Trp-Kyn) metabolism has garnered interest because of its association with HIV-1 pathogenesis and neuropathogenesis (Lovelace *et al.*, 2017; Routy *et al.*, 2015). Once Trp is catabolized, it produces several downstream metabolites via the kynurenine (Kyn) pathway such as Kyn, kynurenic acid (KA), and quinolinic acid (QUIN) (Lovelace *et al.*, 2017). In the brain, Trp-Kyn metabolism occurs mainly in infiltrating macrophages and resident microglial cells (Guillemin *et al.*, 2003a; Guillemin *et al.*, 2005). Given that HIV infects macrophages that traverse the blood-brain barrier, a potential link between the Trp-Kyn metabolism and HIV neuropathogenesis might exist. Furthermore, dysregulated inflammation is a major driver of disease progression (Deeks *et al.*, 2013; Roberts *et al.*, 2010), HIV persistence (Klatt *et al.*, 2013), neuropathogenesis, and the development of milder forms of HAND in the modern-ART era (Heaton *et al.*, 2010; Williams, Ipser, *et al.*, 2020). Dysregulated inflammation is observed in plasma and cerebrospinal fluid (CSF) of people living with HIV (Burdo *et al.*, 2013; Guha *et al.*, 2023; Roberts *et al.*, 2010). This inflammation is closely associated with metabolic changes, especially in Trp metabolism (Keegan *et al.*, 2016). Furthermore, the enzyme, IDO-1, which is responsible for Trp degradation into Kyn is upregulated by inflammatory molecules and cytokines like interferon-gamma (IFN- γ) (Werner-Felmayer *et al.*, 1989; Yasui *et al.*, 1986). Subsequently, Trp degradation via the Trp-Kyn pathway results in metabolites which may have excitatory and inhibitory properties (Baran *et al.*, 2000; Braidy *et al.*, 2009). Thus, these excitatory properties play a role in the HIV infection in the CNS (Heyes *et al.*, 1991; Martin *et al.*, 1992). The HAND population often exhibits lowered Trp and Kynurenine/Tryptophan (Kyn/Trp) ratio levels in plasma (Keegan *et al.*, 2016).

Although there is an association between Trp-Kyn metabolism and HIV neuropathogenesis (Lovelace *et al.*, 2017), the association between Trp-Kyn metabolism and HIV-1 viral proteins are unclear; especially the association between HIV-1 Vpr sequence variation and the Trp-Kyn metabolism. In a previous systematic review conducted by our group, Vpr's association with clinical outcomes in people living with HIV was evident (Asia *et al.*, 2022). However, to our best knowledge, this is the first study to investigate the influence of Vpr amino acid sequence variation on the Trp-Kyn metabolism. Thus, we aimed to assess whether Vpr sequence variation, especially

the neurological-associated amino acids at positions 22, 41, 45, and 55, associates with metabolites of Trp-Kyn metabolism in a South African cohort.

2. Methods

2.1. Study Participants

The Prospective Urban and Rural Epidemiology (PURE) study focuses on cardiovascular diseases in people living with HIV and the underlying mechanism that can contribute to the development of CVD such as inflammation, in individuals from 20 countries with varying income levels, encompassing high, middle, and low-income nations (Teo *et al.*, 2009). As a subset of this extensive research, men and women aged 30 and above of African descent were enlisted from both urban and rural regions in the North-West Province of South Africa. The participants were excluded if they had chronic medication use and existing chronic conditions. Baseline data collection, encompassing n = 2,010 participants, was voluntarily conducted in 2005. Subsequent follow-up data was gathered in 2010 (n = 1,288) and 2015 (n = 923). Participants were re-diagnosed in 2010 to confirm their HIV status. Therefore, individuals who screened positive for HIV-1 and were treatment-naïve at the time of data collection in 2010 were included in this study (n = 103). This selection allowed us to explore metabolic profiles without the interference of antiretroviral treatment (ART), given that ART is recognized for its impact on metabolic profiles in people living with HIV (Keegan *et al.*, 2019). The study's protocol received approval from the Health Research Ethics Committee of the North-West University in South Africa under the reference numbers NWU-00106-22-A1 and NWU-00106-22-A1-01.

2.2. HIV Status

Before establishing the HIV status of the participants, they underwent counselling sessions provided by trained counsellors. The HIV status was determined using the First Response rapid HIV card test, manufactured by Premier Medical Corporation Limited in Daman, India, in accordance with the protocol established by the South African Department of Health. As a confirmation step, an SD BIOLINE HIV 1/2 3.0 card test from Standard Diagnostics, INC in Korea was used. Participants who received a positive result were provided with post-counselling and were then referred to the nearest clinic or hospital for further assessment. At the clinic or hospital, their CD4⁺ cell count was analysed using the flow cytometric method with the Beckman COULTER EPICS XITM machine from Fullerton, USA.

2.3. Analysis of metabolites in blood samples

Fasting blood samples were collected and were centrifuged at $2000 \times g$ for 15 minutes at a temperature of 10°C , all within 2 hours of collection. After centrifugation, the samples were transferred into microfuge tubes, promptly frozen by placing them on dry ice, and then stored at -80°C in the laboratory until they were ready for analysis. In cases where samples were collected from rural areas, they were also snap-frozen on dry ice but stored at -18°C for a maximum of five days until they could be transported to the laboratory. Upon arrival, they were then stored at -80°C for subsequent analysis.

We investigated the Trp-Kyn metabolism metabolites, specifically Trp, Kyn, Kyn:Trp ratio (in direct measure of IDO activity), KA, and QUIN, due to their potential involvement in the pathophysiology of Vpr-mediated neuropathogenesis and HAND, as discussed in scientific literature references (Baran *et al.*, 2000; Braidy *et al.*, 2009; Guillemin *et al.*, 2003a; Guillemin *et al.*, 2005; Keegan *et al.*, 2016; Lovelace *et al.*, 2017; Werner-Felmayer *et al.*, 1989; Yasui *et al.*, 1986). These metabolic profiles were determined using high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) as described in the following sections.

2.3.1. Chemicals

Kynurenic acid-d5 (2.5mg), L-Kynurenine-d4 [2-aminophenyl-3,5-d2] (5mg), 2,3-pyridinedicarboxylic acid-d3 (Major) (1mg) were purchased from TRC Research Chemical. D-Tryptophan (Indole-D5, 98%) (50mg) was purchased from Cambridge Isotope Laboratories. D-Kynurenine free base (25mg), 2,3-dicarboxylic acid, 99% (25g), D-tryptophan, =98.0% (HPLC) (1g) was purchased from MERCK. UPLC water and acetonitrile, 99%, was purchased from Honeywell. Formic acid for LC-MS was purchased from Merck.

2.3.2. Sample preparation for targeted metabolomics

Trp, Kyn, Kyn/Trp ratio, QUIN, and KA were quantified by liquid chromatography–tandem mass spectrometry (LC-MS/MS; Agilent 1200 series HPLC system) using a targeted approach. Proteins were precipitated from HIV plasma samples by adding 300 μl ice-cold acetonitrile to 100 μl HIV plasma samples in addition to 100 μl internal standard mixture (10 ppm, Kynurenic acid-d5, L-Kynurenine-d4 [2-aminophenyl-3,5-d2], 2,3-pyridinedicarboxylic acid-d3 (Major), D-Tryptophan (Indole-D5, 98%)) (Lindeque *et al.*, 2013). Matrix-appropriate external calibrators were prepared similarly. External calibrators were spiked with Kyn, QUIN, and Trp, which were serially diluted to create a calibration curve. Samples were vortexed and incubated on ice for 10 minutes to facilitate protein precipitation. Samples were then centrifuged at $12\,000 \times g$ for 10 min. The supernatant was collected, dried under nitrogen gas then stored at -80°C until analysis. For analysis, samples

were removed from the -80°C freezer and left at room temperature to equilibrate. The samples were then re-dissolved in 50% HPLC water: 50% ACN, left for 30 minutes at room temperature and then vortexed. The resuspended sample was transferred to a glass vial with a vial insert. Samples were then analyzed on the LC-MS/MS. Metabolic profiles were measured via multiple reaction monitoring (MRM) using electrospray ionization mass spectrometry in both positive and negative modes. The MRM transitions and chromatographic conditions on the LC-MS/MS were optimized (with the help of commercial standards) to detect and quantify the target metabolites accurately. The ratio of Kyn to Trp was used for the estimation of IDO activity.

2.3.3. LC-MS/MS analyses

A targeted LC-MS/MS analysis was performed on HIV-positive plasma samples using a 1200 series HPLC system coupled to a 6470-triple quadrupole-mass spectrophotometer (Agilent Technologies). The chromatographic separations were achieved by injecting 1µL sample on an Acquity UPLC CSH C18 1.7µm 2.1x100mm column, kept at 80°C. Mobile phases consisted of HPLC water (solvent A) and acetonitrile (solvent B) with both containing 0.1% formic acid.

For the positive polarization, the chromatographic gradient was set up as t=0, 1% mobile phase B; t=1, 2% mobile phase B; t=2, 5% mobile phase B; t=3, 10% mobile phase B; t=4, 40% mobile phase B; t=5, 50% mobile phase B; t=6, 60% mobile phase B; t=7, 80% mobile phase B; t=8, 100% mobile phase B; t=10, 1% mobile phase B; lastly, 100% mobile phase B for 5 minutes (post-run) to ensure that all analytes are washed out of the column for the next sample analysis. The flow rate was always 0.2 mL/min except at t=1 until t=2, 0.1 mL/min. Source parameters were set as capillary voltage of 3500 V; nitrogen gas at a flow rate of 5 L/min at 300°C; and nebulizer pressure of 45 psi. MRM parameters were set up for all compounds (Supplementary Table 1).

For the negative polarization, the chromatographic gradient was set up as t=0, 1% mobile phase B; t=2, 1% mobile phase B; t=3, 37% mobile phase B; t=4, 40% mobile phase B; t=5, 45% mobile phase B; t=6, 50% mobile phase B; t=7, 100% mobile phase B; t=8, 1% mobile phase B; lastly, 100% mobile phase B for 5 minutes (post-run) to ensure that all analytes are washed out of the column for the next sample analysis. The flow rate was 0.1 mL/min at t=0; t=2; t=3 and 0.2 mL/min at t=4; t=5; t=6; t=7; t=8. Source parameters were set as capillary voltage of 4000 V; nitrogen gas at a flow rate of 8 L/min at 250°C; and nebulizer pressure of 20 psi. MRM parameters were set up for all compounds (Supplementary Table 1).

2.4. Analysis of immune markers in blood samples

Samples were prepared as described above (*Section 2.3*). We selected specific immune markers for investigation, including soluble urokinase plasminogen activator receptor (suPAR), interleukin (IL) 6, high-sensitivity C-reactive protein (hsCRP), soluble CD163 (sCD163), and neutrophil gelatinase-associated lipocalin (NGAL). These markers were chosen based on their potential relevance to Vpr-mediated neuropathogenesis and HAND pathophysiology, as indicated in scientific literature (Asia *et al.*, 2022; Gianella *et al.*, 2019; Rubin *et al.*, 2018; Williams *et al.*, 2019a). To measure suPAR levels, plasma samples with EDTA were used, and the suPARnostic® ELISA kit from ViroGates in Copenhagen, Denmark, was employed. Particle-enhanced turbidimetric assay was used to analyze hsCRP levels (Cobas Integra 400 plus (Roche Diagnostic, Basel, Switzerland)), while IL-6 was determined in plasma through the electrochemiluminescence immunoassay method using an Elecsys 2010 (Roche, Basel, Switzerland) apparatus. For sCD163 and NGAL, ELISA assays (R&D systems DuoSet) were conducted following the manufacturer's instructions, and all samples were analyzed in duplicate. The intra- and inter-assay coefficients of variation for all tests fell within acceptable ranges of < 8% and < 10%, respectively.

2.5. Viral protein analysis

RNA was isolated from 200 µl of prepared plasma using the Quick-RNA™ Viral Kit from Zymo Research. The total RNA was then subjected to reverse transcription-polymerase chain reaction (PCR) using the ProtoScript® II First Strand cDNA Synthesis Kit from New England Biolabs, and the resulting DNA was prepared for subsequent PCR analysis. For the amplification of the Tat exon 1/Vpr region (HXB2 position 4900–6351), a primer pair consisting of Vif-1 (5'GGGTTTATTACAGGGACAGCAGAG) and CATH-4R (5'-GTACCCCATAGACTGTGACC) was employed. The PCR amplification process involved an initial denaturation step at 94°C for 2 minutes, followed by 40 cycles of denaturing at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 68°C for 2 minutes, and a final extension step at 68°C for 10 minutes. Following PCR amplification, purification of all PCR products was carried out using the Nucleospin® Gel and PCR clean-up kit in accordance with the manufacturer's instructions (Machery-Nagel GmbH & Co.KG, Germany). Subsequently, all PCR products were subjected to sequencing using the BigDye Terminator v.3.1 Cycle Sequencing Ready Reaction Kit from ThermoFisher Scientific and were analyzed using the ABI Prism 3130xl automated DNA sequencer from Applied Biosystems, located in Foster City, CA. To analyze the obtained sequences, the GeneStudio™ Professional sequence analysis software (Version 2.2) was

utilized. The nucleotide sequences were translated into amino acid sequences using the Expasy translate method (Gasteiger *et al.*, 2003), and the key mutations in the Vpr region were identified and highlighted for further examination. Sequences are available in GenBank under the accession numbers OR621303- OR621349.

2.6. Statistical analysis

All analyses were conducted using SPSS (version 27, IBM, USA). *P*-values were considered statistically significant for all analyses at less than 0.05. All variables were assessed for normality by the visual inspection of QQ plots using descriptive statistics. Data distribution for age, the metabolites Kyn/Trp ratio, QUIN and KA and the immune markers NGAL and sCD163 were skewed. Therefore, the data of variables that were skewed were log-transformed before statistical analyses. After log transformation, all data presented acceptable skewness and kurtosis values within the range of -2 and 2.

As the primary aim, we wanted to evaluate whether specific metabolites levels could be related to single amino acid variants. Therefore, we stratified participants into groups: I22 vs. L22, N/S41 vs. G41, Y45 vs H45 and A55 vs T55. At position 41, we compared G vs. N and G vs. S, respectively. Chi-squared tests were used to test group differences for sex, smoking, alcohol use and locality between Vpr amino acid variants. Independent sample T-tests determined differences in study characteristics (age, CD4⁺ count, BMI) and metabolite levels between Vpr amino acid variants. A Bonferroni correction was accounted for the number of metabolites tested ($\alpha/n = .05/5 = .01$) in the primary aim. A Pearson correlation analysis was used to determine covariates by determining correlations between sociodemographic variables (age, sex, smoking status, alcohol use, and locality), and specific metabolites. Analyses of covariance (ANCOVA) were performed with levels of metabolites as the dependent variables to compare the metabolite levels between Vpr variants, adjusting for covariates. A Bonferroni correction was accounted for the number of tests ($\alpha/n = .05/3 = .017$) within the model. Multiple regression analysis was used to determine associations between Vpr amino acid variants and metabolite levels after adjusting for potential covariates.

As a secondary aim, we wanted to determine the association between peripheral immune markers and metabolites. Pearson correlations were used to determine correlations between metabolite and immune marker levels/ CD4⁺ count. A Bonferroni correction was accounted for the number of immune markers tested ($\alpha/n = .05/5 = .01$) in the secondary aim. Thereafter, using the enter

method, multiple regression analysis was used to determine associations between immune marker and metabolites levels after adjusting for potential covariates.

3. Results

3.1. Study characteristics

This pilot study encompassed a sample of $n = 32$ treatment-naïve South African subtype C participants, with an average age of 48.08 (± 7.066) years. Only 25% of the participants were males. While the primary study did not record any viral load data, it should be noted that all participants were treatment-naïve at the time of sample collection. CD4⁺ count data was available for 53% of the participants, showing a mean value of 282.76 (± 161.024) cell/mm³. Participants had a mean BMI of 26.64 (± 10.2). Data on smoking status and alcohol consumption were available for 94% ($n = 30$) of the participants, with 56% and 60% of these participants being current or former smokers and alcohol consumers, respectively. Approximately half of the participants were recruited from rural regions in South Africa ($n = 18$, 56%). When participants were stratified according to Vpr amino acid variants (Table 1), no significant differences were observed for any demographic variables (age, sex, CD4⁺ count, BMI, smoking status, alcohol use and locality) (all $p > .05$). After determining correlations between sociodemographic variables and specific metabolites, it was indicated that, no correlations existed for any of the considered variables (age, sex, smoking status, alcohol use, and locality). Nevertheless, we opted to incorporate sex and age as covariates in the pertinent analyses as will be described below, given their established influence on HIV pathogenesis [60, 61].

3.2. Metabolite levels between Vpr amino acid variants

We aimed to examine whether we could link metabolite levels to specific amino acid variants (Table 1). We stratified participants according to Vpr amino acid variants at position 22 ($n = 29$), 41 ($n = 27$), 45 ($n = 30$), and 55 ($n = 28$). After applying Bonferroni corrections of $p = .05/5$, none of the metabolite levels showed significant differences between the Vpr variants at positions 22, 41 or 45. However, QUIN levels were nearing significance for higher levels in the A55 group compared to the T55 group ($p = .022$) (Table 1). After applying Bonferroni corrections of $p = .05/3$, this result remained unchanged in the Vpr 55 group ($p = .023$) after adjustment for covariates (age and sex). Additionally, following the adjustment for age and sex, QUIN levels were nearing significance for higher levels ($p = .023$) in the G41 group when compared to the S41 group.

[Insert Table 1]

Further, we aimed to evaluate the relationships between Vpr amino acid sequence variation and metabolite levels while adjusting for covariates (age and sex) as shown in Table 2. Here, variation at position 41 (between G41 and S41) showed significant associations with QUIN levels ($p = .023$). When investigating the Vpr amino acids at position 55, variation at this position (between A55 and T55) demonstrated significant associations with QUIN levels after ($p = .023$). No significant associations were detected for the other Vpr amino acid signatures or metabolites.

3.3. Correlation between immune markers and metabolites in the specific Vpr groups

Given the significant associations between QUIN levels and variation at Vpr amino acid position 41 and 55, we sought to investigate potential correlations between QUIN and immune markers in these respective groups. Following the application of a Bonferroni correction ($p = .05/5$), we observed a significant positive correlation between suPAR and QUIN levels in the Vpr 41 group ($p = .005$) (Figure 1C). In the Vpr 55 group, QUIN was not associated with any of the investigated immune markers (all $p > .01$) (Figure 1F- Figure 1J). Subsequently, we aimed to assess the association between QUIN and suPAR levels while adjusting for covariates. Even after adjusting for covariates, including age and sex, QUIN was significantly associated with suPAR within Vpr 41 group (adj $R^2 = .356$, $\beta = .664$; $p = .003$). Given the absence of viral load data for the entire cohort, adjustment for this variable was not possible. However, CD4⁺ count data was accessible for 53% of the participants. Consequently, we sought to explore the extent to which these disease characteristics may have contributed to QUIN levels in our study. In the overall cohort with available CD4⁺ data ($n = 17$), QUIN levels were negatively correlated with CD4⁺ count ($r = -.711$, $p = .001$).

[Insert Figure 1]

4. Discussion

Our study encompassed three main findings. Firstly, among the various Vpr amino acid variants and metabolites examined, only the Vpr G41 and A55 group was nearing significantly higher levels of QUIN when compared to the Vpr S41 and T55 variants respectively. Secondly, amino acid variation at positions 41 and 55 were found to be significantly associated with QUIN levels. Lastly, in the Vpr G41 group, QUIN correlated with higher suPAR levels. These results collectively underscore the importance of specific amino acid substitutions in influencing QUIN and

specifically, suPAR levels, potentially contributing to our understanding of their roles in the pathogenesis and neuropathogenesis of HIV-1.

The findings of this study report significant associations between QUIN levels and Vpr variation at positions 41 (between G41 and S41) and 55 (between A55 and T55), respectively. These findings may indicate that Vpr variation at these specific positions could increase QUIN levels. Indeed, we showed that the Vpr G41 and A55 variant showed higher levels of QUIN compared to the S41 ($p = .023$) and T55 groups ($p = .023$), respectively. The higher QUIN levels could potentially be indicative of negative clinical outcomes, as peripheral and CSF QUIN levels have previously been associated with neurological outcomes in psychiatric disorders, including HAND (Anderson *et al.*, 2018; Skorobogatov *et al.*, 2021). Although QUIN cannot freely cross the BBB (Fukui *et al.*, 1991), it is a product of 3-hydroxykynurenine, which can cross the blood-brain barrier freely. Additionally, 3-hydroxykynurenine is a downstream metabolite of tryptophan, which can also readily cross the blood-brain barrier (Schwarcz *et al.*, 2010). As a result, the production of QUIN in the brain may persist, maintaining its neuroexcitatory properties (Braidy *et al.*, 2009). During immune activation in the CNS, over 98% of the Kyn and QUIN present in the brain are produced within the CNS (Kita *et al.*, 2002). However, studies have demonstrated that human monocytes and monocyte-derived macrophages can produce up to 19 times more QUIN than activated microglia (Guillemin *et al.*, 2003b). In line with this, the synthesis of QUIN by microglia in patients with epilepsy was approximately only 15% of the QUIN produced by monocyte-derived macrophages retrieved from brain tissue (Espey *et al.*, 1997). Lastly, it's important to highlight that QUIN concentrations consistently tend to be higher in the bloodstream compared to the CNS across various pathological conditions, with blood-to-cerebrospinal fluid (CSF) ratios of 14:1 in humans, 19:1 in rodents, and even up to 52:1 in nonhuman primates (Morrison *et al.*, 1999). This observation is also pertinent to the CNS because infiltrating activated macrophages could potentially serve as the primary source of QUIN during brain inflammation. Therefore, an investigation into systemic QUIN levels may provide valuable insights into how these levels may be reflected in the CNS. These findings may have translation value to other health conditions. Other studies have also identified associations between plasma Trp-Kyn metabolites and various health conditions, including the presence of depression (Drivsholm *et al.*, 2021), HIV-related gut microbiota alterations, and gut-adipose tissue (Gelpi *et al.*, 2022). Moreover, these metabolites have been shown to predict poor CD4+ T-cell count recovery and increased mortality among

individuals with HIV infection. Additionally, they may play a role in discriminating modest detection of active tuberculosis in adults with HIV (Olsson *et al.*, 2022; Wang, Mehra, *et al.*, 2021).

A study investigating treatment experienced people living with HIV found that the A55 amino acid to be linked with adverse neurological outcomes (Dampier *et al.*, 2017). According to Dampier and colleagues, people living with HIV who possess the A55 amino acid variant tend to have higher global deficit scores (Dampier *et al.*, 2017). However, an opposing viewpoint has been proposed, suggesting positive clinical outcomes. A study associated the Vpr A55 amino acid variant with a lower plasma viral load in treatment naïve people living with (Kamori *et al.*, 2017). Our findings suggest that the A55 variant, by means of the Trp-Kyn metabolism may be associated with negative clinical outcomes by enhancing inflammation as QUIN correlated with suPAR in participants with this variant only. However, further studies are needed to investigate this variant in larger cohorts to validate the findings reported here.

Previous studies have found that the G41 variant is associated with positive clinical outcomes and is observed in treatment experienced long-term non-progressor people living with HIV and non-stroke control groups (McMullen *et al.*, 2021; Zhao *et al.*, 2002). Furthermore, McMullen and colleagues found that the S41 variant is associated with lower CD4 counts and ischemic stroke (McMullen *et al.*, 2021). Conversely, an earlier study by Dampier and colleagues found that the S41 variant in treatment-experienced participants was associated with positive clinical outcomes, as participants exhibited decreased neurocognitive deficits (Dampier *et al.*, 2017), aligning with our findings. Although limited studies have investigated Vpr amino acid variation at position 41, it is evident that there is no clear consensus regarding the influence of G41/S41 amino acids on clinical outcomes in people living with HIV. However, our findings suggest that G41 may be considered a high-risk amino acid in people living with HIV since the G41 group had higher QUIN levels which correlated with suPAR in this group only, which are believed to be characteristic of negative clinical outcomes (Braidy *et al.*, 2009; Smith *et al.*, 2001). It is possible that amino acid 41 is located within the second alpha-helix (38-50), a structurally significant region for key mechanisms such as the regulation of apoptosis, subcellular transport (Morellet *et al.*, 2009) and virion incorporation of Vpr (Singh *et al.*, 2000). Consequently, modifying this configuration and changing the amino acids at this position could potentially disrupt the arrangement of the Vpr alpha helices, thus affecting its functional potential. However, further investigation using modelling and structural techniques is needed to assess the potential functional implications of different amino acids at these positions. While QUIN is known to be neuroexcitatory and linked to

worsened neurological outcomes (Braidy *et al.*, 2009), it is important to note that higher suPAR levels have also been associated with decreased neurological performance in people living with HIV (De Almeida *et al.*, 2022; Gianella *et al.*, 2019). Considering the association of suPAR with neurological outcomes, it's worth considering that suPAR may play a role in tryptophan-kynurenine metabolism as an alternate mechanism in the development of HAND. suPAR is known to contribute to an elevated inflammatory environment, and a proinflammatory milieu can stimulate tryptophan-kynurenine metabolism, particularly through the activity of IDO-1. This suggests that suPAR may play a potential role in the breakdown of tryptophan, leading to the subsequent production of the downstream product QUIN (Werner-Felmayer *et al.*, 1989; Yasui *et al.*, 1986). These findings imply that the inflammatory-associated marker suPAR could further contribute to neurotoxicity due to its significant correlation with the neurotoxic metabolite QUIN. Additionally, suPAR has been shown to be a novel, independent predictive marker of myocardial infarction in people living with HIV (Rasmussen *et al.*, 2016) which may further contribute to the continuous increase in mortality rates in people living with HIV (Lawn *et al.*, 2007; Sidenius *et al.*, 2000).

While our investigation focused on peripheral metabolite levels, we believe that these findings could be relevant to the activity of these metabolites in the CNS. The activation of the Trp-Kyn metabolism in the brain due to cytokine stimulation may have parallels in peripheral tissues (Skorobogatov *et al.*, 2021). In theory, assessments of peripheral metabolites such as QUIN or KA could indirectly offer insights into the conditions within brain tissue (Skorobogatov *et al.*, 2021). Furthermore, in a recent systematic review conducted by our group comprising 22 studies investigating Vpr amino acid substitutions and clinical outcomes, 10 of the studies included treatment-experienced participants, 8 did not report treatment status, and only 4 studies reported on participants who were treatment-naïve (Asia *et al.*, 2022). Therefore, our study provides insight into a relatively scarce population of treatment-naïve individuals within the HIV population.

5. Limitations

This study possesses several noteworthy limitations that deserve attention. First, the sample size was restricted, potentially influencing the reported findings. Therefore, it is essential to interpret our findings considering this limitation. Due to the limited sample size, there is need for future research to confirm our results with larger study cohorts. While we consider this study as preliminary, it provides valuable insights into the link between Vpr sequence variations in a cohort of treatment-naïve individuals, offering a clearer understanding of the metabolic profile without

the potential confounding effects of combination cART. Second, we also examined peripheral metabolic and immune markers to gain insights into their potential role in central nervous system (CNS) function (Burdo *et al.*, 2013; Roth *et al.*, 2021). Furthermore, other peripheral markers, including neurofilament light (NFL), which were not investigated in this study, might have offered additional insights into the neuropathology of HIV-1. Previous studies have established a significant correlation between peripheral NFL levels and those within the CNS. Plasma NFL has been shown to detect both severe and subclinical neuronal injury in HIV (Gisslen *et al.*, 2007). Therefore, incorporating this marker would have been ideal for fully appreciating the translational value of our findings within the CNS. Lastly, studies have identified that plasma Trp-Kyn metabolites predict poor CD4+ T-cell count recovery and increased mortality among individuals with HIV infection. Seeing as CD4+ count and viral load, were unavailable for a substantial subset of participants in our study, we could not consider these variables as covariates. In a subset of participants with CD4+ count data, we observed a significant negative correlation between CD4+ count and QUIN levels. This suggests that CD4+ count may have influenced the reported levels of QUIN in this study. However, further investigation is required, and it is essential to acknowledge that this limitation may have impacted our findings.

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6. Conclusion

Here we report evidence that the Vpr amino acid sequence variations may have an influence on metabolic and inflammatory systems, particularly in the Trp-Kyn metabolism. The associations between these sequence variations and Kyn metabolite levels, alongside the significant correlations with immune markers, provide insights into potential mechanisms contributing to the HIV-1 pathogenesis and potentially neuropathogenesis. This research underscores the importance of understanding the genetic nuances of HIV and their broader systemic implications, emphasizing the need for continued exploration in this area to better understand the pathogenesis and neuropathogenesis in HIV-1 people living with HIV.

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8. Declarations

Ethics approval and consent to participate

All participants provided written informed consent, and their information was anonymized to the greatest extent possible. The study protocol received approval from the Health Research Ethics Committee of North-West University (NWU-HREC) in South Africa under the reference numbers NWU-00106-22-A, NWU-00106-22-A1, and this study NWU-00106-22-A1-01.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

Not applicable.

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Author contributions

LKA and MEW contributed to the conceptualization, investigation, methodology, formal analysis, data curation, and writing. MEW also handled project administration, funding acquisition and supervision. EJVV contributed to formal analysis, writing (both original draft and review & editing), and supervision. LK provided resources and contributed to writing (review & editing). ZL contributed to formal analysis, training, and writing (review and editing).

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ANNEXURE D:

**THE INFLUENCE OF HIV-1 VIRAL PROTEIN R (VPR) SEQUENCE VARIATION ON C-
REACTIVE PROTEIN (CRP) PERIPHERAL LEVELS. THE INTERNATIONAL CONGRESS OF
IMMUNOLOGY (IUIS), CAPE TOWN, SOUTH AFRICA.**

The influence of HIV-1 Viral Protein R (Vpr) sequence variation on C-reactive protein (CRP) peripheral levels. The International Congress of Immunology (IUIS), Cape Town, South Africa.

Background

Vpr is commonly known as an accessory protein in HIV-1 pathogenesis. Previous preclinical studies have focused on the structure and function of Vpr and have found that variation in its amino acid sequence is implicated in HIV-1 pathogenesis. However, limited research has investigated the influence of Vpr sequence variation on the underlying mechanism of inflammation in clinical samples. Of particular interest is C-reactive protein (CRP), a general inflammatory marker that has been widely associated with neurocognitive outcomes in people living with HIV (PLWH). Therefore, this study evaluated the influence of Vpr sequence variation on peripheral CRP levels.

Methods

This study used a cohort of 70 South African PLWH who were not using antiretroviral therapy at the time of sample collection. Vpr sequence variation was determined by Sanger sequencing. Mutations at certain positions of the Vpr protein have been linked to worse clinical outcomes in PLWH. Therefore, we categorized participants into high-neutral and low-neutral risk amino acid groups. These groups were compared to determine whether Vpr risk influenced peripheral CRP levels. Serum CRP was measured by particle-enhanced turbidimetric assay.

Results

We investigated Vpr sequence variation at amino acid positions 45, 55, 63, 77, and 85. The Vpr amino acids were rated high-risk (Tyr45, Ala55, Iso63, Arg77, Arg85) and low-risk (His45, Thr55, Thr63, Gln77, Pro85). When groups were stratified according to risk, there was no significant difference between peripheral CRP levels ($p = 0.403$).

Conclusion

This study investigated, for the first time, the relationship between Vpr specific sequence variation and CRP levels, a general indicator of inflammation. Ongoing work from our group is investigating whether individual amino acid variation may influence inflammation levels. The findings from this study may help explain inflammation for specific Vpr amino acid sequences during infection and potentially why we see differential neuropathogenesis between subtype-specific infections.