

HIV-1 infection and the central nervous system: Investigating markers of neural activation and measures of HIV-1 reservoir

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Declaration

The work I present in this thesis is my own unless stated otherwise or referenced.

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Abstract

Despite successful antiretroviral therapy (ART), mild cognitive impairments remain prevalent amongst people-living-with-HIV (PLWH). However, the underlying mechanisms driving this remain unclear. The persistence of an inaccessible HIV reservoir and low-level viral replication may be associated with immune activation and neuroinflammation, which can lead to neuronal injury and cognitive impairment. Furthermore, HIV-cure and ART-interruption strategies which disturb the HIV reservoir could result in detrimental central nervous system (CNS) sequelae.

This thesis examines the following hypotheses:

1. Lower biomarker concentrations of HIV reservoir size are associated with lower biomarker concentrations of neural activation
2. Higher biomarker concentrations of HIV viral transcription are associated with higher biomarker concentrations of neural activation

In cross-sectional analyses of virologically suppressed PLWH, associations were assessed between cerebrospinal fluid and plasma neurofilament light chain protein (NfL), and between surrogate measures of HIV reservoir size and neuroinflammation, measured *in vivo* using translocator protein (TSPO) binding on positron emission tomography neuroimaging (a marker of microglial activation). In longitudinal analyses, I assessed the impact of “shock-and-kill” using vorinostat + HIV-specific vaccination (a HIV-remission strategy) and ART interruption on measures of HIV reservoir size, HIV replication and plasma NfL, in individuals who initiated ART during primary HIV infection.

My results demonstrated that cerebrospinal fluid and plasma NFL correlated moderately but I was unable to confirm an association between HIV reservoir size and neuroinflammation, as measured by TSPO binding. Using plasma NfL, the absence of associations between HIV reservoir size and replication with neuronal injury following this “shock-and-kill” strategy or ART interruption supports the presumption of CNS safety of these approaches and will

inform the design of future HIV-cure studies. With the imminent introduction of novel therapeutics to control and cure HIV, continued CNS safety monitoring remains essential, and the implementation of CNS assessment using the aforementioned biomarkers was demonstrated to be feasible.

Abbreviations

AHI	Acute HIV infection
AIDS	Acquired immunodeficiency syndrome
ANI	Asymptomatic neurocognitive impairment
ARSAC	Administration of Radioactive Substances Advisory Committee
ATI	Analytical antiretroviral treatment interruption
BOLD	Blood-oxygen-level-dependent
BRC	Biomedical Research Centre
cART	Combination antiretroviral treatment
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 4
CDC	Centers for Disease Control and Prevention
CHI	Chronic HIV infection
CI	Confidence interval
CRF	Circulating recombinant form
CT	Computerised tomography
CTL	Cytotoxic T-lymphocyte
CXCR4	C-X-C chemokine receptor type 4
ddPCR	Droplet digital polymerase chain reaction

DNA	Deoxyribonucleic acid
DTI	Diffusion tensor imaging
DVR	Distribution volume ratio
ECDC	European Centre for Disease Prevention and Control
ECL	Electrochemiluminescence
ELISA	Enzyme-linked immunosorbent assay (ELISA)
fMRI	Functional magnetic resonance imaging
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorders
HDACi	Histone deacetylase inhibitor
HDS	HIV Dementia Scale
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
IHDS	International HIV Dementia Scale
IL	Interleukin
INSTI	Integrase strand-transfer inhibitor
IPDA	Intact proviral DNA assay
IQ	Intelligence quotient
IQR	Interquartile range
kDa	Kilodalton

LRA	Latency reversing agent
MND	Mild neurocognitive disorder
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MRS	Magnetic resonance spectroscopy
NfL	Neurofilament light chain protein
NHS	National Health Service
NIHR	National Institute of Health Research
NNRTI	Non-nucleoside reverse-transcriptase inhibitor
NRTI	Nucleos(t)ide reverse transcriptase inhibitor
PBMC	Peripheral blood mononuclear cell
PBR	Peripheral benzodiazepine receptor
PET	Positron emission tomography
PI	Protease inhibitor
PLWH	People living with HIV
POB	Plasma-over-blood
Q4PCR	Quadruplex qPCR
qPCR	Quantitative polymerase chain reaction
QVOA	Quantitative viral outgrowth assay
REC	Research Ethics Committee

RNA	Ribonucleic acid
ROI	Regions of interest
SD	Standard deviation
Simoa	Single-molecule array
SIV	Simian immunodeficiency virus
SUV	Standardized uptake value
TDF	Tenofovir disoproxil fumarate
TSPO	Translocator protein
V_T	Total volume of distribution

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Chapter 1

Introduction

Chapter 1: Introduction

1.1 Hypotheses of thesis

Mild forms of cognitive impairment remain prevalent amongst people living with HIV (PLWH) despite the widespread use of combination antiretroviral treatment (cART). It has been suggested that the interactions between the HIV reservoir and low-level HIV viral transcription with central nervous system immune activation and neuroinflammation could result in neuronal injury and neurological sequelae, such as cognitive impairment.

This thesis will test the following two hypotheses:

- 1. Lower biomarker concentrations of HIV reservoir size are associated with lower biomarker concentrations of neural activation**
- 2. Higher biomarker concentrations of HIV viral transcription are associated with higher biomarker concentrations of neural activation**

1.2 An introduction to HIV

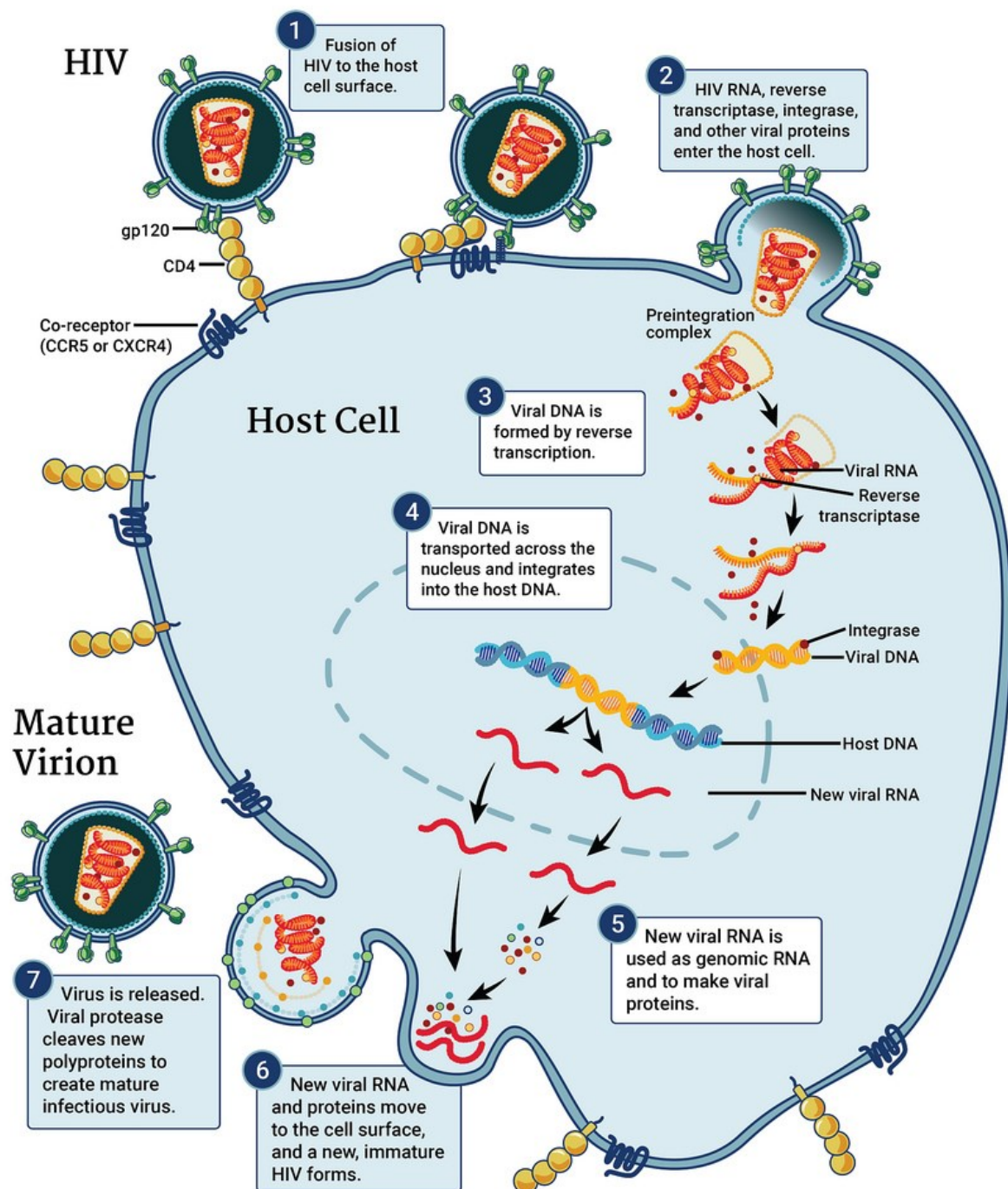
In 1983, a T-lymphotropic retrovirus, later termed human immunodeficiency virus (HIV), was detected in a lymph node from a patient with lymphadenopathy[1], and soon after was confirmed as the aetiologic agent of acquired immune deficiency syndrome (AIDS). Without effective antiretroviral treatment, the long-term prognosis for PLWH is poor, with profound immunodeficiency emerging after several years. Individuals develop characteristic infectious and/or oncological complications known as AIDS-defining illnesses and the typical person progresses to death over a period of about 10 years[2].

However, since the mid-1990s enormous advances have been made in HIV therapeutics and clinical care[2], with dramatic improvements in life expectancy for PLWH on effective cART[3]. Nonetheless, a cure for HIV has not yet been found, and the number of PLWH today is the highest since the start of the epidemic.

1.3 HIV biology

HIV is a retrovirus and a member of the *Lentivirus* genus. HIV utilises the envelope protein gp120 to bind to host cellular receptors (cluster of differentiation 4 (CD4) and a co-receptor (primarily CC-chemokine receptor 5 (CCR5) or CXCR-chemokine receptor 4 (CXCR4), then fuses with the host target cell (predominantly T-lymphocytes, monocytes and macrophages) (Figure 1). Once HIV has entered the host cell, the viral genome and reverse transcriptase enzyme are unpacked, and single-strand viral ribonucleic acid (RNA) is reverse-transcribed into a complementary deoxyribonucleic acid (DNA) provirus (Figure 1). The transcribed DNA is then transported to the nucleus, randomly integrates into the host chromosomal DNA using an integrase enzyme (Figure 1) and can persist in this state for the lifetime of the host cell. By hijacking the host's cellular machinery and enzymes, HIV DNA is transcribed to viral messenger RNA (mRNA). These mRNAs are transported to the cytoplasm where translation occurs to produce proteins which are then cleaved, and mature virions are released (Figure 1).

Figure 1: The HIV replication cycle



Legend: Diagram illustrating the HIV replication cycle. Initially, HIV fuses with the surface of the host cell. A capsid containing the virus' genome and proteins then enters the cell. The shell of the capsid disintegrates, and the HIV enzyme called reverse transcriptase transcribes the viral RNA into viral DNA. The viral DNA is transported into the nucleus, where the HIV enzyme integrase integrates the HIV DNA into the host's genome. The host's normal transcription machinery transcribes HIV DNA into multiple copies of new HIV RNA. Some of this RNA becomes the genome of a new virus, while the cell uses other copies of the RNA to make new HIV proteins. The new viral RNA and HIV proteins move to the surface of the cell, where a new, immature HIV virion forms. Finally, the virus is released from the cell, and the HIV enzyme called protease cleaves newly synthesized polyproteins to create a mature infectious virus. Credit: National Institute of Allergy and Infectious Diseases[4]

HIV is categorised into two main groups, HIV-1 and HIV-2. HIV-1, the main worldwide aetiological agent of AIDS, causes more rapid disease progression in the absence of antiretroviral treatment, whereas HIV-2, which is more commonly seen in western Africa, induces a more indolent course of disease and is less transmissible. My thesis focuses on HIV-1 and any future reference to HIV in my thesis relates to HIV-1, unless stated otherwise.

There are four sub-groups of HIV-1: M, N, O and P. Groups N, O and P are mainly confined to Western Africa, whereas Group M accounts for 99% of the worldwide HIV pandemic and comprises nine subtypes: A – D, F – H, J and K[5]. Subtype B predominates in western Europe, the Americas and Australia, while subtype C is the dominant form seen in Africa and India. Hybrids of these subtypes, known as circulating recombinant forms (CRFs), are becoming increasingly common. The enormous genetic variation seen within HIV is a result of the marked error-prone nature of reverse transcription, which causes a high mutation rate and the continual production of viral variants. The different HIV subtypes and CRFs have evolved in response to varying host and viral factors, and some studies suggest that the different subtypes have different impacts on pathogenicity[6] and disease progression[7]. This extensive genetic diversity in HIV is the reason why developing a preventative vaccine has proven to be such a challenge.

1.4 HIV epidemiology

As of 2019, 38 million people globally were living with HIV, with an estimated 1.7 million people becoming newly infected with HIV in 2019[8]. To date, HIV has infected more than 75 million people worldwide and approximately 33 million people have died from AIDS-related illnesses since the beginning of the epidemic.

The vast majority of PLWH are in low and middle-income countries, with nearly 70% of people with HIV living in sub-Saharan Africa[9]. Globally, the HIV disease burden disproportionately affects women, especially in sub-Saharan Africa where over 57% of PLWH are women. Other key populations frequently affected by HIV disease include men who have sex with men, injecting drug users, sex workers, transgender people and incarcerated people in prisons and other closed settings.

Globally, sexual transmission of HIV following exposure to infectious virus in semen or mucosal surfaces constitutes the most frequent route of HIV transmission. Other routes of HIV transmission include vertical transmission from mother to child, sharing injection drug equipment and blood or blood products transfusion.

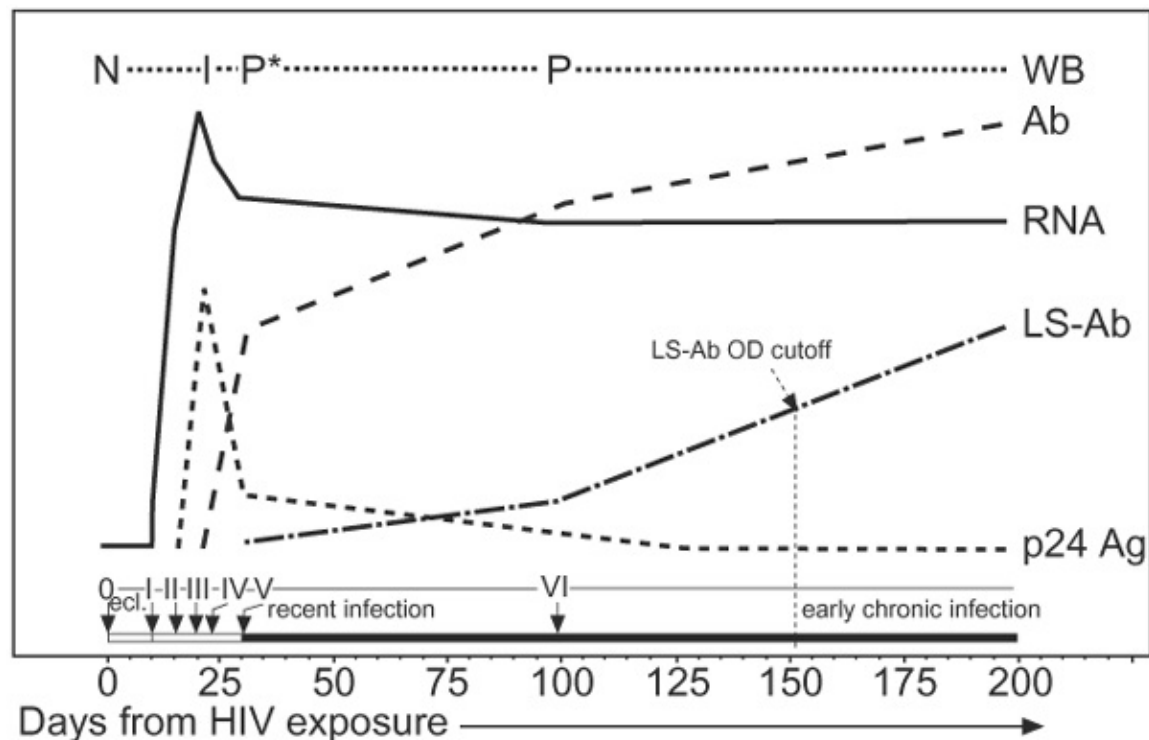
1.5 Natural history of HIV infection

1.5.1 Primary HIV infection

The natural history of untreated HIV infection involves a primary phase lasting up to six months post-infection, and includes the acute phase of HIV infection, which refers to the period immediately following HIV acquisition and prior to the development of HIV antibodies (seroconversion) (Figure 2)[10]. Figure 2 demonstrates the evolution of HIV blood markers and the Fiebig stages during acute HIV infection.

HIV disseminates throughout the body rapidly during acute HIV infection, due to the lack of any pre-existing adaptive immune defences, leading to a surge in viraemia (often peaking at $10^6 - 10^7$ HIV RNA copies/mL) and a significant fall in CD4+ T-cell counts (Figure 3). This phase can manifest in some individuals as an acute HIV syndrome, with (often transient) symptoms that include fever, generalised lymphadenopathy, a non-specific rash, myalgia and/or malaise[11]. In the central nervous system, acute HIV infection is frequently associated with mild central nervous system symptoms including headache and cognitive impairment, that generally abate upon the initiation of cART[12,13].

Figure 2: Schematic display of the progression of HIV markers and Fiebig stages of acute HIV infection



Legend: Schematic display of the progression of HIV markers from time of exposure (day 0) through the first 200 days of infection. As each of the markers appears in the bloodstream, the infection is assigned a new Fiebig stage from 0 (eclipse period, characterized by undetectable viral markers in blood samples; reported to last on average up to 11 days from viral exposure), through Fiebig stage I (definitive HIV RNA viremia), Fiebig stage II (p24 antigenemia), Fiebig stage III (HIV EIA antibody reactive), Fiebig stage IV (Western blot indeterminate, 'I'), Fiebig stage V (Western blot positive without p31 band, 'P') and Fiebig stage VI (Western blot positive, 'P' with p31 band). Image reproduced with permission of the rights holder, Wolters Kluwer Health, Inc[14]*

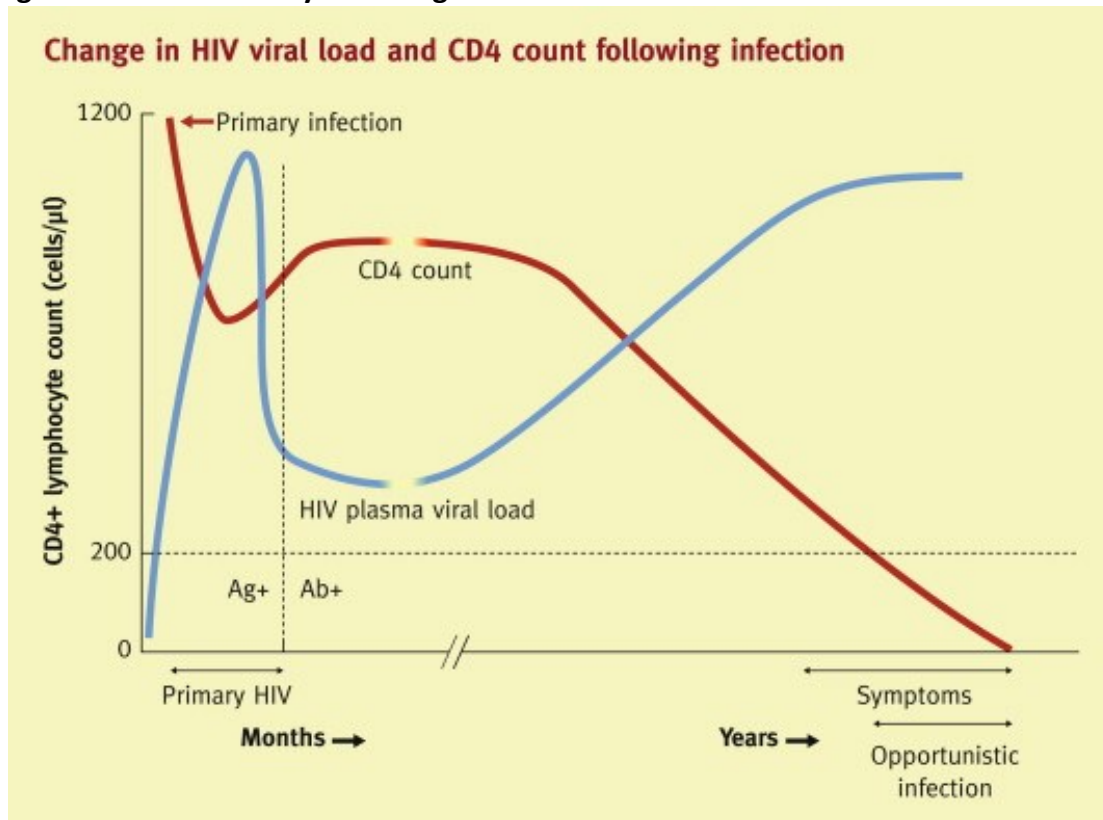
Abbreviations: WB = Western blot; Ab = HIV antibody; RNA = HIV RNA; LS-Ab = HIV antibody determined by less sensitive enzyme immunoassay testing strategy; p24 Ag = HIV p24 antigen

1.5.2 Chronic HIV infection

After several months, the immune system achieves some form of control and the level of HIV replication drops by about $2 \log_{10}$ HIV RNA copies/mL to a viral set-point and CD4⁺ T-cell count somewhat recovers (Figure 3). The individual then enters a clinically latent phase known as chronic HIV infection, where the individual experiences few clinical symptoms but has ongoing systemic HIV replication. Persistent HIV viral replication is associated with a chronic state of immune activation and inflammation, leading to the increased production of pro-inflammatory cytokines[15] and thymus dysfunction[16], which results in a steady decline in CD4⁺ T-cell counts and immunodeficiency. Without cART, CD4⁺ T-cell count is predicted to fall by 50 – 80 cells/ μ L per year (Figure 3)[17]. Once CD4⁺ T-cell counts fall to

very low levels (<200 cells/ μL), most people living with advanced untreated HIV disease will develop acquired immunodeficiency syndromes[18] associated with infectious and/or oncological complications, and eventually progress to death[18,19].

Figure 3: Natural history following infection



Legend: Diagram illustrating the natural change in HIV viral load and CD4+ T-cell count following infection, in the absence of antiretroviral treatment. Image reproduced with permission of the rights holder, Elsevier[20]

1.6 Pathogenesis of HIV in the central nervous system

1.6.1 Pathophysiology

HIV-1 is a neurotropic virus with over half of PLWH developing neurologic manifestations at some point during their disease course[21] and many display evidence of neurologic disease in autopsy studies[22,23].

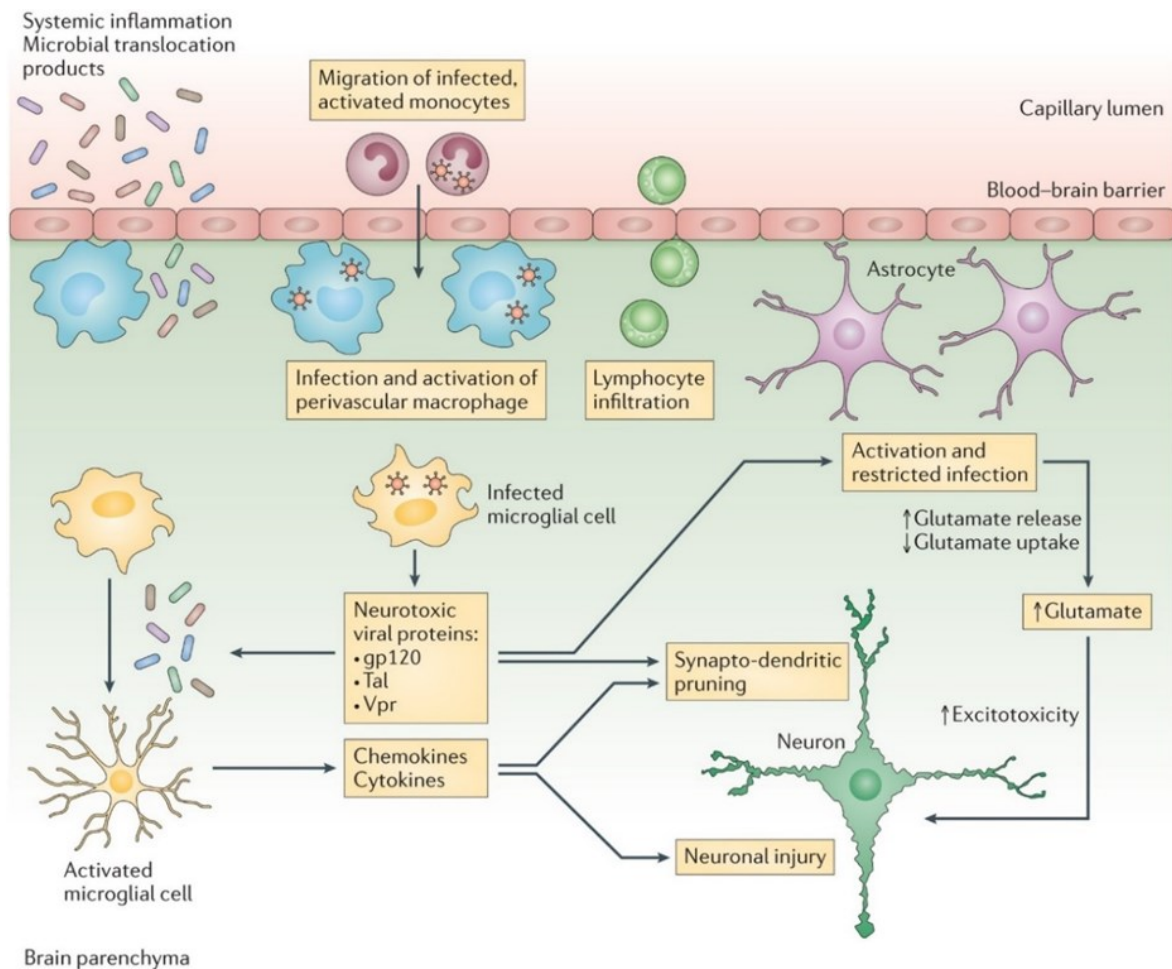
HIV enters the central nervous system early on during acute HIV infection[24], when systemic infection is associated with marked viraemia and an early reduction of blood CD4+ T-cells and elevation of blood CD8+ T-cells[25]. Several different entry mechanisms have

been proposed for central nervous system infection including the cell-mediated “Trojan horse” hypothesis[26]. This is where HIV crosses the blood-brain barrier via infected CD4+ T-cells or monocytes during routine surveillance, then goes on to differentiate into macrophages or infect resident central nervous system immune cells, namely perivascular macrophages, microglial cells and astrocytes[27,28] (Figure 4). Alternatively, there is the free virus transfer hypothesis whereby free HIV virions in the blood compartment enter via the blood-brain barrier disrupted by uncontrolled HIV infection[29], which then go on to infect resident brain immune cells. Once infected, central nervous system perivascular macrophages, microglial cells and monocytes release inflammatory cytokines, chemokines and neurotoxins that can trigger astrocyte activation, perpetuating neuroinflammation and neuronal injury[30] (Figure 4).

Following viral entry into the central nervous system during primary HIV infection, central nervous system immune activation and inflammation can be detected soon after (evidence for this is detailed below)[12,31,32], which appears to trigger a further surge of immune activation within the central nervous system. This activates resident central nervous system immune cells like microglial cells and astrocytes, which disrupts blood-brain barrier epithelium and further facilitates monocyte recruitment into the central nervous system [33,34], thus, perpetuating infection in the central nervous system and neuronal injury. While generating an immune response towards an infection may be beneficial, persistent neuroinflammation can lead to synaptic dysfunction and neuronal injury, which can culminate in neurological sequelae such as cognitive dysfunction[28].

Neurons and oligodendrocytes are not directly infected by HIV; however, they can be affected by the pro-inflammatory cytokines and neurotoxic HIV viral proteins (such as gp120, Tat and Vpr) released by neighbouring central nervous system immune cells infected with HIV, which can lead to neuronal injury and excitotoxicity (Figure 4).

Figure 4: Neuropathogenic mechanisms for HIV viral entry into the central nervous system and mechanisms that contribute to neuronal injury



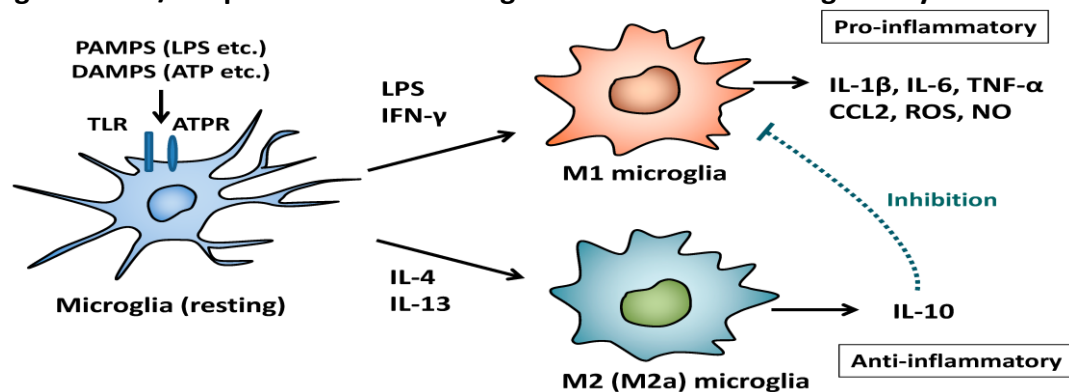
Legend: Diagram to represent the potential mechanisms of HIV entry and central nervous system neuronal injury. Image reproduced with permission of the rights holder, Elsevier[35].

Cerebrospinal fluid inflammatory metabolites measured using magnetic resonance spectroscopy (MRS) and soluble and cellular markers of immune activation increase during the first few months to years of untreated HIV disease[36,37], likely setting the scene for neurological damage and persistent infection in the later stages of HIV disease. Magnetic resonance imaging (MRI) studies have demonstrated that structural central nervous system changes start during primary HIV infection; a study of individuals within 100 days of estimated HIV acquisition demonstrated decreased parenchymal volume on brain MRI and reduced white matter integrity on diffusion tensor imaging (DTI)[38].

1.6.2 The role of microglia

Microglia cells are the main resident innate immune cells in the brain, and can serve as brain macrophages[39]. They originate from erythromyeloid progenitors in the yolk sac during embryogenesis and colonise the developing central nervous system[40]. Microglial cells play a major role in immune surveillance, synaptic pruning and the elimination of microbes, dead cells and protein aggregates such as amyloid plaques[41–43]. Both resting and activated microglial cells display ramifications which closely connect with neurones, astrocytes and blood vessels. Resting cells are constantly surveying the environment and can become activated to varying degrees, due to changes in their microenvironment. They are motile and can quickly travel to the location of brain injury[43]. When activated, microglial cells may evolve into a pro-inflammatory M1 phenotype, characterised by the production of pro-inflammatory cytokines and reactive oxygen species, or may evolve into a state of alternative activation, in which microglia take on an anti-inflammatory M2 phenotype, involved in wound repair and debris clearance [44](Figure 5). It is now becoming increasingly apparent that the binary definitions of activated microglia may be an oversimplification and multiple activation phenotypes of the microglia may co-exist, depending on the surrounding conditions[45]. Microglial cells are crucial for healthy brain function and microglial dysfunction can lead to excessive cytokine, chemokine and neurotoxin secretion, which can contribute to neuroinflammation, with neurologic consequences[46].

Figure 5: M1/M2 polarisation of microglia and their immunoregulatory functions



Legend: In the presence of lipopolysaccharides LPS and IFN-γ, microglial cells polarize to M1 phenotype and produce pro-inflammatory cytokines/mediators. In contrast, IL-4 and IL-13 induce alternative activation of microglia to M2 phenotype which down-regulates M1 functions by anti-inflammatory cytokine, IL-10. Image reproduced with permission of the rights holder, MDPI[47]. Abbreviations: LPS = lipopolysaccharides, IFN-γ = interferon-gamma, IL = interleukin. Image reproduced with permission of the rights holder, MDPI[47]

Evidence suggests that brain microglial cells and macrophages are highly susceptible to HIV infection[48]. Due to the low turnover of microglial cells, once infected with HIV, they may continue to harbour latent integrated virus for prolonged periods of time[49], before undergoing cell division and producing new infectious virions. In contrast, macrophages within the brain such as perivascular macrophages, are bone-marrow derived, do not undergo cell division in the central nervous system and are often replenished from peripheral monocytes crossing the blood-brain barrier[48]. The latent integration of HIV into host cells will be described in more detail later in this chapter.

1.6.3 The role of astrocytes

Astrocytes comprise approximately 30% of cells in the human central nervous system and play an important role in maintaining central nervous system homeostasis and function, and synaptic pruning[50]. Like microglia, astrocytes can also produce pro- and anti-inflammatory responses depending on the microenvironment and mode of injury[51]. Astrocytosis, the rapid activation of astrocytes can lead to an increase in the size and number of astrocytes and the secretion of cytokines, cell surface molecules, growth factors and neurotoxins[52]. Along with microglia, astrocyte dysfunction is thought to cause neuroinflammation[53], which can lead to neurological sequelae, such as cognitive dysfunction.

Astrocytes can be infected with HIV, but the frequency of infection is estimated to be <5% of total astrocytes[54,55]. While astrocytes are unable to support viral replication, data suggests that astrocytes can transfer HIV via cell-to-cell contact, which contributes to ongoing HIV infection, immune activation and HIV persistence in the brain[56,57].

1.7 HIV reservoir

1.7.1 Latency

To date, the main HIV treatment approach for HIV is cART with the aim being to achieve plasma HIV RNA load <50 copies/mL. Antiretroviral treatment is described in further detail later on in this chapter. cART alone is unable to cure HIV disease[58,59], and upon cART cessation, plasma viral load rebounds in the majority of PLWH within eight weeks[60–63]. The source of rebounding virus is cells latently infected with HIV, termed the HIV

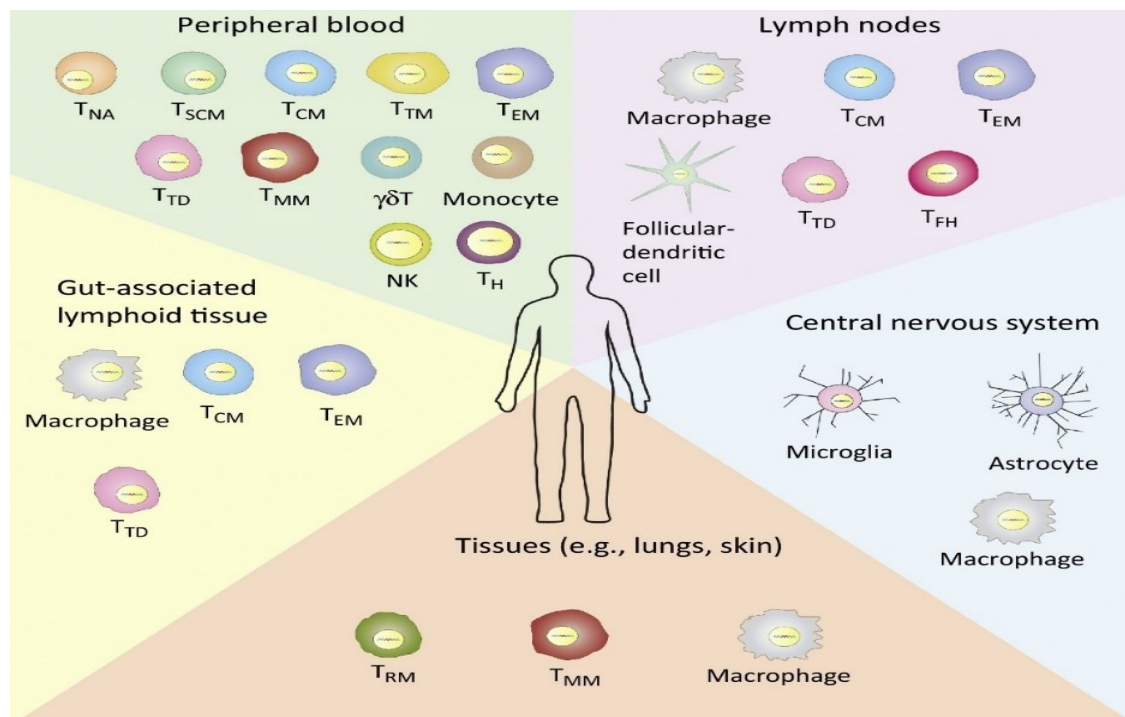
reservoir[58,64–66], formed when HIV genetic material integrates into the genome of the infected cell[65,67]. Reservoirs are established soon after HIV acquisition[68], remain stable despite cART[65,69,70] and persist for the lifetime of the infected cell. The host cells do not routinely express viral antigens and thus evade immune system surveillance and clearance[71]. Several mechanisms maintain viral latency including complex transcriptional pathways that may prevent reactivation[72], the upregulation of anti-apoptotic genes[73] and restrictions to post-initiation HIV RNA transcriptional pathways[74].

1.7.2 HIV reservoir locations

The main HIV latent reservoir is thought to be located in infected memory CD4⁺ T-cells in circulation and lymphoid tissue[65,75], whereas naïve CD4⁺ T-cells are infected less frequently[76]. When not infected with HIV, most CD4⁺ T-cells exist in a resting state, such as central memory or naïve T-cells. However, once infected these resting cells transform into more short-lived activated effector T-cells and HIV replicates preferentially in activated T-cells[77]. This usually kills the T-cells, but occasionally some survive and revert back to a resting state, resulting in long-lived latently infected memory T-cells.

Additional cellular reservoirs for HIV include monocytes, macrophages and microglial cells, with reservoir anatomic compartments proposed to include the central nervous system, lungs, skin, adipose tissue and genitourinary tract (Figure 6)[78].

Figure 6: Latent HIV reservoir sites



Legend: In addition to the main HIV reservoirs composed of CD4⁺ memory T-cells in blood, lymph nodes and gut-associated lymphoid tissue, other anatomical focuses of latent HIV infection include the central nervous system, lungs and skin. Image reproduced with permission of the rights holder, Elsevier[78].

1.7.3 HIV reservoir in the central nervous system

Up until now, much of the research efforts into examining the HIV reservoir has focused on CD4⁺ T-cells. The question as to whether the central nervous system is a true HIV reservoir and its importance in HIV-remission strategies remains a hotly debated topic. Blankson *et al* have identified five key requirements that cells must have to be considered a biologically significant HIV reservoir[58]:

The cell must:

1. be long-lived
2. be significant in number
3. contain integrated HIV DNA provirus, that is capable of replication
4. have the potential to be activated to produce new viral particles
5. have mechanisms to suppress the HIV replication and induce latent infection

A literature review performed by Gray *et al* in 2014 established that the central nervous system fulfilled most of these criteria to be considered a HIV reservoir, as described in greater detail below[79].

Brain resident immune cells which include microglial cells, macrophages and astrocytes express CD4 and CCR5 co-receptors that enable them to be infected by HIV[80,81] and are infected in the early stages of disease[82]. Astrocytes and microglia are long-lived[79] and when quiescent, are not killed as a consequence of HIV infection or immune monitoring, hence can become viral reservoirs of HIV. Studies in individuals on virologically suppressive cART have identified HIV DNA in brain tissue evaluated at autopsy [22,23,83] and in cerebrospinal fluid[84,85]. In neurocognitively normal individuals, HIV DNA can be found in 4% of astrocytes and 20% of macrophages[86]. Furthermore, apparent latent HIV infection in microglial cells, astrocytes and macrophages in the brain have been demonstrated in numerous neuropathological and *in vitro* studies[83,86–92]. However, this remains a contentious issue as some have argued that the demonstration of such integrated virus merely reflects the phagocytic capacities of these cells. While animal models have demonstrated the capacity of latently infected macrophages from the brain to produce replication competent virus[93], similar studies in humans cannot be performed due to ethical and technical issues.

1.7.4 HIV reservoir dynamics

In the absence of antiretroviral treatment, there is ongoing systemic viral replication and evolution, which continually replenishes integrated HIV DNA genomes in the HIV reservoirs. HIV reservoir size is smallest at the beginning of HIV infection during primary HIV infection, but as the course of disease progresses, HIV reservoir size increases in the absence of cART until it reaches a set-point; the level of which is likely to be an interaction between viral and host factors. Following the introduction of virologically suppressive cART, plasma HIV RNA load decreases to undetectable levels (<50 HIV RNA copies/mL) and in turn, there is evidence to suggest that HIV DNA levels rapidly decay by >85% in the first 12 months, followed by a slower decay (23% decrease annually)[94,95]. Several independent studies have demonstrated that the viral sequence of the majority of the integrated HIV genome in

reservoirs of PLWH on virologically suppressive cART was almost identical to the circulating HIV RNA sequences seen just before cART initiation[96,97]. This implies that in the absence of cART, HIV DNA in cells latently infected with HIV continually decays and reseeds, but this stops with the initiation of cART. Following cART interruption, HIV DNA transcription and translation from the HIV reservoir initiates plasma HIV RNA rebound, but whether or not short-term cART interruption is associated with further HIV reservoir reseeding is debatable. In a randomised controlled trial comparing continuing cART versus short-term (3 months) cART interruption in infants who had initiated early cART for at least 24 months, HIV DNA levels were similar at 15 months post-cART re-initiation compared with pre-cART interruption levels, implying that either significant HIV DNA reseeding did not take place or that any reseeding that had taken place had decayed upon cART re-initiation[98].

Data from Siliciano *et al* estimated that in virologically suppressed PLWH who initiated cART during chronic HIV infection, the mean half-life of the latent reservoir in resting CD4+ T-cells was around 44 months, and an estimated 74 years of cART would be required to eliminate the latent reservoir[65]. While data from one study suggests that early cART initiation soon after HIV acquisition can significantly decrease the HIV reservoir size[99], other data demonstrates that cART initiation during Fiebig I stage (within 14 days of HIV acquisition) does not prevent reservoir seeding and upon cART cessation, viral load rebounded in all these individuals[100]. There is some evidence to suggest that intact HIV DNA may decay more quickly compared with defective HIV DNA[101,102], whereas other studies have shown the opposite[103]. Data also suggests that the ability to induce HIV DNA wanes over time as the reservoir gradually becomes relatively more latent and resistant to reactivation. If this is true, this may mean that virus populations in PLWH on long-term cART are less likely to initiate virus replication upon cART interruption.

1.7.5 Quantifying the HIV reservoir

To date, many methods have been proposed to quantify the latent HIV reservoir, but the optimum method of measuring replication-competent virus in the body has not yet been established[104]. Table 1 describes the characteristics of the main assays currently used to quantify the latent HIV reservoir[105].

Table 1: HIV reservoir assays

Assay	Assay characteristics	Sample size	Assay input	Primers ^a	Unit of comparison	Limit of detection ^b (LOD)	Dynamic range ^c (logs)	Normalization method	Explanation for negative values	Ref.
Viral outgrowth	95% CI for individual determinations = ± 0.7 logs based on Poisson statistics and 2 replicates/cell number; coefficient of variation = 0.95^d	120–180 ml blood	resting CD4 ⁺ T cells	none	infectious units per 10 ⁶ (IUPM) resting CD4 ⁺ T cells	0.02	2.7	none	frequency < LOD	5,6,9, 10,33
Droplet digital PCR for HIV-1 DNA	coefficient of variation depends on template number (Strain et al., submitted); accuracy superior to kinetic PCR (5 fold at low copy numbers)	variable, 5 × 10 ⁶ cells in this study	resting CD4 ⁺ T cells or PBMC	primers: 2539→2562, 2659→2634. probe: 2589→2604	copies per 10 ⁶ cells	2	3.2	normalized using RNase P to quantify host genomic DNA	frequency < LOD; primer mismatch	53
<i>Alu</i> PCR for integrated HIV-1 DNA	detects individual integrated HIV-1 genomes with standard curve to correct for sites too far from an <i>Alu</i> sequence to be detected; coefficient of variation = 0.20	variable, 5 × 10 ⁶ cells in this study	resting CD4 ⁺ T cells or PBMC	primers, outer: <i>Alu</i> primer, 800→782; inner: 525→542, 619-599. probe: 559→596	copies per 10 ⁶ cells	3	3.0	normalized by [DNA] assuming 1 µg = 150,000 cells	all assays above LOD in this study	39, 40, 55, 80
qPCR for HIV-1 DNA rectal biopsies	coefficient of variation for 10 copy standard = 0.18	up to 30 3 mm biopsies	cells from biopsy	primers: 522→543, 640→626. probe: 581→559.	HIV-1 genomes per 10 ⁶ CD4 ⁺ T cells	0.05	5.3	normalized by [DNA] and %CD4 ⁺ T cells (assuming 1 µg = 160,000 cells and all virus in CD4 ⁺ T cells)	all assays above LOD in this study	44
Droplet digital PCR for 2-LTR circles	coefficient of variation depends on template number (Strain et al., submitted); accuracy superior to kinetic PCR (>10 fold at low copy numbers)	variable, 5 × 10 ⁶ cells in this study	resting CD4 ⁺ T cells or PBMC	primers: 9588→9607, 48→28. probe: 556→530	copies per 10 ⁶ cells	2	1.1	normalized using RNase P to quantify host genomic DNA	frequency < LOD; primer mismatch	53
Single copy assay for residual viremia	sensitivity superior to approved clinical assay; internal control for virus recovery	8 ml plasma	plasma	primers: 1299→1323	copies/ml of plasma	0.2	1.1	none	viral RNA < LOD; primer mismatch	46,47, 67

^aHXB2 coordinates.

^bBased in standard sample size. Except for residual viremia, LOD is expressed as infectious units or copies per 10⁶ cells. For residual viremia, the LOD is 0.2 copies/ml of plasma.

^cDynamic range is reported here as the difference in log units between the highest value measured in these study patients and the limit of detection of the relevant assay.

^dBased on repeat measurements in the same patient as reported in reference 10 and assuming no decay in the reservoir.

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Legend: Characteristics of assays currently used to measure HIV reservoir size. Table reproduced with permission of the rights holder, Plos[104]

However, it remains uncertain what is being measured by the different assays and the clinical relevance of these assay measurements. Conventionally the quantitative viral outgrowth assay (QVOA) has long been considered the gold standard for reservoir quantification[69]. However, QVOA is expensive, time consuming, labour intensive and requires high cellular input. Furthermore, it is unclear whether the repeat rounds of activation required to induce additional viral outgrowth accurately reflects the processes occurring in vivo.

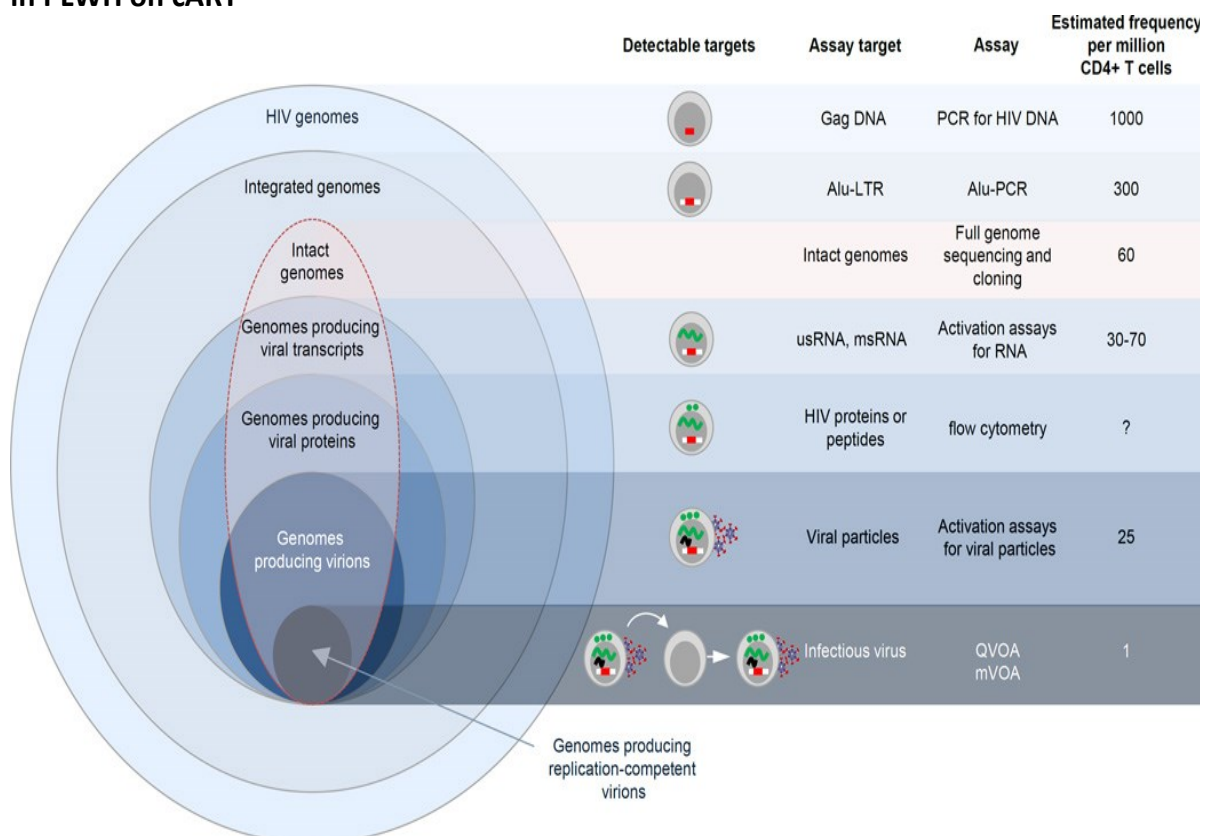
On the other hand, HIV nucleic acid measurements based on polymerase chain reaction assays are relatively cheap and allows a high throughput of sample. HIV DNA measurements in the form of total HIV DNA, integrated HIV DNA and cell-associated HIV DNA can be measured using quantitative polymerase chain reaction (qPCR) and droplet digital polymerase chain reaction (ddPCR). Total HIV DNA and integrated HIV DNA tend to overestimate the HIV reservoir size because they include the measurement of defective non-replication competent HIV DNA, which make up >95% of the quantifiable HIV DNA (Figure 7).

Recently, several new assays have been developed to distinguish intact from defective proviruses but are still undergoing validation. Intact proviral DNA assay (IPDA) is an assay measuring intact HIV DNA by ddPCR with primers and probes able to differentiate intact from defective proviruses. Quadruplex qPCR (Q4PCR) is an assay measuring intact HIV DNA through a limiting-dilution four-probe qPCR, followed by sequence verification of reactions positive for two or more probes[106]. Both these strategies have the added benefits of requiring a relatively small number of cells to estimate the size of genetically intact HIV DNA levels, in a less labour-intensive and low-cost manner compared to QVOA.

Furthermore, it remains unclear whether blood reservoir measurements can be used as surrogate measures of HIV reservoir size within the different tissues, which is where the majority of replication-competent HIV DNA resides[107]. Novel methods have been developed that can quantify virus within tissues directly (e.g. biopsies or imaging using labelled anti-HIV antibodies[108]) or indirectly (e.g. assessing host responses to the virus by quantifying antibody titres[109]).

It is estimated that 0.01 – 1% of circulating CD4+ T-cells contain an integrated genome, of which <5 – 10% are intact and only a small percentage of intact genomes are inducible and can produce infectious virions[110] (Figure 7).

Figure 7: Diagram to demonstrate the assay target and estimated frequency of HIV DNA per million CD4+ T-cells for each of the assays used to quantify the latent HIV reservoir in PLWH on cART



Legend: The frequency of cells that produce replication-competent virus is only a proportion of the cells that are infected with intact (surrounded by the red line) and defective genomes (total pool of infected cells).

Abbreviations: PLWH = people living with HIV, cART = combination antiretroviral treatment, US = unspliced, MS = multiply spliced, QVOA = quantitative viral outgrowth assay, MVOA = murine viral outgrowth assay. Image reproduced with permission of the rights holder, Springer Nature[111].

1.7.6 Reducing HIV reservoir size with early antiretroviral treatment initiation

Initiating cART during primary HIV infection (within 6 months of acquiring HIV infection and before the establishment of the HIV reservoir maximal set-point size) is thought to be a way of reducing long-term HIV reservoir size[99,112,113] and improving immune system recovery[112,114].

The RV254/SEARCH 010 cohort study conducted in Bangkok, Thailand, has extensively examined the effect of very early cART initiation during acute HIV infection. To date, over 600 individuals have enrolled into the study having been diagnosed with antibody-negative acute HIV-1 infection at a median of 19 days after presumed infection[115] and >95% of individuals opted to commence cART within 2 days of diagnosis[116]. Quantitative measurements of HIV DNA in peripheral blood mononuclear cells (PBMCs) were consistently lower but still detectable in these individuals compared to individuals who initiated cART during chronic HIV infection[117]. Furthermore, elevated plasma biomarkers of inflammation were demonstrated in this group of individuals despite the very early initiation of suppressive cART[118], which suggests disturbances associated with HIV infection.

In some individuals, early cART initiation may confer the ability for a minority of people to maintain virological suppression in the absence of cART. A subset of individuals who initiated cART within weeks to months of acquiring HIV infection and remained on cART for several years before interrupting cART for various reasons, were found to be able to effectively control HIV replication despite remaining off cART[119]. The mechanisms underlying this remain unclear but suggest that an interplay between the virus and host immune system can, in some situations, lean towards cART-free remission.

Overall, data indicates that PLWH should initiate cART as early as possible after HIV diagnosis[120]. This is discussed in greater detail in the 'Antiretroviral treatment' section below. Early cART initiation may substantially limit the laying down of HIV reservoirs and reduce quantifiable HIV reservoir measures, but evidence has shown that early cART alone is insufficient to eliminate HIV reservoirs altogether[119] and additional methods are required to substantially deplete HIV reservoirs in PLWH.

1.8 AIDS and AIDS-defining conditions

According to the US Centers for Disease Control and Prevention (CDC) definition, a person is considered to have AIDS if they have laboratory evidence of HIV infection and have either:

- CD4+ T-cell count below 200 cells/ μ L, or

- CD4+ T-cell percentage of total lymphocytes less than 14%, or
- One or more of the diseases that have been listed as an AIDS-defining condition as per the CDC guidelines[121]

The comparable case definition for AIDS according to the European Centre for Disease Prevention and Control (ECDC) relies on having laboratory evidence of HIV infection and any of the clinical conditions as listed in the ECDC AIDS case definition[122].

The CDC and ECDC lists of diseases associated with AIDS include opportunistic infections such as *Pneumocystis jirovecii* pneumonia and toxoplasmosis of the brain, and oncological complications such as invasive cervical carcinoma and primary brain lymphoma[121].

1.8.1 HIV-associated dementia

In the central nervous system, apart from the opportunistic infections and oncological complications of AIDS, a spectrum of HIV-associated cognitive impairments has been observed since the early days of the HIV epidemic. The most severe form, termed HIV-associated dementia, was designated an AIDS-related condition and typically occurs in advanced HIV infection when CD4+ T-cell counts are below 200 cells/ μ L.

1.8.1.1 *Clinical manifestations of HIV-associated dementia*

In the pre-cART era, HIV-associated dementia occurred in 15 - 20% of individuals living with untreated HIV [35,123] with an annual incidence rate of 7%. Characteristically, HIV-associated dementia presents with severe cognitive impairment (reduced mental concentration, depression and memory problems), behavioural changes (personality changes, apathy and social withdrawal) and motor symptoms (leg weakness, slowness of hand movement and gait)[124]. In its more severe form, HIV-associated dementia can cause a vegetative state, paraplegia and death[125].

1.8.1.2 *Neuropathology of HIV-associated dementia*

Pathological correlates of HIV-associated dementia are characterised by the formation of multinucleated giant cells in the brain white and grey matter and perivascular spaces[126,127]. Microglial activation emphasised by the presence of microglial nodule formation and astrogliosis are frequently seen around small blood vessels in the central white matter areas[128]. White matter pathology in HIV-associated dementia includes myelin pallor, axonal damage, astrogliosis, macrophage infiltration and, less frequently, extensive white matter tract destruction[127,129].

HIV-associated dementia brain lesions have been noted throughout the brain but predilections for the basal ganglia, frontal cortex and white matter, and also the hippocampus, brainstem, cerebellum and thalamus have been observed[130].

1.9 Antiretroviral treatment

The introduction of cART enables the suppression of HIV plasma viraemia to levels below the clinical limit of detection (<50 copies HIV RNA/mL)[131] and this is accompanied by a recovery in CD4+ T-cell count and immune function. Consequently, AIDS-defining illnesses are rarely observed in people taking antiretroviral treatment[132] and life expectancy in PLWH on suppressive cART has near-normalised[19,133]. Effectively treated HIV infection is now a chronic manageable disease and the focus is shifting from the management of immunosuppression and opportunistic infections, to the sequelae of chronic viral infection, its treatment and ageing[134].

In terms of the optimal timing of initiation of cART for adults living with HIV, initial observational studies showed poorer outcomes in terms of mortality and disease progression in people who delayed initiating cART until CD4+ T-cell counts were <200 cells/ μ L[135,136]. Even once cART was started, long-term cART use was associated with challenges such as drug toxicity, side effects, pill fatigue, difficulties with adherence and cost. Episodic cART, guided by CD4+ T-cell counts was trialled to overcome these challenges. However, this strategy was halted following the results from the SMART randomised controlled trial which observed that CD4+ T-cell count-guided episodic use of antiretroviral

treatment was associated with an increased risk of opportunistic infections and death[137]. Finally, it was the results of the START randomised controlled trial that informed clinical practice of the benefits of initiating cART for all PLWH, regardless of CD4 T-cell count[120]. The START study enrolled individuals with CD4+ T-cell counts above 500 cells/ μ L and randomly allocated the individuals to initiate cART immediately or defer until CD4 T-cell count fell below 350cells/ μ L or the development of a condition that warranted cART initiation. The START study demonstrated a decrease in both AIDS and non-AIDS events in individuals who initiated immediate cART, and importantly, did not find increased rates of cART-related adverse events in these individuals. Following the START study findings, WHO and national antiretroviral treatment guidelines were changed to remove the previous CD4+ T-cell count threshold and recommended the initiation of cART for all PLWH[138,139].

However, there remained no specific guidance on the optimal timing of cART initiation in asymptomatic people presenting with primary HIV infection. The SPARTAC randomised controlled trial attempted to address this question and demonstrated that 48 weeks of cART initiated during primary HIV infection delayed time to disease progression compared with standard of care at the time (which was deferred cART initiation until CD4 T-cell count fell below 350cells/ μ L or the development of a condition that warranted cART initiation), but this overall delay was not significantly longer than the overall duration of the treatment itself[140]. Importantly, the SPARTAC trial demonstrated that short-term cART was safe when initiated during primary HIV infection, which may be particularly relevant in the design of future HIV-remission studies involving cART interruption in individuals who initiated cART during primary HIV infection.

Whilst the morbidity and mortality benefits of cART in PLWH are clear, the reality is that global access to cART remains limited in certain parts of the world due to healthcare, economic, geographical, educational and gender inequalities. The majority of PLWH are in resource-limited countries which are least able to meet the high costs of providing HIV care, which include trained healthcare providers, cART and access to laboratories for diagnostics and monitoring.

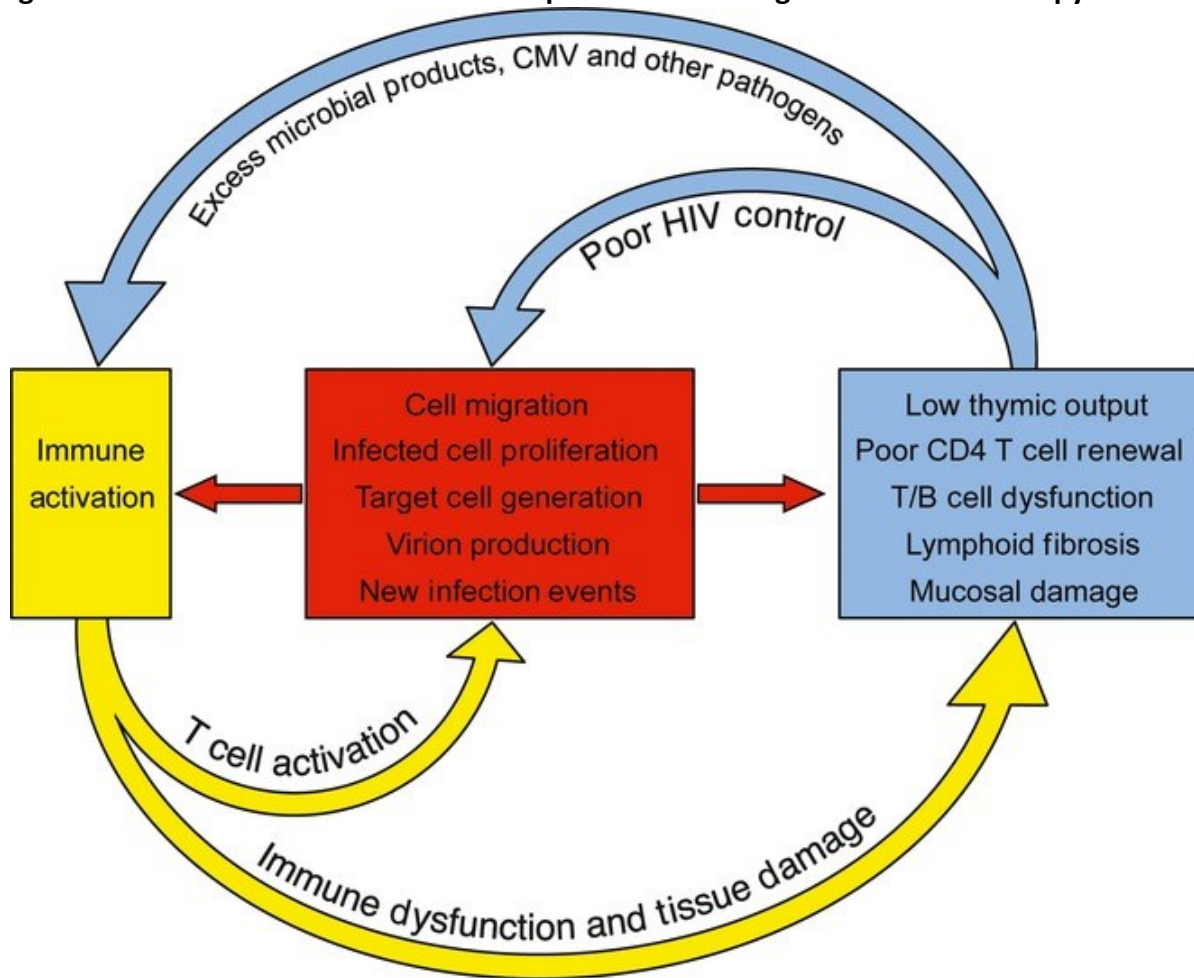
Thus, despite the clear benefits of cART, significant challenges persist, which has spurred the scientific field to pursue options beyond cART for the management of HIV.

1.10 Non-AIDS associated co-morbidities

In areas with high uptake of modern antiretroviral treatment, the prevalence of AIDS has dramatically reduced. However, it is becoming increasingly clear that while PLWH on virologically suppressive cART rarely present with AIDS-defining conditions, these individuals continue to be at greater risk of developing non-AIDS related neurological[141], cardiovascular[142], bone[143], liver[144] and kidney disease[145].

This is compounded by the growing evidence to suggest that despite an improvement in immune function following the initiation of antiretroviral therapies, PLWH on suppressive cART continue to have persistently heightened systemic and intrathecal immune activation and inflammation, compared to that measured in HIV-negative populations[146–150]. Several important confounders are hypothesised to contribute to the increased levels of immune activation and inflammation seen in PLWH on cART, which include non-infectious co-morbidities (e.g. cardiovascular disease and diabetes mellitus), co-infections (e.g. Cytomegalovirus, Epstein-Barr virus and Hepatitis C) and lifestyle factors (e.g. tobacco and recreational drug use), which have been shown to be more prevalent in some HIV-positive populations[151]. Additionally, specific HIV-related factors such as antiretroviral drug toxicity, longer duration of untreated HIV infection, lower nadir CD4+ T-cell count and lower CD4:CD8 ratio may influence levels of immune activation and inflammation. More recently, data is emerging to suggest that a complex interaction exists between the HIV reservoir and low-level viral transcription with the development of immune activation and inflammation, which can ultimately result in end-organ disease (Figure 8). The exact direction of causality is uncertain; it is unclear whether the higher levels of T-cell activation in PLWH result in higher levels of HIV reservoir, or whether higher viral reservoir burdens cause elevated immune activation. Ultimately, it is likely that even in PLWH on virologically suppressive cART, both pathways are active and that a vicious cycle exists, driving the maintenance of both immune activation and HIV persistence (Figure 8).

Figure 8: Immune activation sustains HIV persistence during antiretroviral therapy



Legend: During long-term antiretroviral therapy, the residual immune dysfunction results in immunodeficiency, excess amounts of pathogens, and chronic immune activation. The immune activation in turn leads to migration of CD4+ T cells to foci of HIV replication, generation of activated and susceptible target cells, and production of virus from latently infected cells (all of which enable more efficient cell-to-cell spread of HIV and replenishment of infected cells). The inflammatory environment also leads to proliferation and maintenance of latently infected cells. The residual HIV replication in turn contributes to sustained tissue damage and immune activation. Image reproduced with permission of the rights holder, John Wiley and Sons (2013)[152].

While many factors are hypothesised to contribute to the higher risk of non-AIDS associated co-morbidities seen in PLWH on virologically suppressive cART, the precise pathogenesis remains to be elucidated. The COmorBidity in Relation to AIDS (COBRA) study was designed to investigate the possible factors associated with non-AIDS associated co-morbidities, by comparing biomarkers in PLWH on virologically suppressive cART with lifestyle-similar HIV-negative control participants. Furthermore, in subgroup analyses, biomarker data from age-matched blood donors were included. On the one hand, results from the COBRA study suggests that both PLWH and lifestyle-similar HIV-negative individuals display signs of age

advancement using an algorithm based on 10 blood biomarkers, when compared with blood donors[153]. On the other hand, markers of immune activation (CD4+ T-cell activation (HLA-DR⁺CD38⁺) and CD4+ T-cell exhaustion (PD1⁺) were elevated in the PLWH on virologically suppressive cART compared to both groups of HIV-negative individuals (lifestyle-matched HIV-negative controls and blood bank donors)[154], suggesting excess immune activation in PLWH that is not explained by lifestyle behaviours. It is likely that a combination of factors (HIV- and non-HIV related) are likely to be responsible, with different factors playing key roles in different populations of PLWH.

1.10.1 Cognitive impairment in the cART era

1.10.1.1 *Clinical manifestations of HIV-associated cognitive impairment*

Frank HIV-associated dementia is now rarely seen in people taking cART[141], however the global prevalence of all cognitive disorders in PLWH remains unchanged due to the increasing prevalence of PLWH with milder forms of cognitive impairments, with reported prevalence rates of 15 – 69% seen in a variety of clinical and geographical settings[155–161]. In the cART era, cognitive impairment in PLWH typically affects the domains of attention, fine movement, executive function, processing speed and learning domains[162–166], as opposed to the reduced cognitive speed, motor slowing and behavioural changes typically seen in PLWH with cognitive impairment in the pre-cART era[163].

1.10.1.2 *Neuropathology of HIV-associated cognitive impairment in the cART era*

The neuropathology correlates of HIV-associated cognitive impairment in the cART era appears different compared to the neuropathological features seen in HIV-associated dementia. Gray *et al* examined brain autopsy specimens of PLWH on virologically suppressed cART and predominantly observed myelin pallor, astrogliosis and microglial activation, as opposed to the multinucleated giant cells and microglial nodules classically seen with HIV-associated dementia[167]. Antony *et al* investigated neuroinflammation in PLWH on virologically suppressive cART who had no obvious central nervous system pathology noted on autopsy. Here the group found evidence of microglial activation in the

basal ganglia and hippocampus in these individuals, at levels comparable and surprisingly, in some cases even exceeding those seen in individuals with HIV-associated dementia[168].

1.10.1.3 Impact of cognitive impairment

Symptomatic cognitive impairment can significantly reduce the quality of life for PLWH[169], with major functional impact on the ability to maintain employment[170] and quality of life[169]. Additionally, cognitive impairment may impede adequate medication adherence[171], which can affect the long-term effectiveness of cART for the individual and enable onward HIV transmission. On a global scale over 95% of PLWH are adults, many of whom are in the prime of their working lives[8]. The presence of symptomatic cognitive impairment can affect the productivity of these individuals and their ability to contribute to the economy, further impacting resource-limited countries who bear the brunt of HIV disease and place even greater demands on mental health services, which are already at capacity in many parts of the world. Furthermore, given that effectively treated PLWH are living longer, the prevalence of PLWH with symptomatic cognitive impairment is predicted to rise further with time[158], with significant impact on individuals and society.

1.10.1.4 Impact of early cART on cognitive impairment

In a study investigating PLWH with untreated acute HIV infection (median 19 days post-HIV exposure), most individuals displayed normal cognitive function, and the introduction of early cART slightly improved their cognitive function upon retesting three months later[172]. However, about a quarter of participants in this study demonstrated evidence of cognitive impairment during early untreated acute HIV infection, which was associated with higher cerebrospinal fluid HIV RNA, and early cART did not restore cognitive function in these individuals when retested up to six months later, suggesting limited reversibility of cognitive impairment in some individuals with acute HIV infection[172].

1.10.1.5 Factors contributing to cognitive impairment

The pathogenesis of cognitive disorders in people living with effectively treated HIV is likely multifactorial and there are multiple potential factors which may aggravate cognitive impairment.

1.10.1.5.1 Non HIV-related factors

Non HIV-related factors which have been postulated to contribute to the development of cognitive impairment include:

- Neurodegeneration relating to the ageing process, which contributes to reduced cognitive reserve.
 - In many settings, cohorts of PLWH are increasing in age[173], largely caused by the increased life expectancy of PLWH on suppressive cART[133]
 - In the UK and other similar countries, the increasing age of PLWH is also driven by the rising incidence of new HIV diagnoses in people aged over 50 years (9% (667/7,439) in 2006 to 17% (1018/6095) in 2015)[174]
- Co-morbidities
 - Association between cognitive impairment and co-morbidities such as cardiovascular disease[175], kidney disease[175] and diabetes mellitus[176] have been observed in HIV-positive cohorts
- Co-infections
 - Co-infections, such as with cytomegalovirus[177] and past history of syphilis [178] have demonstrated associations with cognitive impairment in HIV-positive cohorts
- Lifestyle factors
 - In PLWH, the following lifestyle factors have been shown to be associated with cognitive impairment

- tobacco[179]
- alcohol[180]
- substance use[181]
- Mental health conditions such as depression, anxiety and post-traumatic stress disorder have been associated with reduced cognitive performance in PLWH[182–185]
- Traumatic brain injury[186]
- Polypharmacy[187,188]

1.10.1.5.2 HIV-related factors

- The HIV reservoir in the central nervous system has been discussed earlier in this thesis.
 - A hypothesis that is increasingly gaining traction in the scientific field is that the HIV reservoir and low-level viral transcription in the central nervous system may contribute to central nervous system immune activation and neuroinflammation which can then lead to neuronal injury, culminating in cognitive impairment.
 - Associations between the detection of cells latently infected with HIV[85] and HIV proteins[189] in the cerebrospinal fluid of people living with HIV on long-term virologically suppressive cART and cognitive impairment has been observed, strengthening the argument that such a link exists.
- Cerebrospinal fluid HIV escape[190]
 - Rarely, cerebrospinal fluid HIV RNA can be detected in people on cART with plasma HIV RNA suppression, and this may be associated with new or worsening cognitive disorders.

- Lower nadir CD4+ T-cell count[191–193] and decreasing CD4:CD8 ratio[194] have been shown to be independent predictors of cognitive impairment
- HIV subtype (clade)
 - Some studies have suggested that certain HIV subtypes may be associated with a higher risk of developing cognitive disorders, while others have not confirmed this association. It has been suggested that PLWH with HIV subtype D are more likely to have cognitive impairment compared to PLWH with subtype A[7], and that subtype B may be more neurovirulent than subtype C[195].
- The legacy effects of untreated HIV infection may also contribute to persistent cognitive impairment due to:
 - Irreversible atrophy and neuronal loss caused by the period(s) of uncontrolled HIV replication
 - The effects of previous AIDS-defining illnesses and neurotoxic antiretroviral agents

1.10.1.5.3 Antiretroviral reduced central nervous system penetration or toxicity

Concentrations of antiretroviral drugs within the brain can be variable depending on blood-brain barrier integrity or drugs being actively effluxed out of the central nervous system due to membrane transporters[196]. While there is good evidence to suggest that poor central nervous system penetration of cART can be associated with detectable cerebrospinal fluid HIV RNA[197,198], the evidence that antiretroviral drugs with good central nervous system penetration improve cognitive function is debatable; some studies have reported improvements in cognitive performance with better central nervous system-penetrating cART[199,200], while others have reported no significant improvements[201] and even deterioration in cognitive performance[202].

There is no doubt that cART is beneficial for the overall health for PLWH, but evidence is emerging that some antiretroviral drugs may negatively impact cognitive function and it is likely that there is a therapeutic window between drug efficacy and toxicity in the central nervous system. An *in vitro* study performed by Robertson *et al* exposed rat neuronal cultures to 15 antiretroviral agents at a spectrum of concentrations, which found extensive neuronal death was uncommon and only seen at the highest concentrations of certain drugs (particularly efavirenz) but mitochondrial dysfunction and neuronal injury was observed at drug concentration levels comparable to standard clinical dosing[203]. Clinical evidence of cART toxicity was further demonstrated in a study performed by Robertson *et al*, who observed improvements in cognitive function after planned interruption of antiretroviral therapy[204]. Given the historical clear benefits of cART initiation on cognitive performance, the results from this study were surprising and in contrast with the investigators' original hypothesis.

1.10.1.5.4 Chronic immune activation and inflammation in the central nervous system

While long-term suppressive cART is associated with immune reconstitution, studies have demonstrated that intrathecal immune activation and inflammation remain persistently higher in PLWH on virologically suppressive cART compared with HIV-negative control populations[148,205–207]. Notably, Ulfhammer *et al* observed that over 50% of PLWH on cART had persistently raised cerebrospinal fluid neopterin concentrations (a soluble marker of macrophage activation) despite more than 10 years of suppressive cART[148].

Additionally, in a study of PLWH on virologically suppressive cART, the presence of low-level cerebrospinal fluid HIV RNA (detectable using the single-copy assay) was associated with higher cerebrospinal fluid neopterin, highlighting the potential for persistent viral replication despite cART to cause ongoing central nervous system immune activation[208]. Neuroimaging using magnetic resonance spectroscopy corroborates the finding of persistent immune activation in the central nervous system during virologically suppressive cART[209].

Furthermore, a study by Eden *et al* has demonstrated an association between elevated concentrations of cerebrospinal fluid neopterin and reduced cognitive performance in PLWH

on virologically suppressive cART, implying that cognitive disorders may be a result of ongoing inflammation despite effective cART[205]. Neuroimaging studies corroborate these findings by demonstrating increased microglial activation on positron emission tomography (PET) neuroimaging in neuro-asymptomatic PLWH on effective cART compared with control participants, and associations between increased microglial activation in some brain regions and reduced cognitive performance[210,211].

Flow cytometry studies of cerebrospinal fluid have demonstrated that similar to the picture seen in the blood compartment, CD8⁺ T-cells predominate in the cerebrospinal fluid of PLWH, resulting in a lower CD4:CD8 ratio in the cerebrospinal fluid, compared to HIV-negative individuals[212,213]. Lower CD4:CD8 ratios in both peripheral blood and cerebrospinal fluid have been associated with cognitive impairment in PLWH[214]. Chronic untreated HIV infection is associated with a reduction in naïve T-cells, and an expansion in the proportion of memory T-cells[212,214] and activated CD4⁺ and CD8⁺ T-cells in the blood and cerebrospinal fluid compartments[212,214,215]. Virologically suppressive cART improves but does not fully normalise T-cell activation and individuals with more severe cognitive impairment have been shown to have higher proportions of activated T-cells in the cerebrospinal fluid compartment[214]. However, in this analysis confounders such as detectable plasma HIV RNA load, nadir CD4⁺ count and duration of untreated HIV disease were not taken into account.

There is growing evidence to support early cART initiation for PLWH diagnosed soon after acquiring HIV infection, to restore immune function and reduce end-organ damage. Intrathecal immune markers of inflammation (neopterin, monocyte chemotactic protein/CCL2 and interferon γ -induced protein 10/CXCL-10) have been shown to be higher in some individuals with very early HIV infection before cART initiation (median time since estimated HIV acquisition 8 days)[12]. Various studies have demonstrated biomarker benefits in individuals initiating very early cART during acute HIV infection; evidence suggests normalisation of cerebrospinal fluid inflammatory biomarkers, but not plasma inflammatory markers after 2 years of cART[216] and normalisation of inflammation as measured by choline on brain MRS following six months cART initiated very soon after HIV acquisition[31].

1.10.1.5.5 Chronic viral replication in the central nervous system

During untreated HIV infection, a steady invasion of virus into the central nervous system is maintained, as described above. In untreated PLWH, cerebrospinal fluid HIV RNA is normally 1 log₁₀ lower compared to plasma HIV RNA (and approximately 2 log₁₀ lower compared with plasma HIV RNA in individuals with acute HIV infection[12]), and controlling peripheral blood HIV RNA is the most effective way of preventing ongoing viral entry into the central nervous system.

Early genetic sequencing of virus obtained from the cerebrospinal fluid in patients with primary HIV infection demonstrates that central nervous system HIV is similar to virus obtained from the blood[217], but over time central nervous system HIV evolves into a compartmentalised virus[218] that can replicate independently of blood HIV virus.

Compartmentalised central nervous system HIV is usually more macrophage-tropic, enabling it to persist in central nervous system perivascular macrophages and monocytes, given the generally low numbers of CD4+ T-cells in the central nervous system [218].

Furthermore, it is speculated that reduced cART penetration through the blood-brain barrier into the central nervous system can lead to subtherapeutic cART concentrations in the central nervous system, allowing viral escape in this compartment. Sub-therapeutic cART in this compartment may exert partial drug pressure on replicating virus, which could then lead to drug resistant mutations and loss of viral control in the central nervous system.

1.10.2 Diagnosing cognitive disorders

1.10.2.1 *Clinical diagnosis of cognitive dysfunction*

If cognitive impairment is suspected in clinical practice, formal neuropsychometric assessment is vital. The tests often cover multiple cognitive domains including memory, attention, executive function, motor function and language. These tests usually include estimates of premorbid function, intelligence quotient (IQ) testing, co-morbidities which can contribute to cognitive impairment and assessments of mental health including depression and anxiety, to help determine whether an individual has significant cognitive impairment.

Cognitive test results are then compared with normative controls[163], usually stratified by age but occasionally also by ethnicity and education attainment level. Importantly, the impact of the factors mentioned previously which can affect cognitive function and practice effect needs to be taken into consideration when interpreting cognitive function results.

Whilst these tests are the gold standard, they can take several hours to complete and require highly trained staff to implement the tests. Common assessments include Digit Span, Trail Making and Grooved Pegboard tests[219].

1.10.2.2 Research assessments of cognitive dysfunction

Research cognitive assessments can take the format of formal neuropsychological assessments (see above), computerised cognitive tests and brief screening tools.

1.10.2.2.1 Computerised cognitive tests

Computer-based cognitive tests have been developed as an alternative method to formal neuropsychological assessments and can be completed in much less time. Some computerised batteries have been shown to strongly associate with formal neuropsychological assessments and are highly specific for the detection of cognitive impairment, including subtle sub-cortical deficits which are frequently seen in individuals with HIV-associated cognitive disorders[220]. Furthermore, bespoke battery combinations can be designed to specifically evaluate the cognitive domains of interest, based on the condition being investigated.

1.10.2.2.2 Brief screening tools

The HIV Dementia Scale (HDS)[221] was first devised to assess PLWH in the pre-cART era. The HDS does not require any equipment, is simple to implement and tests memory, attention, psychomotor speed and constructional skills domains. In 2005, the International HIV Dementia Scale (IHDS)[222] was developed to overcome the cross-cultural challenges of administering the HDS. The IHDS is another rapid screening tool to assess motor speed, psychomotor speed and memory recall domains using three simple tasks. It is also easy to implement, does not require equipment and has been shown to be a useful screening tool to identify individuals at risk of HIV-associated dementia in both industrialised and

developing nations. However, both tests perform poorly as a screening tool for milder forms of HIV-associated cognitive dysfunction[223], largely because they were designed to test motor and cognitive slowing, key features of the subcortical injury seen in people with HIV-associated dementia, as opposed to impairment in learning and executive function domains, which are more commonly seen in people with milder HIV-associated cognitive impairment.

1.10.2.3 Definitions of cognitive dysfunction

Over the years, several research diagnostic criteria have been used to define HIV-associated cognitive impairment. In 1991, the American Academy of Neurology (AAN) criteria defined two levels of neurologic manifestations of HIV disease[159]:

- HIV-associated dementia (HAD)
- minor cognitive motor disorder (MCMD)

Several problems were noted with this definition, in that the number of cognitive domains to be tested for diagnosis and the degree of cognitive impairment required for a diagnosis were not defined, thus introducing inconsistencies in the clinical estimation of severity. Furthermore, there was a degree of overlap between the criteria for HAD with mild functional decline and MCMD, and the criteria did not include milder forms of cognitive impairment which did not interfere with activities of daily living.

To address these issues, in 2007 a standardised research definition of cognitive impairment in PLWH was proposed, known as the HIV-associated neurocognitive disorders (HAND) or Frascati criteria, which is still widely used today. The Frascati criteria stipulates neuropsychometric testing of the following domains, with two tests per domain: verbal and language, attention and working memory, abstraction and executive function, memory (learning and recall), information processing speed, sensory-perceptual skills and motor skills.

Mild cognitive impairment is defined as at least two of the domains tested scoring one standard deviation below the normative test scores. Self-reported functional assessments such as the Lawton's Activities of Daily Living Scale are then used to divide those with mild

cognitive impairment into Mild Neurocognitive Disorder (MND) if cognitive impairment affects the individuals' activities of daily living, or Asymptomatic Neurocognitive Impairment (ANI) if it does not (Table 2). HIV-associated dementia (HAD) criteria are met when at least two of the domains tested are two standard deviations below the normative test scores (Table 2). Importantly, for a diagnosis of HAND, cognitive impairment needs to be acquired and unlikely caused by other co-morbidities, however, in reality, it is often challenging to extricate the multifactorial causes of cognitive impairment in most clinical cohorts.

Table 2: Frascati criteria for the diagnosis of HIV-associated neurocognitive disorders

	Cognitive function	Interference with daily function
Asymptomatic neurocognitive impairment (ANI)	Impairment in 2 or more cognitive domains with performance <1 standard deviation below the mean	None
Mild neurocognitive disorder (MNI)	Impairment in 2 or more cognitive domains with performance <1 standard deviation below the mean	Mild
HIV-associated dementia (HAD)	Marked impairment in 2 or more cognitive domains with performance <2 standard deviations below the mean	Severe

Legend: Cognitive performance is assessed through detailed neuropsychological testing assessing a minimum of five cognitive domains: verbal/language, attention/working memory, abstraction/executive function, memory (learning and recall), speed of information processing, sensory perceptual skills and motor skills. Adapted from Antinori et al. Neurology (2007)[41].

The aim of the Frascati definition was to identify the more minor cognitive impairments seen in PLWH on cART, as opposed to the more severe dementia phenotype seen in the pre-cART era. Nonetheless, the Frascati criteria has limited use in the clinical setting as the clinical significance of identifying people with ANI is uncertain. Some studies have shown that individuals with ANI have an increased risk of progression to symptomatic neurocognitive impairment[224], but confounders were often more commonly seen in the individuals who experienced a deterioration in cognitive classification. Furthermore, some have argued that the HAND criteria over-diagnoses cognitive disorders with up to 50%

cognitive impairment reported in some cohort studies, which is not the clinical picture seen in real-life settings.

To address some of these limitations, additional definitions have been proposed including:

- Gisslén criteria [225]
 - The Gisslén criteria is a modification of the original HAND criteria, but instead a threshold of 1.5 standard deviations rather than 1 standard deviation below the normative mean is used to define ANI and MND.
- Global Deficit Score criteria[226]
 - The Global Deficit Score is calculated by converting demographically adjusted cognitive test results to deficit scores and then averaging them to determine impairment. The purpose of converting to deficit scores is to place more weight on impaired performance, with less weight placed on normal and high-performing scores, which would counteract impairment if a simple average were taken across the domains tested.
- Multivariate normative score[227]
 - In this method, the control group is utilised as a reference to calculate a multivariate statistic, accounting for performance in all the cognitive domains and covariance between the different tests. A study comparing the Frascati criteria, Gisslén criteria and multivariate normative score demonstrated that the multivariate normative score method potentially optimises the balance between sensitivity and specificity for the diagnosis of HIV-associated cognitive impairment[228].

Overall, these newer definitions appear to reduce the number of people with HIV categorised as having cognitive impairment, but further clinical validation is warranted.

1.10.3 Neuroimaging cognitive impairment

1.10.3.1 *Computerised tomography and magnetic resonance imaging*

Cerebral imaging techniques using computerised tomography (CT) and MRI are widely available in resource-rich settings and are frequently used in the routine clinical care of PLWH to investigate central nervous system opportunistic infections, malignancy and neurological disorders, such as cognitive impairment and seizures, and for research purposes to investigate HIV-associated neurological conditions. Structural imaging focuses on assessments of cerebral volume and cerebral microstructure.

In early and asymptomatic HIV disease, structural cerebral imaging is often normal, but hyperintense lesions in the frontal lobe (on T2-weighted MRI) and foci of demyelination in the white matter on MRI may be detected, that gradually increase over time as HIV disease progresses[229]. As HIV disease progresses, generalised cerebral atrophy has been recognised as a hallmark feature of HIV-associated dementia [229]. Associations have also been detected between impaired neuropsychological test scores with atrophy in various brain regions including the basal ganglia, caudate nucleus, corpus callosum, cortex and hippocampus in PLWH[230–234]. A longitudinal study assessing structural brain MRI changes in individuals who initiated early cART (median duration of infection was 15 days) demonstrated atrophy in the putamen and caudate, despite the presumed neuroprotective effects of 2 years of cART[235].

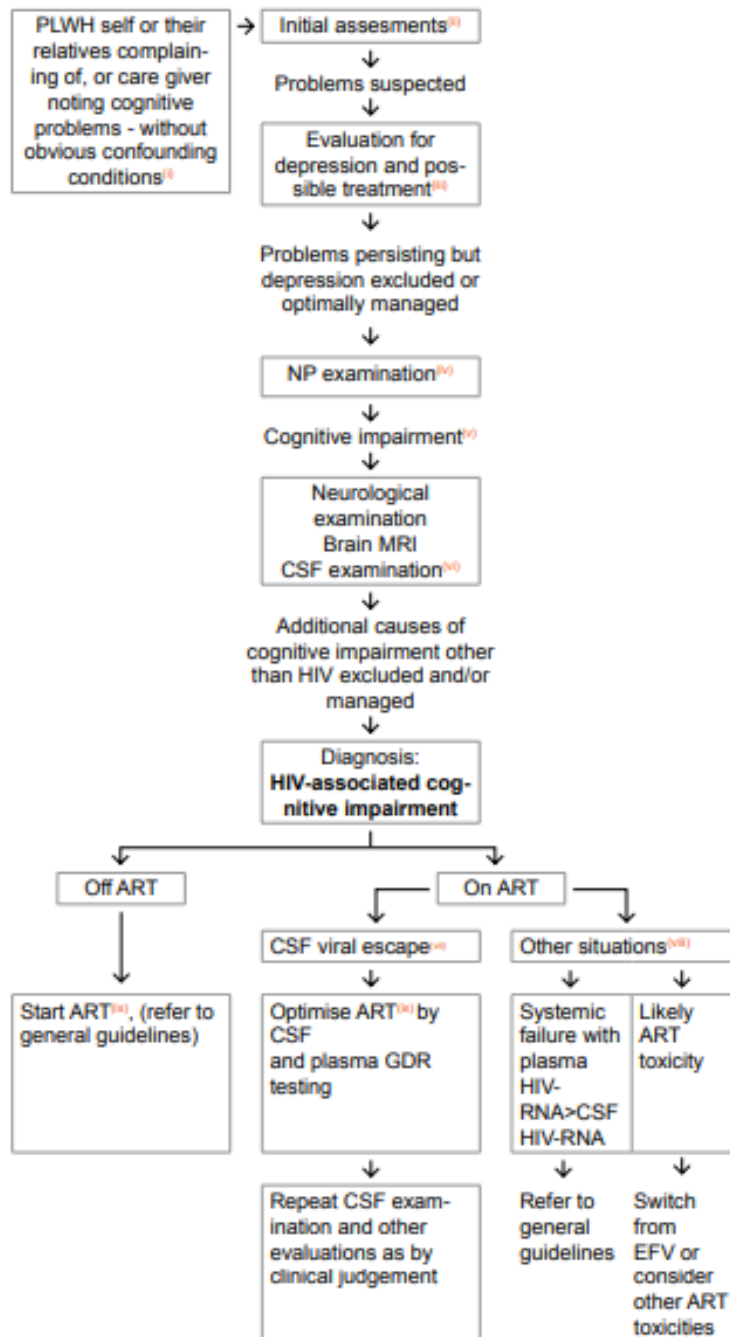
Other neuroimaging modalities that have been used to investigate cognitive impairment in PLWH is described later in this chapter.

1.10.4 Treatment of HIV-associated cognitive impairment

cART remains the mainstay of treatment for HIV-associated cognitive impairment, regardless of stage of HIV disease. The European AIDS Clinical Society guidelines algorithm for the diagnosis and management of cognitive impairment in PLWH is detailed below (Figure 9). To date, numerous adjunctive therapeutic agents aimed at reducing cognitive

impairment in PLWH on suppressive cART have been investigated, but in general, none have shown a convincing beneficial effect [161,236].

Figure 9: Algorithm for Diagnosis and Management of Cognitive Impairment in PLWH



Legend: Algorithm for the diagnosis and management of cognitive impairment in people living with HIV, as per the European AIDS Clinical Society (2019) guidelines[237].

Abbreviations: PLWH = people living with HIV, NP = neuropsychometric, MRI = magnetic resonance imaging, CSF = cerebrospinal fluid, ART = antiretroviral treatment, GDR = genotypic drug resistance, EFV = efavirenz

A deeper understanding of the underlying pathogenic mechanisms of the development of HIV-associated cognitive disorders in the context of virologically suppressive cART is crucial to guide future screening, surveillance and novel therapeutic approaches.

1.10.5 Challenges in the diagnosis of HIV-associated cognitive dysfunctions

In reality cognitive testing is time consuming to perform, and prone to many confounding issues such as the factors mentioned above, day to day variability in an individual's performance, practice effect and social and cultural factors[163].

A major challenge in this field is to establish the expected cognitive performance of a given population, given that many populations of PLWH differ from established control populations in terms of lifestyle behaviours and the prevalence of other co-morbidities. As mentioned above, various factors can impact the prevalence of reported cognitive impairment, if the control populations used to generate the cognitive normative data differ from the PLWH being assessed.

Furthermore, the broad range in prevalence rates of mild cognitive impairment reported in PLWH on modern cART also likely reflects the different definitional criteria that have been used over the years to diagnose HIV-associated cognitive disorders[159,163] (see above).

Importantly, cognitive testing is unable to differentiate between impairment caused by static residual damage from historical events or from ongoing brain injury, a distinction that has crucial implications in terms of therapeutic strategies[205]. Markers that dynamically reflect processes taking place in the brain would represent an important tool for evaluating ongoing active central nervous system disease, as well as response to therapy.

1.11 HIV cure

1.11.1 Why a cure is needed

Despite the clear benefits of cART for PLWH, several important factors are driving research into a cure for HIV. Modern cART regimens are generally safe and tolerable, but they are not

completely risk-free and some PLWH are unable to tolerate long-term cART due to toxicity and side effects. Higher rates of cardiovascular[238], bone[239] and kidney disease[240] are associated with some of the frequently used antiretroviral drugs. Even the more recently introduced class of antiretroviral drugs, the integrase strand transferase inhibitors (INSTIs), have been associated with weight gain and central nervous system side effects[241,242]. A substantial proportion of people who received early antiretroviral drugs would have developed drug resistance and now require complex multidrug cART regimens to maintain viral suppression. As the population of PLWH grow older and develop co-morbidities, they will need to start taking additional drugs and the issues that come with polypharmacy, meaning adherence and drug-drug interaction will become an increasing. Given that lifelong cART is currently needed to control viral replication, adherence issues and cART interruption due to 'pill fatigue' can expose an individual to the development of drug-resistant virus, the complications of uncontrolled systemic HIV replication and the risk of HIV transmission. Stigma also remains a major issue for many PLWH, preventing some PLWH from taking cART or attending HIV clinic visits and can affect mental health and well-being[243].

Virological suppression with effective cART eliminates the risk of HIV transmission and test-and-treat strategies with the aim of reducing transmission is the universal goal. However, despite massive global efforts, as of 2019, only 68% of PLWH worldwide were currently receiving antiretroviral treatment[8]. Without increasing the percentage of PLWH receiving cART, the HIV pandemic will unlikely be controlled[244]. At this stage, nearly all countries have successfully rolled-out national antiretroviral treatment programmes, but HIV outcomes have been disparate[245]. In some communities with high HIV prevalence rates, only small reductions in HIV incidence rates have been seen despite widespread availability of cART[246].

cART and the provision of HIV care is costly and places significant burdens on healthcare systems. This is especially challenging for some developing nations, who have disproportionately high HIV prevalence rates. Theoretically, a short-term, inexpensive and effective intervention that can lead to sustained periods of virological suppression in the absence of cART would reduce the strain on public health systems, enabling time and money to be spent on other aspects of HIV care and prevention. For these reasons,

optimising methods to eliminate the HIV reservoir size to achieve a cure for HIV is a much sought-after goal.

1.11.2 Defining cure

Ideally an HIV-cure intervention would result in the complete eradication of all replication-competent HIV DNA in an individual; this is known as a ‘sterilising cure’. It will be very challenging to confirm that every single infected host cell that contained replication-competent HIV DNA has been eradicated[247], and a potentially ‘cured’ individual may never really know whether any virus particles that are capable of re-initiating systemic infection persist. Several case reports of individuals starting very early cART or undergoing allogeneic bone marrow transplantation demonstrated undetectable virus, but HIV RNA load eventually rebounded in most cases within weeks to months of stopping cART[100,248–250]. This difficulty with predicting if and when a rare infected cell will instigate viral rebound advocates for strategies that support durable host-mediated immune control of any remaining reservoir.

Many interventions currently under trial are aiming to achieve a ‘functional cure’, whereby the virus only remains present at such low levels that it can no longer be transmitted or cause disease. Realistically, functional cures will likely need to be defined as a sustained maintenance of HIV RNA load below the level of quantification on standard assays used in clinical practice, for a certain period of time.

1.11.3 HIV-cure strategies

Broadly, there are five main approaches to achieving an eradicated cure or remission, as described in Table 3.

Table 3: Comparison of different strategies for curing HIV.

Approach	Summary	Cost	Scalability	Successes
Early cART initiation	Start cART early in acute infection	Low	Yes	Post-treatment controllers
Shock and kill	Stimulate HIV expression from the reservoir followed by clearance of HIV expressing cells	Low	Yes	HIV expression from reservoir using HDACi
Block and lock	Induces a state of deep latency	Low	Yes	Delay in virus rebound after stopping cART in a humanised mouse model
Immunotherapy	Alter immune system to clear infected cells	Low	Maybe	SIV clearance in Rhesus macaques
Genetic modifications	Alter the host genome to resist or limit HIV	High	No	Berlin patient

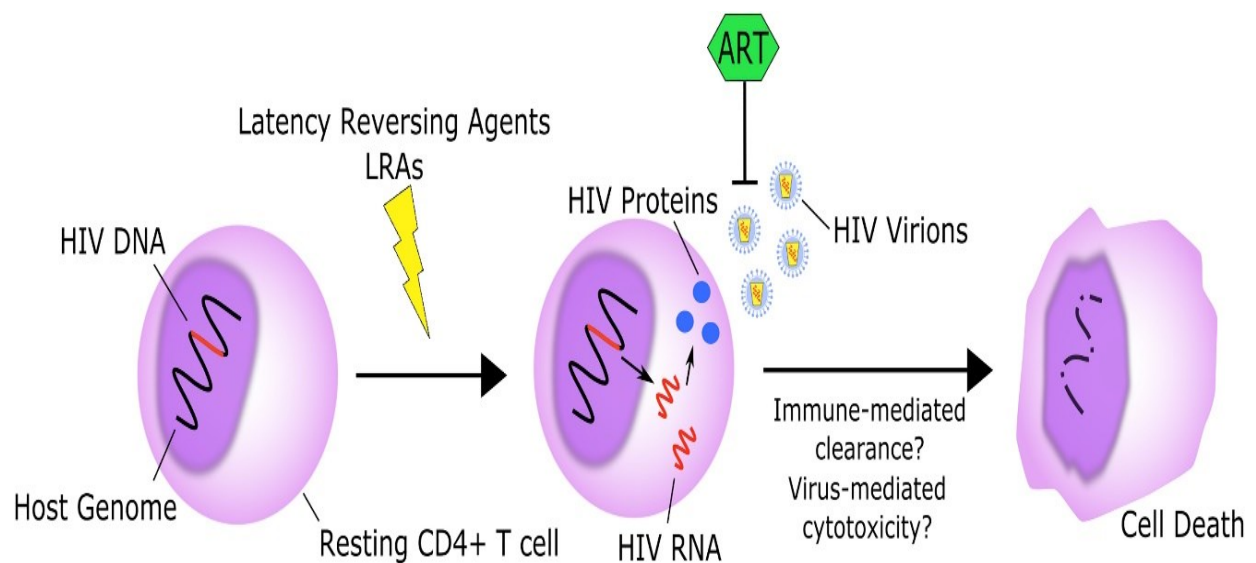
Abbreviations: cART = combination antiretroviral treatment, HDACi = histone deacetylase inhibitors, SIV = simian immunodeficiency virus. Adapted and reproduced with permission of the rights holder, Taylor & Francis[251]

I have discussed early cART initiation earlier in this chapter and I will discuss “shock and kill” below. The block and lock, immunotherapy and genetic modification approaches are beyond the scope of this thesis.

1.11.3.1 “Shock and kill”

Several HIV remission interventions are currently being investigated but one such method currently gaining traction in the clinical trials circuit is “shock and kill”, which involves forcing viral transcription from latently infected cells using latency reversing agents (LRAs) (the “shock”), followed by the targeted removal of these HIV-expressing cells using a “kill” mechanism such as CD8⁺ cytotoxic T-lymphocyte (CTL) clearance[252–254] (Figure 10).

Figure 10: "Shock and kill" to eliminate cells latently infected with HIV



Legend: The "shock and kill" strategy employs the use of latency-reversing agents to increase HIV transcription, protein expression and virion production. Following that, the cell may die through virus-mediated cytopathic consequences or through immune-mediated clearance. Image reproduced with permission of the rights holder, Elsevier[255].

Various methods of stimulating the latent reservoir have been investigated and more recently, clinical trials have been focussing on the use of histone deacetylase inhibitors (HDACi), which open up chromatin structures and enable HIV transcription. Several HDACis have been observed to stimulate HIV mRNA transcription (vorinostat and disulfiram)[256–258] and possibly can even stimulate viral particle production (panobinostat and romidepsin)[259,260]. However, HDACis alone have not been shown to reduce HIV reservoir size[256], and additional boosts to the immune system for the HIV-targeted killing of the infected targets are likely to be needed (the "kill").

"Kill" strategies currently under investigation include priming the immune system with HIV-specific vaccination prior to HDACi administration or immunotoxin approaches that would use HIV envelope expression on the surface of HIV-infected cells coupled with cytotoxic agents that bind the HIV envelope. In a proof-of-principle study, Hansen *et al* demonstrated that a simian immunodeficiency virus (SIV)-directed cytomegalovirus vectored vaccine led to viral control in a subset of SIV-infected Rhesus macaques and a reduction in concentrations of replication-competent virus in the reservoir[261]. However, similar results have not been replicated in human studies.

Overall, “shock and kill” strategies are scalable, but the two-step approach complicates research efforts as many combinations of different “shock” and “kill” approaches may need to be trialled before a successful pairing is identified.

1.11.4 Analytical antiretroviral treatment interruption

To date, the only way of truly assessing whether a HIV remission strategy has worked is by conducting an analytical antiretroviral treatment interruption (ATI)[251], where the aim is to assess the impact of novel therapeutic interventions on duration to viral load rebound or altered viral load setpoint[262].

However, there are concerns with this approach given the SMART study[137] results, which identified an increase in all-cause mortality associated with cART interruption for prolonged periods with CD4+ T-cell count re-initiation criteria, thought to be driven by inflammation and immune activation. Nonetheless, recent research exploring novel cure strategies including an ATI component has suggested that with careful participant selection, frequent viral load monitoring (usually at least weekly) and a low threshold for cART restart, ATI may be a safe way of accurately identifying HIV remission[262].

1.11.5 Central nervous system safety and efficacy concerns

Only very limited data are currently available on the central nervous system safety of HIV-cure and ATI strategies, and many of the long-term consequences of LRAs are unknown[263,264]. Following the reactivation of latent virus, the release of HIV viral proteins which can be neurotoxic or the LRAs themselves could cause neuronal injury. Additionally, viral rebound following the LRAs, the LRAs and immunotherapies could lead to systemic and central nervous system inflammation and immune activation, which could cause neuronal injury. The cells in the central nervous system which most likely harbour the HIV reservoir are macrophages and microglial cells, and the ‘kill’ aspect might mean the removal of these cells which have crucial function for brain health. Also, many of the LRAs under trial are non-specific, which carry a significant risk of reactivating other integrated

resting viruses in a resting phase[265–267]. Unlike other organs, the brain is encased in a bony cavity with little or no room for expansion; hence any inflammation in the brain could lead to substantial brain neuronal injury with catastrophic consequences.

In a study of SIV-infected macaques that had long-term virological suppression with central nervous system-penetrant cART[268], following the administration of LRAs, viral reactivation in the macaque brain was evident, with one macaque experiencing significant viral rebound (1 log₁₀ higher in the cerebrospinal fluid compared to plasma), increased central nervous system immune-activation and neuronal injury biomarkers as well as neurological symptoms necessitating euthanasia[268]. Upon sequencing the rebound virus, the most prevalent SIV genotype in the cerebrospinal fluid was distinct compared to the peripheral genotypes, suggesting central nervous system compartmentalisation of virus despite long-term peripheral viral suppression.

With regards to ATI, viral rebound in the central nervous system following ATI could theoretically precipitate central nervous system immuno-activation and neuro-inflammation, leading to neuronal injury. Central nervous system neurological injury associated with immune activation are frequently reported in individuals during primary HIV infection[269], which is substantially reversed with cART. However, a treatment interruption approach with viral rebound may precipitate a recurrence of the neurological injury, and the impact of repeated episodes of neurological injury and immune activation on the brain is unknown.

On the other hand, the central nervous system is a sanctuary site; when intact, the blood-brain barrier selectively permits entry of compounds from the bloodstream into the central nervous system. Thus, HIV cure interventions that poorly penetrate the blood-brain barrier may have subtherapeutic concentrations within the central nervous system, leading to incomplete eradication of HIV reservoirs in this compartment. Additionally, the majority of HIV cure trials to date have focussed on maximising remission effects on resting CD4+ T-cells, which is presumed to make up the largest proportion of cells harbouring replication-competent integrated HIV DNA. However, whether the HIV remission agents currently under trial affect non-CD4+ T-cells that harbour latent HIV is unknown. While pursuing HIV

remission interventions which target the majority of cells harbouring latent HIV is sensible, upon cART interruption, it would only require one latently infected cell that was not susceptible to the remission strategy to reactivate and start producing infectious virions again, thus defeating the purpose of that remission strategy.

Ultimately, the ideal HIV “shock and kill” strategy in the central nervous system would effectively reactivate cells latently infected with HIV in the brain without causing toxicity or excessive immune activation, inflammation or neuronal injury. The safety, efficacy and durability of current cART regimens means that although there is a strong mandate for HIV remission, the risks versus benefits must be carefully evaluated, especially given that participants taking part in HIV remission studies at present tend to be young and healthy. Any significant increased harm associated with HIV remission interventions must be carefully explored, as these critical safety concerns could seriously jeopardise the future direction of HIV remission research.

1.12 Neural and viral biomarkers

Neuropsychometric testing, structural neuroimaging and monitoring of clinical symptoms have long been the mainstay for the diagnosis of HIV-associated cognitive disorders. However, these performance-based assessments and clinical symptoms are generally descriptive, lack sensitivity and specificity, do not have the objectivity of quantitative biomarkers and cannot easily distinguish between active and historical brain injury. Routine brain biopsies are not feasible, thus there has been a strong push to identify reliable fluid and molecular neuroimaging biomarkers to diagnose neuro-HIV processes associated with neural activation, to monitor disease progression and response to treatment, and to evaluate risk of developing disease and prognosis. To date, a one-size-fits-all biological marker that can accurately predict ongoing neural activation and neurodegeneration has not been found, and several studies have concluded that a combination of biomarkers will most likely be needed to accurately predict this[161,163,270,271].

1.12.1 Cerebrospinal fluid biomarkers of neural activation

Cerebrospinal fluid is an ideal compartment for monitoring changes in the central nervous system environment given its contiguity to the brain parenchyma and spinal cord, with which it exchanges molecular components. Furthermore, the increased concentrations of central nervous system proteins in this medium compared to blood makes it superior in studies of neurodegenerative disorders, despite the more invasive nature of cerebrospinal fluid sampling.

The three main categories of cerebrospinal fluid biomarkers examined in HIV studies are viral, immune and neuronal markers (Table 4). Several reports have exemplified biomarkers of neuronal injury as the most useful of cerebrospinal fluid biomarkers for characterising the different stages of central nervous system HIV infection and resultant active brain injury[272–275], of which neurofilament light chain protein (NfL) has been shown to be the most sensitive[276].

Table 4: Examples of cerebrospinal fluid biomarkers examined in HIV studies

Category of CSF biomarker	Frequently used biomarker	Other biomarkers
Viral	HIV RNA	HIV DNA, genotypic sequencing, T-cell vs macrophage tropism, drug resistance mutations, co-receptor utilisation
Immune and inflammatory	Neopterin	White blood cell count, CCL2 (MCP-1), CXCL10 (IP-10), β 2M, sCD14, sCD163, sVCAM; MMP-9, YKL-40 (CHI3L1)
Neural	NfL	t-tau, p-tau, A β 1-42, sAPP α , sAPP β

Abbreviations: CSF = cerebrospinal fluid, NfL = neurofilament light chain protein, MCP-1 = monocyte chemokine protein; IP-10 = interferon protein; β 2M = beta-2- macroglobulin; TNF α = tumour necrosis factor alpha; sCD14 = the soluble lipopolysaccharide ligand; sCD163 = a macrophage chemokine; sVCAM = soluble vascular adhesion molecule; MMP-9 = matrix metalloproteinase; CHI3L1 = chitinase-3-like protein 1; t-tau = total tau and p-tau = phosphorylated tau; A β 1-42 = amyloid beta 1–42; sAPP α and sAPP β = soluble degradation products of the amyloid precursor protein. Adapted with permission from Price et al[273]

1.12.1.1 Neurofilament proteins

Neurofilaments are a heteropolymer family of neuronal intermediate filaments involved in maintaining the structural and functional integrity of axons[277–279]. Neurofilament proteins form major structural component of axons, and the expression of these proteins is particularly high in large myelinated axons where they influence conduction speed[280]. Neurofilaments comprise about 85% of the cytoskeleton proteins and include four main subunits with different molecular weights: neurofilament light (68 kDa), neurofilament medium (150kDa), neurofilament heavy (190 – 210 kDa) and α -internexin (66kDa), of which neurofilament light chain protein (NfL) is the most abundant and most soluble[279] (Figure 11).

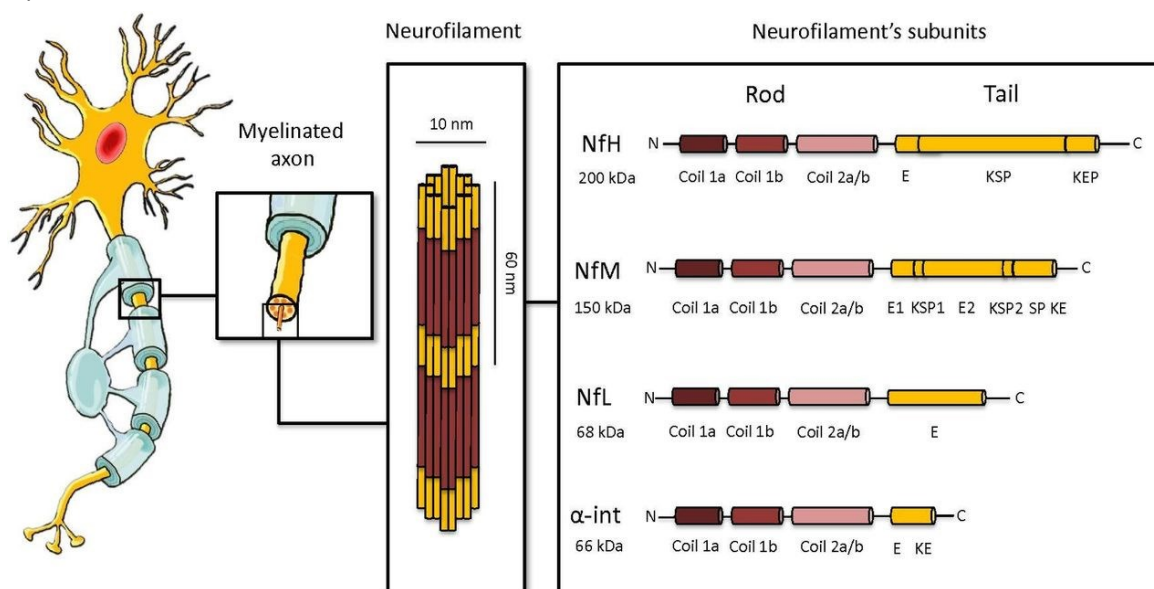


Figure 11: Overview of the structure of neurofilament proteins

Legend: Overview of the structure of neurofilament proteins. Large calibre myelinated axons abundantly express neurofilaments. Neurofilaments are cylindrical structures with a diameter of 10 nm calibre are exclusively located in neuro-axonal bundles. They confer structural stability to the axons, enable the radial growth of the myelinated axons thus allowing a higher conduction velocity. In the central nervous system, neurofilaments are composed of neurofilament light chain, neurofilament middle chain, neurofilament heavy chain and α -internexin. Image reproduced with permission of the rights holder, BMJ Publishing Group Ltd[281].

In conditions involving cortical neuronal injury, neurofilament proteins can be utilised as a biomarker of axonal damage. Following an insult, neurofilament proteins from the damaged neuro-axonal units are released proportional to the severity of injury into interstitial fluid and enters the cerebrospinal fluid, where they can then be measured[269].

Neurofilament proteins reflect active and ongoing neuro-axonal injury but are not disease-specific; elevated concentrations of cerebrospinal fluid NfL concentrations have consistently

been detected in several conditions including cerebrovascular disease, multiple sclerosis, frontotemporal and vascular dementias, traumatic brain injury and cardiac arrest[277,282–284]. In the general population, cerebrospinal fluid NfL is predicted to increase annually by 3.1%, which may be related to increased neuro-axonal breakdown or decrease in cerebrospinal fluid flow, as part of the normal ageing process.

1.12.1.1.1 Cerebrospinal fluid NfL in untreated HIV

In untreated HIV disease, cerebrospinal fluid NfL varies according to the stage of systemic disease and neurological status. Cerebrospinal fluid NfL concentrations are significantly elevated in subjects with HIV-associated dementia and an inverse association is seen between cerebrospinal fluid NfL and CD4+ T-cell count in neuro-asymptomatic individuals with untreated HIV[269,285]. Intrathecal markers of immune activation and blood-brain barrier integrity, and reduced cognitive performance have been shown to be independently associated with elevated cerebrospinal fluid NfL in untreated PLWH[276,286], indicating an association between inflammation, neuro-axonal injury and cognitive impairment.

Cerebrospinal fluid NfL data during acute and primary HIV infection is more contradictory: in one study of individuals in acute HIV infection (median 18 days since HIV exposure) and prior to cART initiation, 1/32 (3%) subjects demonstrated elevated cerebrospinal fluid NfL concentrations, compared to 31% of individuals with chronic HIV infection[287]. In another study of individuals in primary HIV infection (median 3 months after estimated HIV acquisition), 36/82 (44%) individuals displayed significantly elevated cerebrospinal fluid NfL concentrations compared to an age-matched HIV-negative cohort. These results might be explained by the different median time since exposure to HIV, indicating that central nervous system neuronal injury occurs at some point after acute infection, following sustained uncontrolled viral replication and central nervous system inflammation. There is data to suggest that following the rise in cerebrospinal fluid NfL during primary HIV infection, cerebrospinal fluid NfL normalises as HIV infection becomes chronic and CD4+ T-cell counts are restored[276]. Additionally, elevated cerebrospinal fluid NfL concentrations during primary HIV infection are not accompanied by elevations in other neuronal biomarkers associated with HIV-associated dementia, suggesting that the early neuronal

injury seen during primary HIV infection is less severe or has a different pathogenic mechanism compared with HIV-associated dementia[276].

1.12.1.1.2 Cerebrospinal fluid NfL in treated HIV

Cerebrospinal fluid NfL concentrations decrease and near-normalise following the initiation of suppressive cART[269,274,276,288,289] but a proportion of individuals on suppressive cART have persistently elevated cerebrospinal fluid NfL above the upper reference limit[269,288], with one study demonstrating cerebrospinal fluid NfL concentrations in individuals with treated HIV equivalent to HIV-negative controls who were 3.9 years older[285]. Additionally, parallel improvements in cognitive performance have been noted with virological suppression and normalisation of cerebrospinal fluid NfL[205,269]. This gives rise to the suggestion that there may be an association between viral reservoir size, viral transcription and extent of neuro-axonal injury.

1.12.1.1.3 Cerebrospinal fluid NfL in cART interruption

A retrospective study of eight participants with cerebrospinal fluid HIV RNA <50 copies/mL and normal cerebrospinal fluid NfL who subsequently interrupted cART demonstrated that while none of the participants displayed neurological symptoms, three participants had significant increases in cerebrospinal fluid NfL, indicating neuro-axonal injury[290]. All eight participants experienced rapid plasma HIV RNA and slower cerebrospinal fluid HIV RNA rebound, followed by plasma and cerebrospinal fluid neopterin elevations (in all the participants) and then lastly, cerebrospinal fluid NfL elevations in three participants, 58 – 80 days post- treatment interruption[290]. The sequence of biomarker trajectories seen in this study suggests that post-cART interruption, viral rebound may have led to inflammation, which caused neuro-axonal injury in a proportion of individuals. However, it remains unclear why neuro-axonal injury was triggered in these three participants, whereas the other five participants who also experienced plasma and cerebrospinal fluid viral load rebound to similar levels did not experience neuro-axonal injury.

1.12.1.1.4 Plasma NfL in HIV infection

The invasive nature of cerebrospinal fluid collection via lumbar puncture limits the more widespread use of cerebrospinal fluid NfL. Until recently, NfL was mainly measured using the conventional enzyme-linked immunosorbent assay (ELISA) technology or electrochemiluminescence (ECL)-based assays, which lacks the analytical sensitivity to reliably quantify blood NfL concentrations, normally 50 – 100 times lower compared to cerebrospinal fluid NfL[279]. However, Simoa technology has recently been introduced which is 126-fold and 25-fold more sensitive than ELISA and ECL, respectively, for the quantification of NfL[291]. The Simoa method is based on fluorescent microbeads coated with high-affinity capture antibodies that bind NfL, followed by a fluorescently labelled detector antibody[292]. The increased analytical sensitivity of Simoa is due to its unique detection method, whereby assay beads with captured NfL and fluorescent detector antibody are loaded onto a disk containing >200,000 microwells, each capable of holding one bead only. At high analyte concentrations, the total fluorescence can be measured and correlated with analyte concentration. At low analyte concentrations, the individual fluorescent microwells can be quantified, thus lowering the limit of quantification significantly.

Using Simoa, the analytical sensitivity of blood NfL quantification in the blood has improved to a level that blood NfL concentrations can be reliably quantified even in young and healthy individuals[293,294], such that changes in blood NfL concentration related to the normal ageing process or after mild injury can be detected. The introduction of blood NfL measurement removes the barriers facing repeated cerebrospinal fluid sampling, and thus allows more frequent measurements given that blood samples are easier to obtain than cerebrospinal fluid samples.

Preliminary studies across a variety of neurological conditions indicate a moderate to strong correlation between cerebrospinal fluid and blood NfL measurements[295–298]. In a cross-sectional study on stored samples, paired cerebrospinal fluid and blood NfL were quantified from 121 PLWH (13 in primary HIV infection, 22 suppressed on cART, 11 with HIV-associated dementia and 75 neuro-asymptomatic individuals with untreated HIV stratified fairly equally according to CD4+ T-cell count: CD4+ count >350 cells/ μ L, CD4+ count 200 –

349 cells/ μ L, CD4+ count 50-199 cells/ μ L and CD4+ count <50 cells/ μ L) and 19 HIV-negative individuals[293]. NfL was quantifiable in all the samples from all the participants, including the HIV-negative participants, and a strong correlation between cerebrospinal fluid and blood NfL was shown ($r = 0.89$). Plasma NfL displayed a similar pattern to cerebrospinal fluid NfL with age-related increases and highest values seen in individuals with HIV-associated dementia and significant elevations seen in neuro-asymptomatic individuals with untreated HIV and low CD4+ T-cell counts[293]. However, the correlation between cerebrospinal fluid and plasma NfL in treated HIV infection (which is at the lower end of detection of the assay) has not yet been established.

Plasma NfL has the potential to be a surrogate biomarker of active neuronal injury, albeit it lacks specificity. It could be a screening or evaluation tool for individuals with cognitive disorders or cerebrospinal fluid escape, and to identify individuals who may benefit from cerebrospinal fluid sampling and neuroimaging. Plasma NfL could also potentially be used to assess risk, predict progression and quantify severity of disease in those with HIV-associated cognitive disorders. Furthermore, plasma NfL could be used to monitor the central nervous system safety of individuals undergoing HIV eradication interventions that may cause central nervous system viral rebound and neurological symptoms.

1.12.2 Neuroimaging biomarkers of neural activation

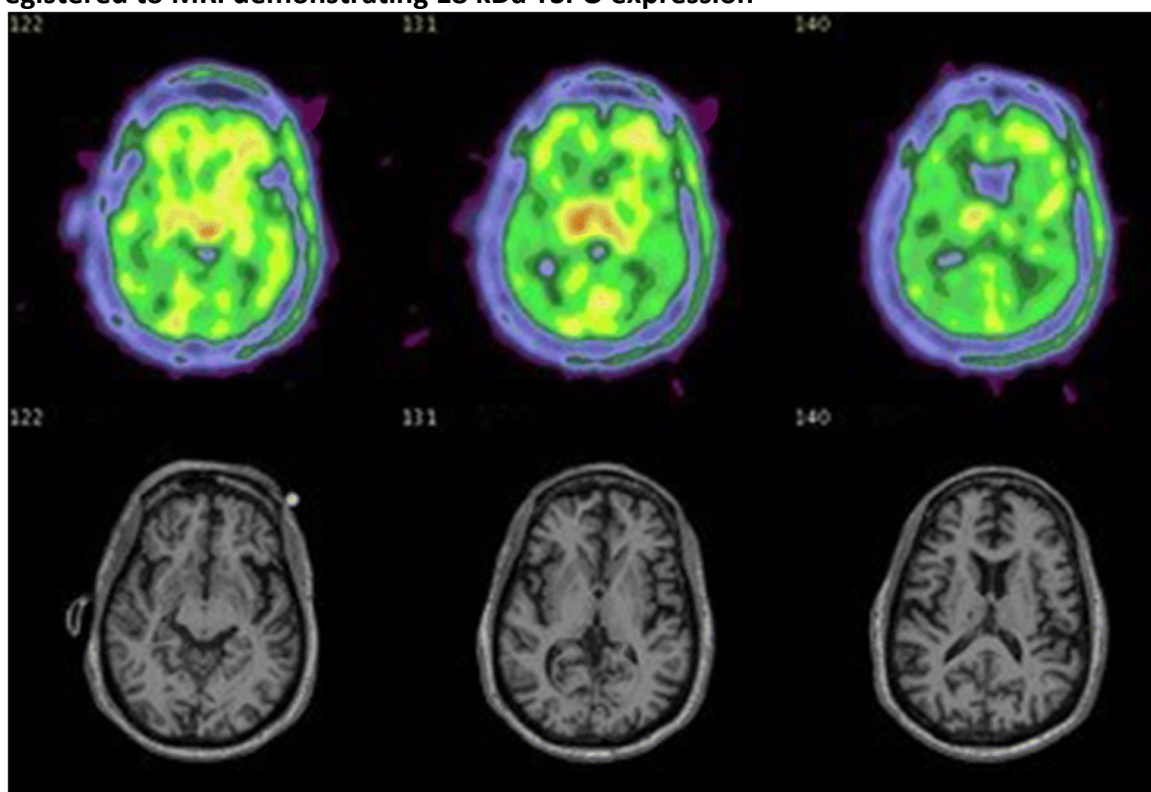
Neuroimaging has the advantage of being able to define anatomical areas of the brain displaying abnormal signal, whereas cerebrospinal fluid and blood measurements present a diluted signal from the brain as a whole. Undeniably, neuroimaging is costly, and thus sample sizes in neuroimaging studies have invariably been smaller compared to studies investigating fluid biomarkers only. However, over the past three decades, molecular neuroimaging studies using positron emission tomography (PET) have increasingly been applied to evaluate neuroinflammation, and more recently, in the investigation of PLWH with cognitive impairment.

1.12.2.1 Positron emission tomography (PET) imaging

During a PET scan, an individual is injected with a radioligand that binds a cellular marker of interest. After a specific period of time, images are acquired to identify the regions of increased radioligand binding (Figure 12). PET brain imaging studies commonly utilise radiotracers specific for the 18kDa translocator protein (TSPO) to investigate neuroinflammation (Figure 12).

Originally described as peripheral benzodiazepine receptor (PBR), TSPO is mainly found on the outer mitochondrial membrane and was initially investigated due to its role in binding benzodiazepines. Subsequently, the TSPO receptor was found to be expressed throughout the body, including the brain and histochemical studies identified that TSPO PET tracer binding co-localised with activated microglia[299–302]. The exact function of TSPO remains unknown, but it is postulated to be involved in cellular processes such as steroidogenesis and inflammation[303]. PET imaging using TSPO radiotracer binding has become the mainstay of molecular imaging of neuroinflammation and has been investigated in a range of conditions including Alzheimer’s disease[304] and multiple sclerosis[305]. Since 2005, several studies have utilised TSPO radiotracers to investigate the potential role of neuroinflammation in the development of HIV-associated cognitive disorders in people living with HIV.

Figure 12: Representative standard uptake value ratio image of a [^{11}C] PBR28 PET scan registered to MRI demonstrating 18 kDa TSPO expression



Abbreviations: PET = positron emission tomography, MRI = magnetic resonance imaging, TSPO = translocator protein. Image reproduced with permission of the rights holder, Springer Nature[306].

1.12.2.1.1 TSPO PET imaging methods

The original first-generation TSPO radioligand, [^{11}C] PK-11195, is still frequently used in neuroimaging studies of neurodegeneration. However, [^{11}C] PK-11195 has high levels of non-specific binding and poor penetration through the blood-brain barrier, causing a low signal to noise ratio [307]. Thus, second generation radiotracers including [^{11}C] PBR28 and [^{11}C] DPA713, were developed with greater TSPO affinity binding, better penetration of the blood-brain barrier and overall higher signal to noise ratios[308].

Further studies have since demonstrated that binding affinities for these second-generation TSPO radiotracers are highly dependent on the single nucleotide polymorphism (SNP) rs6971, an alanine (Ala)-to-threonine (Thr) substitution in the TSPO gene[309]. Individuals who are homozygous for the threonine substitution (Thr/Thr) are designated low-affinity binders and have minimal specific TSPO radiotracer uptake, heterozygotes (Ala/Thr) have reduced TSPO radiotracer uptake and are considered medium-affinity binders, while individuals who are homozygous for Ala/Ala display high specific TSPO radiotracer

binding[310]. In the case of [^{11}C] PBR28, high affinity binders display x50-fold higher binding compared to low affinity binders[311]. Thus, TSPO genotypic analysis prior to PET imaging is essential, and individuals who are low-affinity binders are frequently excluded from second-generation TSPO radiotracer studies.

In order to account for intra-individual differences, the concentration of the TSPO radiotracer in the brain parenchyma can be normalised to the concentration in blood plasma using a metabolite-corrected plasma input function. This requires an arterial line insertion, frequent blood sampling and is costly and time consuming. Alternatively, radiotracer binding values can be normalised to a reference region, however this is problematic as TSPO receptors are expressed throughout the brain and in many conditions, true disease-free regions of the brain have not been identified. Depending on the condition being investigated, the cerebellum, whole brain and cortical grey matter have been used as pseudo-reference regions[312–314].

TSPO PET imaging studies in PLWH

The TSPO PET imaging studies performed in PLWH to date are summarised in Table 5. Results from these studies have been inconsistent, likely related to the different methodologies employed and small sample sizes. Some studies enrolled PLWH with and without cognitive impairment, whilst others only enrolled cognitively unimpaired PLWH. Criteria for defining cognitive impairment was inconsistent, with some studies utilising the Frascati criteria, whilst others used the CogState™ computerised assessment tool or Memorial Sloan-Kettering dementia scale. The studies also approached PET radiotracer uptake measurement analysis differently; firstly, some studies used the regions-of-interest (ROI) based approach, while others used voxel-based analysis, and secondly, different pseudo-reference regions were chosen for normalisation purposes in the different studies.

Overall, the studies have reported varying results when comparing TSPO radiotracer uptake amongst PLWH compared to control participants. Hammoud *et al*[315], Wiley *et al*[316], Garvey *et al* [211](using the ROI approach), Coughlin *et al*[317] and Boerwinkle *et al*[318] observed no significant difference in TSPO ligand binding between cognitively unimpaired PLWH and control participants, but Garvey *et al* (using a voxel-based approach) observed

increased tracer uptake within the corpus callosum, anterior and posterior cingulate, temporal cortex and frontal cortex in cognitively unimpaired PLWH versus control participants and Vera *et al* [210] observed increased TSPO binding in the parietal lobe, occipital lobe and globus pallidus.

When comparing cognitive unimpaired and impaired PLWH, Wiley *et al* and Hammoud *et al* demonstrated no significant difference, but Coughlin *et al* noted increased tracer uptake in the frontal cortex of cognitively impaired PLWH. Furthermore, when comparing cognitive impaired PLWH with control participants, Wiley *et al* noted no significant differences but Hammoud *et al* found increased TSPO binding in the thalamus, putamen, frontal cortex, temporal cortex and occipital cortex.

Table 5: Summary of TSPO PET imaging studies in people living with HIV

	Hammoud <i>et al</i>, 2005[315]	Wiley <i>et al</i>, 2006[316]	Garvey <i>et al</i>, 2014[211]	Coughlin <i>et al</i>, 2014[317]	Vera <i>et al</i>, 2016[210]	Rubin <i>et al</i>, 2018[319]	Boerwinkle <i>et al</i>, 2020[318]
Radiotracer	PK11195	PK11195	PK11195	DPA-713	PBR28	DPA-713	PBR28
Participants (mean age in years, % male)	10 PLWH (45, 100%) 5 control participants (41, 80%)	12 PLWH (49, NA) 5 control participants (44, NA)	7 PLWH (48, NA) 9 control participants (31, NA)	23 PLWH (46, NA) 12 HABs 12 control participants (40, NA)	12 PLWH (42, 100%) 8 HABs 10 control participants (41, 100%)	21 PLWH (48, 81%) 13 HABs Nil control participants	24 PLWH (57, 75%) 13 HABs 13 control participants (59, 69%)
cART status	7 on cART 3 cART naïve	All on cART	All on cART	All on cART	All on cART	All on cART	All on cART
Plasma viral load	NA	9: <50cp/mL 3: >50cp/mL	All <50cp/mL	All <50cp/mL	All <50cp/mL	All <50cp/mL	All <50cp/mL
Cognitive status of PLWH	5 unimpaired, 5 impaired	6 unimpaired, 6 impaired	All unimpaired	8 unimpaired, 12 impaired	All unimpaired	7 unimpaired, 14 impaired	All unimpaired
Radiotracer measurement methodology	Standardised uptake value ratio	Binding potential	Binding potential	Volume of distribution	Distribution volume ratio	Volume of distribution	Binding potential
Reference region	White matter	Cluster with normal ligand kinetics	Normal behaving cortical grey matter	Total grey matter	Cortical grey matter	Total grey matter	Cerebellum cortex, total grey matter, unsegmented white matter

Significant findings of cognitively unimpaired PLWH vs control participants (p<0.05)	No differences noted	No differences noted	ROI analysis: No differences noted Voxel-based analysis: corpus callosum, anterior and posterior cingulate, temporal cortex, frontal cortex	No differences noted	ROI analysis: parietal lobe, occipital lobe, globus pallidus	NA	No differences noted
Cognitive domains significantly correlating in PLWH (p<0.05)	NA	NA	Executive function	NA	Global cognition, speed, accuracy/visual learning and memory/verbal learning	Verbal memory, processing speed /attention/ concentration, executive function, working memory, motor function, visual memory, visual construction	Global cognition, executive function

Abbreviations: PLWH = people living with HIV; NA = not available, cART = combination antiretroviral treatment; pVL = plasma viral load. Adapted with permission of the rights holder, Springer Nature[306]

In terms of the correlation between cognitive domains impairment and TSPO uptake, Garvey *et al* [211] showed a negative correlation between executive function and increased [¹¹C]PK-11195 TSPO uptake in the anterior cingulate, posterior cingulate and corpus callosum and Rubin *et al* [319] noted an association between poorer cognitive performance across a number of cognitive domains including verbal memory, processing speed, attention, concentration, executive function, working memory and motor function and increased [¹¹C]DPA-713 TSPO binding at the frontal, temporal and occipital cortex and hippocampus. On the other hand, Vera *et al* [210] observed a correlation between reduced global cognitive performance and greater [¹¹C] PBR28 TSPO uptake in the hippocampus, amygdala and thalamus.

On the whole, these results suggest that there is an association between TSPO radiotracer uptake with cognitive impairment. The inconsistencies in the precise domains and regions involved may indicate a global, rather than focal nature of neuroinflammation.

1.12.2.2 *Magnetic resonance spectroscopy (¹H-MRS)*

The widely available MRS uses the isotope (¹H) to measure changes in N-acetyl aspartate (NAA) – a neuronal integrity marker[320], choline (Cho) – a marker of cellular proliferation and inflammatory response[321], creatine (Cr) – a marker of brain energy metabolism, myo-inositol (MI) – a measure of glial activation and glutamine(Gln)/glutamate(Glu) – markers of neurotoxicity in different areas of the brain[322]. Numerous studies have demonstrated regional metabolite changes consistent with neuro-axonal injury (reduced NAA) and glial activation (increased MI) with cognitive impairment[323–326], with data to suggest that glial activation occurs prior to neuronal injury[327]. Higher levels of Cho and MI have been noted in neurocognitively intact PLWH[209], but greater increases in Cho and MI, and neuronal loss (NAA/Cr and Gl/Cr) have been noted in PLWH with cognitive impairment[209]. Overall, MRS may afford greater sensitivity compared to conventional structural MR imaging and can be used to monitor response to therapeutics.

1.12.2.3 Diffusion tensor imaging

More recently, studies incorporating diffusion tensor imaging (DTI) have increasingly been used to investigate white matter structural integrity. DTI assesses the diffusion of water molecules within white matter, and changes in axial and radial diffusivity measurements may be utilised to differentiate between axonal and demyelinating damage, respectively. DTI study results in PLWH have produced variable results but in general many studies investigating people living with treated and untreated chronic HIV infection have shown evidence of increased mean diffusivity and decreased fractional anisotropy (suggesting loss of white matter integrity), however the locations of the changes have been variable in the different studies[322]. During very early HIV infection (around the time of cART initiation), DTI data suggests minimal brain structural disturbances[328]. Overall, DTI may be more sensitive than structural T2-weight MRI for detecting signals, in PLWH with normal-appearing white matter on conventional structural MRI.

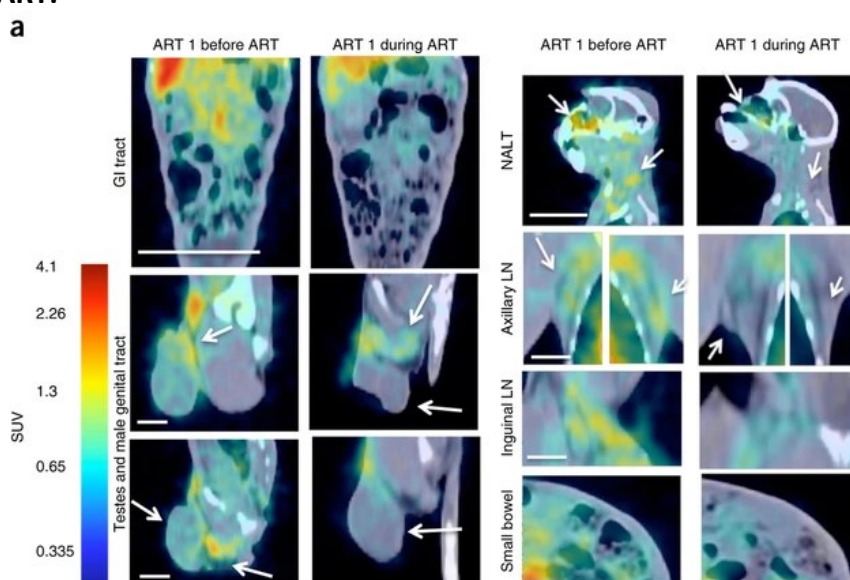
1.12.2.4 Blood oxygen-level dependent (BOLD) fMRI

Changes in the BOLD functional MRI (fMRI) response at different brain regions signify connections between changes in neuronal activity and cerebral blood flow for a particular task[329]. Rises or falls in brain activation during a stimulus compared to a resting state are presumed to be related to the cognitive function being investigated[330]. A systematic meta-analysis review demonstrated that PLWH had greater functional activation in the caudate nucleus and left inferior frontal gyrus compared to HIV-negative control participants, and this was associated with neurocognitive impairment[331]. Overall, BOLD fMRI studies have found that PLWH require the recruitment of additional brain areas to meet the demands of functional tasks[322] and there is evidence in support of HIV-associated premature ageing, with reduced brain activity in PLWH equivalent to HIV-negative controls who were 15-20 years older, reflecting a decrease in brain reserves in PLWH[332].

1.12.3 A novel biomarker of simian immunodeficiency virus (SIV) replication

Recently, a study reported that a novel, real-time *in vivo* PET imaging method utilising a [^{64}Cu]-labelled SIV Gp120-specific antibody radiotracer readily detected SIV replication in the gastrointestinal and respiratory tracts, lymphoid tissue and reproductive organs of viraemic rhesus macaques[108]. Aviraemic, antiretroviral-treated macaques displayed reduced but persistently detectable viral signals in the colon, lymph nodes, small bowel, nasal turbinates, genital tract and lungs (Figure 13). Given the difficulties with accessing brain tissues, a non-invasive biomarker than can identify viral replication within the central nervous system would be invaluable in the longitudinal investigation as to whether residual viral replication drives neuroinflammation, neuronal injury and cognitive impairment. Unfortunately, this particular radiotracer molecule was too large to cross the blood-brain barrier and was unable to identify replicating virus within the central nervous system in this study. Various nanobodies are being investigated for brain-penetrating capabilities to enable to investigation of viral replication and viral reservoir within the central nervous system[333].

Figure 13: PET/CT results from a chronically infected macaque, before and at 5 weeks of ART.



Legend: Standard uptake value (SUV) maps of gastrointestinal tract, genital tract, nasal-associated lymphoid tissue, lymph nodes and small bowel. Arrows indicate areas for which specific PET signals decreased during ART. Abbreviations: PET = positron emission tomography, CT = computed tomography, ART = antiretroviral treatment, NALT = nasal-associated lymphoid tissue. Image reproduced with permission of the rights holder, Springer Nature[108].

1.13 Summary

In summary, mild cognitive impairment in PLWH on virologically suppressive cART remains prevalent. The HIV reservoir and ongoing HIV viral transcription are postulated to drive immune activation and neuroinflammation in the central nervous system, which contributes to neuronal injury and can lead to cognitive impairment. Furthermore, given the interest in HIV remission strategies that may lead to viral rebound, central nervous system monitoring is paramount to prevent the development of neurological sequelae.

In order to further explore the interactions between the HIV reservoir, viral transcription and neural activation markers, I will test my hypotheses using clinical, neuroimaging and soluble biomarker data.

1.14 Thesis aims

The aims of this thesis are to:

- i. Assess the overall correlation between cerebrospinal fluid and blood NfL across a variety of disease processes, and to assess the impact of various factors on the overall correlation
- ii. Assess and compare the correlation between cerebrospinal fluid and blood NfL in PLWH on virologically suppressive cART and in lifestyle-similar HIV-negative individuals, and to identify factors associated with cerebrospinal fluid and plasma NfL in these two cohorts
- iii. Evaluate the associations between measures of HIV reservoir size and HIV viral replication with markers of microglial activation, neuronal injury and cognitive function in neuro-asymptomatic PLWH
- iv. Evaluate the impact of a HIV “shock and kill” intervention on the associations between measures of HIV reservoir size and HIV viral replication with markers of neuronal injury in PLWH
- v. Evaluate the impact of cART interruption on the associations between measures of HIV reservoir size and HIV viral replication with markers of neuronal injury in PLWH

Chapter 2

Materials and Methods

Chapter 2: Materials and Methods

2.1 Clinical studies

Work performed during my PhD utilised data and biological samples obtained from one clinical study which I completed during my PhD (PET in acute HIV infection (AHI)) and four previously completed clinical studies (COBRA, SPARTAC, RIVER and PET in chronic HIV infection (CHI)). The separate studies are comprehensively described below.

2.1.1 [¹¹C] PBR28 PET in treated acute HIV infection (PET in AHI)

2.1.1.1 *Study design*

The PET in AHI study was a single-site, cross-sectional observational study investigating cerebral [¹¹C] PBR28 binding by PET-CT imaging in individuals who initiated cART during acute HIV infection. Comparisons were made with cerebral [¹¹C] PBR28 binding in individuals who commenced cART during chronic HIV infection (see PET in CHI study below) and in control participants. Additionally, associations between cerebral [¹¹C] PBR28 binding with participant demographics, clinical parameters, routine clinical laboratory parameters, research biomarkers and cognitive function test scores were determined.

2.1.1.2 *Participants and recruitment procedures*

I was responsible for the recruitment of participants into the PET in AHI study. Adult male participants aged ≥18 years enrolled into the **HIV Reservoir Targeting with Early Antiretroviral Therapy** (HEATHER) study were approached and invited to enrol into the PET in AHI study. The HEATHER study is a prospective observational study of a cohort of individuals who initiated cART within three months of HIV acquisition and were recruited at three HIV clinical centres based in London, UK: St Mary's Hospital, Guys and St Thomas' Hospital and Chelsea & Westminster Hospital[334]. Participants were enrolled into the PET in AHI study between Oct 2016 and June 2019. Participants were required to be able to communicate in spoken English, receiving cART for ≥3 months with plasma HIV RNA <50 copies/mL at screening and high affinity binders to [¹¹C] PBR28 on TSPO genotypic testing.

Exclusion criteria included active opportunistic infection, significant co-morbidities including neurological conditions and contraindication to arterial cannulation, lumbar puncture or MRI.

Data on cerebral [^{11}C] PBR28 binding in control participants (between the ages of 20 to 60 years) were obtained from previous studies conducted at the Imanova Centre for Imaging Sciences, which has since been acquired by Invicro LLC. Exclusion criteria for these studies were similar to those of the HIV-positive cohort. Notably, the control participants were presumed to be in good health, but HIV testing was not performed as part of the screening procedures. Furthermore, the control participants were not age- and gender-matched to the participants in the PET in AHI and PET in CHI studies, and apart from age, gender, weight and TSPO affinity binding status, no other background information was available for the control participants, including lifestyle behaviour, e.g., alcohol intake and recreational drug use.

2.1.1.3 *Study procedures*

At the screening visit, the following details were collected from the participants: demographics, socioeconomic status, medical history, medication list, recreational drug and alcohol use and anthropometric assessment. HIV-specific information (including current CD4⁺ and CD8⁺ T-cell counts, nadir CD4⁺ T-cell count, current plasma HIV RNA and highest plasma HIV RNA measurement before initiating cART) were obtained from the participants' electronic hospital records. Blood samples were collected and tested for TSPO genotype, haematology and coagulation profile.

Following enrolment, the following procedures were performed: cerebral PET-CT imaging with [^{11}C] PBR28 ligand, cognitive function testing, and blood and cerebrospinal fluid collection.

I performed the lumbar puncture procedures for cerebrospinal fluid collection within three months of the cerebral PET-CT scanning visit. Lumbar punctures were performed as a day case procedure at St Mary's Hospital campus, Imperial College London, using standard

procedures and under aseptic conditions with local anaesthesia (2-5mL 2% lignocaine). 15mL cerebrospinal fluid was collected in universal containers.

2.1.1.4 *Sample collection and analysis*

The following measurements were performed on the participants' fluid samples after the cerebral PET-CT imaging visit:

- i. **Neurofilament light chain protein (NfL):** in plasma and cerebrospinal fluid (see below)
- ii. **Protein, glucose, leukocytes:** in cerebrospinal fluid, measured at the Clinical Biochemistry and Microbiology laboratories at Imperial College Healthcare National Health Service (NHS) Trust, London
- iii. **HIV RNA:** in cerebrospinal fluid, measured at the Virology laboratories at Imperial College Healthcare NHS Trust, London), using the Roche Amplicor assay (Roche Holding AG, Basel, Switzerland), with a lower limit of detection of 20 copies/mL

Remaining blood and cerebrospinal fluid were centrifuged for 10 minutes (2,000G for blood and 400G for cerebrospinal fluid with brake) at 20°C, and the supernatant was divided into ampoules (maximum 1.0mL per ampoule) and stored at -80°C.

2.1.1.5 *Funding, ethical approval and consent*

The PET in AHI study was funded through a National Institute of Health Research (NIHR) Biomedical Research Centre (BRC) grant via the Imanova IMPETUS programme. The study was approved by a UK Research Ethics Committee (REC) (reference number: 16/LO/0096, Camden & Kings Cross, London). Permission was obtained from the UK Administration of Radioactive Substances Advisory Committee (ARSAC) (reference number: RPC 630/3764/34269) for the administration of [¹¹C] PBR28. All participants were screened, consented and assessed for study eligibility by me, they provided written informed consent prior to commencing any study procedures and consented for future use of their stored fluid samples for bioresearch purposes.

2.1.1.6 Specific tasks I performed within this study for my PhD

I contacted eligible participants who had indicated that they would be interested in being approached about the study. I explained the study purpose and procedures to the interested participants and gave them a copy of the participant information sheet. I offered the participants a follow-up telephone call to discuss the study further, before arranging a screening visit appointment. At the screening visit, I reviewed the participant's understanding of the study and eligibility. If eligible and the participant consented to take part, I obtained the participant's written informed consent and undertook the screening visit study procedures: participant blood sampling for full blood count, coagulation profile and TSPO genotype, participant completion of Lawton's activities of daily living questionnaire, a full physical examination of the participant including Allen's test and organised for the participant to complete a practice computerised cognitive function test session using Cogstate™. Once the participant's ongoing eligibility is confirmed from the blood test results (only high-affinity binders on the TSPO genotype testing were eligible to enrol), I organised the participant's cerebral MRI and PET-CT imaging and provided medical supervision cover during the participant's imaging visit at the Invicro LLC research imaging facility. I ensured the cerebral MR scans were reported by an NHS consultant neuroradiologist and if no contraindications to lumbar puncture were identified, the participant was invited to attend a third visit, where I performed the lumbar puncture for cerebrospinal fluid collection, blood sampling and supervised the participant during their computerised cognitive function testing using Cogstate™. I organised for stored plasma and cerebrospinal fluid samples to be shipped to the Fluid Biomarkers for Neurodegenerative Diseases laboratory, UK Dementia Research Institute, University College London, UK for NfL measurement by our collaborators. I analysed the cerebral [¹¹C] PBR28 ligand binding by PET-CT using the MIAKAT pipeline, provided by Invicro LLC. I curated the results database comprising the demographic and clinical data from the study case report forms, plasma and cerebrospinal fluid NfL measurement, the [¹¹C] PBR28 ligand binding by cerebral regions of interest data and cognitive function test scores and performed data clean-up. I then performed the data analysis and interpreted the results. Throughout the course of the study, I was responsible for submitting protocol amendments to the NHS Health Research

Authority (requesting study extensions and minor protocol changes), award extension requests to the funder (National Institute of Health Research (NIHR) Imperial Biomedical Research Centre (BRC)) and annual update reports to the funders (as above) and the UK Research Ethics Committee.

2.1.2 [¹¹C] PBR28 PET in treated chronic HIV (PET in CHI)

2.1.2.1 *Study design*

The PET in CHI study was a single-site, cross-sectional observational study investigating cerebral [¹¹C] PBR28 binding by PET-CT in individuals commenced on cART during chronic HIV infection[210]. The published results compared cerebral [¹¹C] PBR28 binding with control participants, and associations with cognitive function test scores and fluid biomarkers were determined.

2.1.2.2 *Participants and recruitment procedures*

Male patients between the ages of 20 to 50 years attending the HIV outpatient department for routine clinical care at St Mary's Hospital, Imperial College Healthcare NHS Trust were invited to participate. At screening, participants were required to be HIV seropositive for at least two years and receiving stable cART with a plasma HIV RNA of <50 copies/mL. Participants with low affinity binding status to TSPO on genotype testing and/or concurrently using anti-inflammatory drugs, benzodiazepines, recreational drugs or excessive alcohol intake were excluded. Participants with a current or previous history of neurological or psychiatric conditions including depression or anxiety, were excluded. Screening and enrolment into the study took place between 2012 and 2014 and 12 participants were enrolled.

2.1.2.3 *Study procedures*

At the screening visit, details including demographics, socioeconomic status, medical history, recreational drug and alcohol use, medication list, anthropometric assessment were collected. HIV-specific information including current and nadir CD4⁺ and CD8⁺ T-cell counts,

and pre-treatment and current plasma HIV RNA was obtained from the participants' hospital records.

Following enrolment, cerebral PET-CT imaging with [^{11}C] PBR28 ligand was performed (details below) (designated the 'PET visit'). On a separate day, participants returned for blood and cerebrospinal fluid collection and cognitive function testing (designated the 'lumbar puncture visit').

2.1.2.4 *Sample collection and analysis*

Blood samples were collected at the screening visit and the lumbar puncture visit. At screening, blood was tested for TSPO genotype, haematology and coagulation profile.

Cerebrospinal fluid was collected following enrolment and the following parameters were measured: protein, glucose, leukocytes and HIV RNA at the Clinical Biochemistry, Microbiology and Virology laboratories at St Mary's Hospital, Imperial College Healthcare NHS Trust, London. Cerebrospinal fluid HIV RNA was measured using the real-time polymerase chain reaction (RT-PCR) (Qiagen probe PCR), with a lower limit of detection of 20 copies/mL. Concurrent blood samples were collected in EDTA bottles following cerebrospinal fluid collection. Remaining blood and cerebrospinal fluid were centrifuged for 10 minutes (1500G, 3000 rpm) in a 4°C refrigerated centrifuge. Plasma and cerebrospinal fluid samples were then transferred to storage tubes and stored at -80 °C.

2.1.2.5 *Funding, ethical approval and consent*

The PET in CHI study was funded through a Wellcome Trust Translational Medicine and Therapeutics Fellowship (recipient: Dr Jaime Vera (JV)). The study was approved by the National Research Ethics Service (12/LO/1570). Permission was obtained from the UK Administration of Radioactive Substances Advisory Committee (ARSAC reference number: 630/3764/29163) for the administration of [^{11}C] PBR28. All participants provided written informed consent before commencing any study procedures and consented for future use of their stored fluid samples for research purposes.

2.1.2.6 *Specific tasks I performed within this study for my PhD*

I obtained permission from JV to retrieve and utilise the clinical, neuroimaging, cognitive function and laboratory parameter data, and stored plasma and cerebrospinal fluid samples he obtained from his study. Specifically, I obtained and reanalysed the raw data from his cerebral [^{11}C] PBR28 ligand binding on PET-CT using the MIAKAT pipeline provided by Invicro LLC and reanalysed the raw data from his computerised cognitive function test results using the combined PET in AHI and PET in CHI cohorts' normative values, rather than normative values from the general population as reported in his PhD thesis. I retrieved stored plasma and cerebrospinal fluid samples from participants who had consented for their data and samples to be used in future studies and arranged for the measurement of plasma and cerebrospinal fluid NfL at the Fluid Biomarkers for Neurodegenerative Diseases laboratory, University College London. I obtained the previously collected demographic and clinical data from JV, curated the results for plasma and cerebrospinal fluid NfL measurement, cognitive function test scores and the [^{11}C] PBR28 ligand binding by cerebral regions of interest data into a results spreadsheet and performed data clean-up. I then performed the data analysis and interpreted the results.

2.1.3 The COBRA cohort

2.1.3.1 *Study design*

The **CO**mor**B**idity in **R**elation to **A**IDS (COBRA) clinical cohort is a multi-site, prospective, longitudinal study investigating the potential causal link between HIV and age-associated non-communicable comorbidities in people living with HIV aged ≥ 45 years on suppressive combination antiretroviral treatment (cART) and otherwise similar HIV-negative control participants[335]. The objectives of the COBRA study were to: 1) investigate the association between HIV disease and age-associated non-communicable comorbidities, 2) explore causation and pathogenic mechanisms underlying any links identified, 3) elucidate the potential accelerated ageing phenotype and 4) identify biomarkers that may be utilised for prevention, treatment and management of age-associated non-communicable comorbidities.

2.1.3.2 Participants and recruitment procedures

All COBRA participants were required to be aged 45 years or over. HIV-positive participants had to be on cART and had to have plasma HIV RNA <50 HIV RNA copies/mL recorded for at least 12 months prior to enrolment. In total, 134 HIV-positive participants were recruited from HIV treatment centres in Amsterdam, The Netherlands and London, UK. Seventy-nine HIV-negative control participants with similar demographic and lifestyle behaviours to the HIV-positive group were recruited from sexual health clinics and targeted community groups[335]. Specific exclusion criteria included current major depression, confounding neurological diseases, history of severe head injury, history of cerebral infections, current intravenous drug use, daily recreational drug use, excess alcohol intake and severe psychiatric conditions. Study recruitment took place between January 2013 - October 2014.

2.1.3.3 Baseline study visit

At the first study visit (baseline), all participants underwent a comprehensive assessment and details collected included demographics, socioeconomic status, medical history, details of lifestyle, use of medication, anthropometric assessment and detailed cognitive function assessment[335]. Current and historical information on antiretroviral treatment, CD4⁺ and CD8⁺ T-cell count and plasma HIV RNA were obtained for all HIV-positive participants from national Dutch and UK HIV databases (<https://www.hiv-monitoring.nl/nl> and <http://www.ukchic.org.uk/>, respectively)[335].

2.1.3.4 Sample collection and analysis

Biological samples collected at baseline included blood and cerebrospinal fluid. The following biomarkers were measured as part of the original study[335]:

- i. **Blood:** CD4⁺ and CD8⁺ T-cell counts, albumin, creatinine, HIV RNA and neopterin
- ii. **Cerebrospinal fluid:** albumin, protein, leukocytes, glucose, HIV RNA, neopterin and NfL

I measured plasma NfL (see below) separately, as part of my PhD.

2.1.3.5 Funding, ethical approval and consent

The COBRA study was funded through a European Union Seventh Framework Programme grant (agreement number: 305522). This study was approved by the institutional review board of the Academic Medical Center (AMC) (reference number: NL 30802.018.09) and a UK Research Ethics Committee (REC) (reference number: 13/LO/0584 Stanmore, London). All participants gave written informed consent before commencing any study procedures and consented for future use of their stored fluid samples for research purposes.

2.1.3.6 Specific tasks I performed within this study for my PhD

I submitted a proposal plan to the COBRA Steering Committee requesting permission to obtain and use stored plasma samples and previously obtained demographic, clinical and laboratory parameter data. I applied for and was successfully granted funding worth £4,800 from Imperial Health Charity to cover the cost of plasma NfL measurement in the COBRA cohort participants. I organised the retrieval of stored plasma samples from the COBRA cohort participants and arrange courier shipment to the Fluid Biomarkers for Neurodegenerative Diseases laboratory, UK Dementia Research Institute, University College London, UK. Originally, the plan was for me to perform the plasma NfL measurement assays at University College London but due to restrictions following the COVID-19 pandemic, I was unable to gain access to the laboratory at University College London. Therefore, the plasma NfL measurement assay was performed by a research technician at the Fluid Biomarkers for Neurodegenerative Diseases laboratory, University College London, on my behalf. I obtained the plasma NfL measurement results from the reporting laboratory and the demographic, clinical and previously performed laboratory parameter data from the COBRA study biostatistician, then curated and organised the data and performed data clean-up. I then performed the statistical analysis and interpreted the results.

2.1.4 The RIVER trial

2.1.4.1 *Study design*

The **R**esearch **I**n **V**iral **E**radication of HIV **R**eservoirs (RIVER) trial (ClinicalTrials.gov registry number: NCT02336074) was a phase 2, open-label randomised controlled trial investigating a “shock and kill” approach towards an HIV cure[336]. The primary aim of the RIVER trial was to compare cART combined with a “shock and kill” strategy utilising the latency-reversing HDACi vorinostat (“shock”) and a prime-boost vaccination with ChAdV63.HIVconsv and MVA.HIVconsv[337] (“kill”), with cART-alone, on measures of the HIV reservoir in individuals initiating cART during primary HIV infection[336].

2.1.4.2 *Participants and recruitment procedures*

Participants aged between 18 to 60 years with recently acquired HIV infection and cART initiation within one month of confirmed HIV diagnosis were randomly assigned 1:1 to receive ART-alone (control) or ART plus vaccination with ChAdV63.HIVconsv prime and MVA.HIVconsv boost (given eight weeks apart) followed by vorinostat taken orally in 10 doses of 400mg every 3 days for 28 days (ART+V+V)[338]. Recruitment was undertaken at six clinical sites in the UK between December 2015 and November 2017. Participants with viral co-infections (hepatitis B, hepatitis C and HTLV) and concurrent malignancy or opportunistic infections were excluded.

Recent HIV infection was defined as: positive HIV-1 serology within 12 weeks of negative HIV-1 serology or point-of-care test, positive p24 antigen and a negative HIV-1 serology test, negative HIV-1 antibody test with detectable HIV RNA or proviral DNA, Public Health England recency HIV antibody avidity assay reported as incident (<16 weeks prior to enrolment), weakly reactive or equivocal fourth generation HIV antibody-antigen test, or equivocal or reactive HIV antibody test with less than 4 bands on a Western Blot analysis[336].

2.1.4.3 *Study visits*

After enrolment, all participants were commenced on a four-drug cART regimen, including raltegravir. Randomisation to continue cART-alone or to receive cART plus “shock and kill”

took place after participants had been on cART for ≥ 22 weeks. Following randomisation, all participants were followed up for 18 weeks. Study visits included assessment of adverse events, adherence to medication and anthropometric measurement.

2.1.4.4 Sample collection and analysis

Peripheral blood measurements of haematology, biochemistry (including creatinine), HIV RNA, CD4⁺ and CD8⁺ T-cell counts, HIV DNA in CD4⁺ T-cells (total and integrated), single-copy HIV RNA assay, cell-associated unspliced HIV RNA in CD4⁺ T-cells and HIVconsv-specific phenotype and cytokine secretion profiles of CD4⁺ and CD8⁺ T-cells via intracellular cytokine staining to detect IFN- γ , TNF- α , CD107a, IL-2 and CD154 expression were performed at specific study timepoints as part of the study[336]. For the purposes of my PhD, I measured plasma NfL at randomisation, and weeks 12 and 18 post-randomisation.

2.1.4.5 Funding, ethical approval and consent

The RIVER study was funded by the Medical Research Council (grant number: MR/L00528X/1) and all participating sites obtained ethics committee approval (reference number: 14/SC/1372 Oxford A, South Central). All participants provided written informed consent prior to enrolment and consented for their samples to be utilised for future research purposes.

2.1.4.6 Specific tasks I performed within this study for my PhD

I submitted a proposal plan to the RIVER Trial Management Group requesting permission to obtain and use stored plasma samples and demographic, clinical and laboratory parameter data previously obtained within the context of the RIVER trial, for the purposes of my sub-study. I applied for and was successfully granted funding worth £27,584 from the British HIV Association (BHIVA) to cover the cost of plasma sample retrieval, shipment and NfL measurement in the RIVER and SPARTAC (see below) trial participants. I organised the retrieval of stored plasma samples from the RIVER trial participants, stored at the Peter Medawar Building for Pathogen Research, Nuffield Department of Medicine, University of Oxford, UK, and arrange for the plasma samples to be shipped to the Fluid Biomarkers for

Neurodegenerative Diseases laboratory, UK Dementia Research Institute, University College London, UK. I assisted a research technician with performing the plasma NfL measurement assays at University College London. I obtained the plasma NfL measurement results from the reporting laboratory and the demographic, clinical and previously performed laboratory parameter data from the RIVER trial biostatistician, then organised the data and performed data clean-up. I then performed the data analysis and interpreted the results.

2.1.5 The SPARTAC trial

2.1.5.1 *Study design*

The **Short Pulse Anti-Retroviral Therapy At SeroConversion** (SPARTAC) trial (registry number: ISRCTN76742797) was a multi-site, open-label, randomised controlled trial designed to evaluate whether a short course of cART initiated during primary HIV infection can lengthen the time until patients reach a CD4⁺ T-cell count of <350 cells/mm³ or require long-term cART compared with not receiving immediate cART[140].

2.1.5.2 *Participants and recruitment procedures*

Participants were required to be adults with primary HIV infection at enrolment and were recruited across seven countries: UK, Italy, Spain, South Africa, Uganda, Australia and Brazil, between 2003 and 2007. In total, 366 cART-naïve participants with primary HIV infection were randomised 1:1:1 to receive either immediate cART for 12 weeks, immediate cART for 48 weeks or no immediate cART (standard of care at the time)[140]. Individuals in whom cART was indicated were not eligible. Participants randomised to receive cART were recommended to receive two nucleos(t)ide reverse-transcriptase inhibitors (NRTIs) plus a ritonavir-boosted protease inhibitor (PI).

Primary HIV infection was defined as meeting at least one of the following criteria: a positive HIV-antibody test within 6 months after a negative HIV test, a negative HIV-antibody test with a detectable plasma HIV RNA, low level of HIV antibodies (optical density units <0.6) (according to a serological testing algorithm for recent subtype B strain infection only), an equivocal HIV-antibody test with a repeat test two weeks later demonstrating a rise in the level of HIV antibodies or clinical evidence of symptomatic HIV seroconversion illness

supported by antigen positivity and less than four positive bands on Western blot analysis[140].

2.1.5.3 *Study visits*

Participants attended for study visits at weeks 0 (baseline), 4, 12, 16, 24, and every 12 weeks thereafter until November 2010, when the 360th participant had completed 3.5 years of follow up. At the first study visit (baseline), all participants underwent a comprehensive medical assessment and details collected included demographics, medical history, use of medication and anthropometric assessment. At each subsequent study visit, clinical history since the last assessment, adherence to trial medication, use of concomitant medication, HIV and non-HIV related adverse events, AIDS events, occurrence of pregnancy, physical examination (as required) and anthropometric assessments were recorded.

2.1.5.4 *Sample collection and analysis*

Blood samples were collected at each scheduled study visit with the following tests performed as part of the study, at every timepoint: full blood count, biochemistry (including creatinine), CD4⁺ and CD8⁺ T-cell counts, HIV RNA, and at specific timepoints: plasma D-dimer, plasma interleukin-6 (IL-6) and total and integrated HIV DNA in circulating CD4⁺ T-cells. The lower limit of detection for plasma HIV RNA was 50 copies/mL, except in South Africa and Uganda where it was 400 copies/mL. Plasma was also stored for future research purposes at the King's College London Infectious Diseases BioBank. As part of my PhD, I measured plasma NfL at weeks 0 (baseline), 48, 60 and 84.

2.1.5.5 *Funding, ethical approval and consent*

The SPARTAC trial was funded by the Wellcome Trust (grant number: 069598/Z/02/Z). The trial was approved by research ethics committees in each country and all participants gave written informed consent prior to enrolling into the study including consent for the future use of their stored blood samples for research purposes.

2.1.5.6 Specific tasks I performed within this study for my PhD

I submitted a proposal plan to the SPARTAC Trial Management Group and applied for approval to obtain and utilise stored plasma samples and demographic, clinical and laboratory parameter data previously obtained within the context of the SPARTAC trial, for the purposes of this sub-study. I applied for and was successfully granted funding worth £27,584 from the British HIV Association (BHIVA) to cover the cost of plasma sample retrieval, shipment and NfL measurement in the RIVER (see above) and SPARTAC trial participants. I organised the retrieval of stored plasma samples from the SPARTAC trial participants, stored at the King's College London Infectious Diseases Biobank, London, UK and the Peter Medawar Building for Pathogen Research, Nuffield Department of Medicine, University of Oxford, UK. I organised for the baseline, week 48 and week 60 plasma samples to be couriered to the Clinical Neurochemistry Laboratory at Sahlgrenska University Hospital, Sweden, while the week 84 plasma samples were shipped to the Fluid Biomarkers for Neurodegenerative Diseases laboratory, UK Dementia Research Institute, University College London, UK at a later date. I assisted a research technician with performing the plasma NfL measurement assays at University College London. I obtained the plasma NfL measurement results from the reporting laboratory and the demographic, clinical and previously performed laboratory parameter data from the SPARTAC trial biostatistician, then organised the data and performed data clean-up. I then performed the statistical analysis and interpreted the results.

2.1.6 Summary of the clinical studies which provided data included in my PhD thesis and summary of my role within the clinical studies

Table 6 provides a summary of the clinical studies which contributed data presented in my PhD thesis. On the following page, Table 7 summarises my role within these studies for the purposes of my PhD thesis.

Table 6: Summary of the clinical studies included in my PhD thesis

Clinical study	Population studied in my PhD
COBRA	- Adults aged ≥ 45 years living with HIV on suppressive cART - Lifestyle-similar HIV-negative control participants
PET in AHI	Individuals who initiated cART within three months of acquiring HIV infection
PET in CHI	Individuals who initiated cART beyond three months of acquiring HIV infection
RIVER	Individuals who initiated immediate cART during primary HIV infection and were randomly allocated to receive either: - cART-alone - cART plus vaccination and vorinostat
SPARTAC	Individuals who were randomly allocated to initiate immediate cART for 48 weeks during primary HIV infection followed by a protocol-indicated treatment interruption

Abbreviations: cART = combination antiretroviral treatment; PET = positron emission tomography; AHI = acute HIV infection; CHI = chronic HIV infection

Table 7: Summary of clinical studies included in my PhD, my specific role within the studies for the purposes of my PhD thesis and biomarkers measured within the studies and utilised for my PhD thesis

Clinical study	Conceptualised the original idea for the substudy	Sought permission from the study committees to perform the proposed substudy	Obtained funding to conduct the substudy	Submitted protocol amendments and annual updates to Research Ethics committee and funder	Responsible for the recruitment, follow-up of study participants and clinical data collection	Performed blood and CSF collection	Organised sample retrieval and shipment to the appropriate laboratory	Plasma NfL	CSF NFL	Plasma HIV RNA ²	CSF HIV RNA ²	CD4 ⁺ and CD8 ⁺ T-cell counts	Ultra-sensitive plasma HIV RNA	Total HIV DNA in CD4 ⁺ T-cells isolated from PBMCs	Cerebral [¹¹ C] PBR28 binding on PET-CT	Supervised cognitive function testing	Curated the (sub)-study database	Planned and performed data analysis, performed results interpretation
COBRA	X	X	X				X	X	X ¹	X ¹	X ¹	X ¹				X ¹	X	X
PET in AHI				X	X	X	X	X	X	X ³	X	X ³			X	X	X	X
PET in CHI		X					X	X	X	X ³	X ¹	X ¹			X ¹	X ¹	X	X
RIVER	X	X	X				X	X		X ¹		X ¹	X ¹	X ¹			X	X
SPARTAC	X	X	X				X	X		X ¹		X ¹		X ¹			X	X

¹ Performed previously within the original study. ² Commercial HIV RNA assay: depending on the study, the lower limit of quantification was 400 copies/mL, 200 copies/mL, 50 copies/mL or 20 copies/mL. ³ Obtained from the participants' electronic patient hospital records, with consent. Abbreviations: NfL = neurofilament light chain protein; CSF = cerebrospinal fluid; PBMCs = peripheral blood mononuclear cells; PET = positron emission tomography; CT = computed tomography

2.2 Cerebral imaging

2.2.1 Cerebral magnetic resonance imaging

For the purposes of the PET in AHI and PET in CHI studies, T1-weighted whole brain structural images were obtained on a Siemens MAGNETOM® Verio 3.0 Tesla magnetic resonance scanner (Siemens Healthineers, Munich, Germany).

2.2.2 Cerebral positron emission tomography (PET) imaging with [^{11}C] PBR28

Cerebral [^{11}C] PBR28 ligand binding on PET-CT imaging was performed at the Invicro Clinical Imaging Facility, Burlington Danes Building, Hammersmith Hospital, London, UK.

2.2.2.1 *Radiotracer synthesis*

[^{11}C] PBR28 was produced on site at the Invicro Clinical Imaging Facility immediately before use, according to local standard operating procedures. Quality assurance assessments were made using validated procedures in accordance with good manufacturing practices before injection to ensure the manufactured [^{11}C] PBR28 met the prerequisite specifications.

2.2.2.2 *Arterial blood sampling and processing*

Prior to arterial line placement, all participants underwent an Allen's test to confirm ulnar and radial artery collateral circulation in both hands. Following skin infiltration with 1% lidocaine as local anaesthetic, a cannula was inserted into the participant's radial artery to enable regular arterial blood sampling throughout the cerebral PET-CT imaging procedure.

2.2.2.3 *PET imaging data acquisition*

The cerebral [^{11}C] PBR28 PET-CT images were acquired using a Biograph 6 PET-CT scanner (Siemens Healthcare). Participants were injected with an intravenous bolus of [^{11}C] PBR28 over 20 seconds at the beginning of the 90-minute 3D- mode of dynamic PET acquisition; injected activities ranged from 120.45 to 374.45 MBq.

2.2.2.4 *Blood data processing*

Arterial blood was sampled to enable generation of an arterial plasma input function. A continuous sampling system (ABSS Allogg, Mariefred, Sweden) was used to measure whole blood activity each second for the first 15 minutes of each scan. Discrete arterial blood samples were manually withdrawn at 5, 10, 15, 20, 30, 50, 70 and 90 minute timepoints after the scan commenced to measure whole blood and plasma activity. Samples obtained at 5, 10, 20, 30, 50, 70 and 90 minute time points after the scan commenced were analysed using high-performance liquid chromatography (HPLC) to determine the fraction of parent radioactivity in the arterial plasma. The first three discrete blood samples were used to calibrate the continuous blood data, then the continuous and discrete datasets were used to form a whole blood activity curve, covering the duration of the scan. Discrete plasma samples were divided by the corresponding whole blood samples to obtain the plasma-over-blood (POB) data.

An exponential approach to a constant POB model was fitted[339], to generate the metabolite corrected plasma input function. This POB value was then multiplied by the whole blood curve to generate a total plasma curve. Parent fraction data were fitted to a sigmoid2 model:

$$f = \left(\left(1 - \frac{t^3}{t^3 + 10^a} \right)^b + c \right) / (1 + c)$$

where t = time, and a , b and c are fitted parameters. The resulting fitted parent fraction profile was multiplied by the total plasma curve and then smoothed post-peak using a tri-exponential fit to derive the required parent plasma input function. For each scan, a time delay correction was fitted and applied to the input function to account for any time delay between blood sample measurement and the tomographic measurements of the tissue data. Free fraction in plasma (f_p) was measured through ultrafiltration (Amicon Ultra regenerated cellulose MWCO 30 kDa, Millex, Ireland) in triplicate using Tris buffer (0.1M, pH = 7.4) to determine and enable correction for non-specific binding.

2.2.2.5 *PET image processing*

PET data were reconstructed using filtered back projection, corrected for attenuation and scatter (based on low-dose CT acquisition scan). Dynamic data were binned into 26 frames (durations: 8 x 15 seconds, 3 x 1 minute, 5 x 2 minutes, 5 x 5 minutes and 5 x 10 minutes).

Motion correction in the dynamic PET data was performed via frame-to-frame image registration of the non-attenuation corrected PET image to the participants' structural T1-magnetic resonance image using SPM5 (Wellcome Trust Centre for Neuroimaging), with a mutual information cost function.

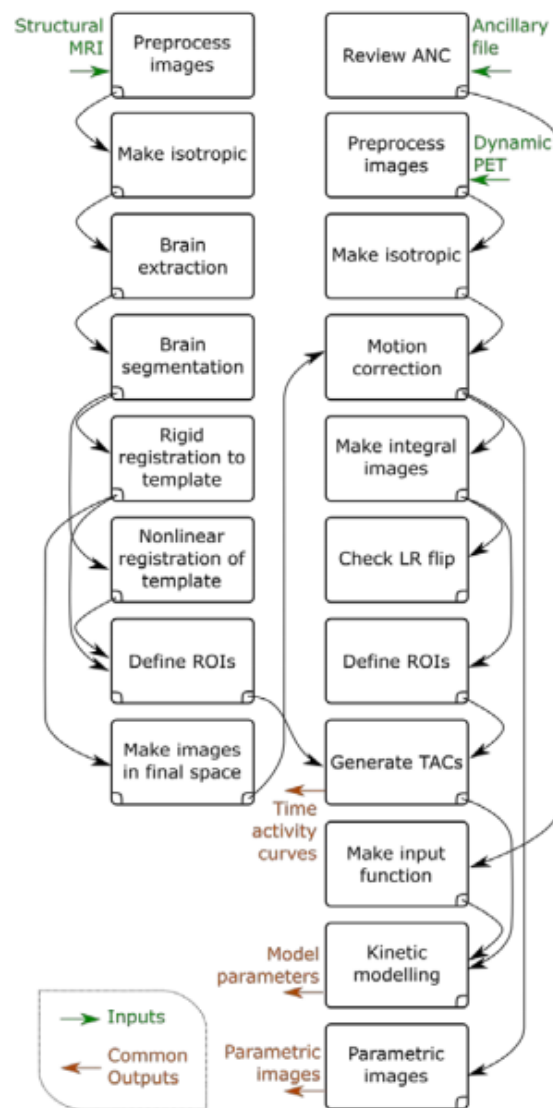
The CIC neuroanatomical atlas version 2.0 was nonlinearly deformed into the participant's space, via structural T1-MRI data mapping, to obtain a personalised anatomical parcellation of regions of interest (ROIs). The following ROIs were chosen to assess levels of binding based on published studies investigating TSPO in people with HIV: *whole brain, occipital lobe, insular cortex, temporal lobe, amygdala, hippocampus, frontal lobe, cingulate cortex, posterior cingulate cortex, anterior cingulate, parietal lobe, subcortical matter, basal ganglia, globus pallidus, striatum, left striatum, right striatum, caudate, left caudate, right caudate, putamen, left putamen, right putamen, thalamus, cerebellum, brainstem, midbrain, pons, medulla and cerebral white matter*. Each ROI was then applied to the dynamic PET data to derive regional time-activity curves.

2.2.2.6 *Kinetic modelling*

A two-tissue compartment model using the metabolite corrected plasma input function was applied to the dynamic PET data using a fixed blood volume correction of 5%. For each ROI evaluated, the total volume of distribution (V_T) was estimated from the rate constant as described previously[339]. Model fitting and parameter estimation was performed by myself using the MIAKAT™ pipeline, implemented in MATLAB™ R2019b (The MathWorks, Natick, MA, USA).

Figure 14 illustrates each process of the MIAKAT analysis pipeline for the analysis of the brain [^{11}C] PBR28 PET data. My role in the analysis was to verify that the processes were being carried out correctly at each step along the pipeline.

Figure 14: Overview of the MIAKAT analysis pipeline



Legend: Detailed schematic of the analysis pipeline. The black arrows denote the dependency on the process on each other; a process can only start when all processes pointing to it are complete. Reproduced with permission from the creators of MIAKAT™.

2.3 Cognitive function testing

For the PET and AHI and PET in CHI studies, cognitive function testing was performed in individuals with HIV using CogState™ (CogState Ltd, Melbourne, Australia), a commercially available computerised cognitive test battery designed to measure cognitive performance, covering seven cognitive domains. The CogState™ tool has been validated and shown to be a sensitive diagnostic tool for the assessment of HIV-associated cognitive impairment [340,341].

2.3.1 Test situation

The CogState™ test battery consisted of a series of tasks in the form of card games and shape-based puzzles to minimise language differences.

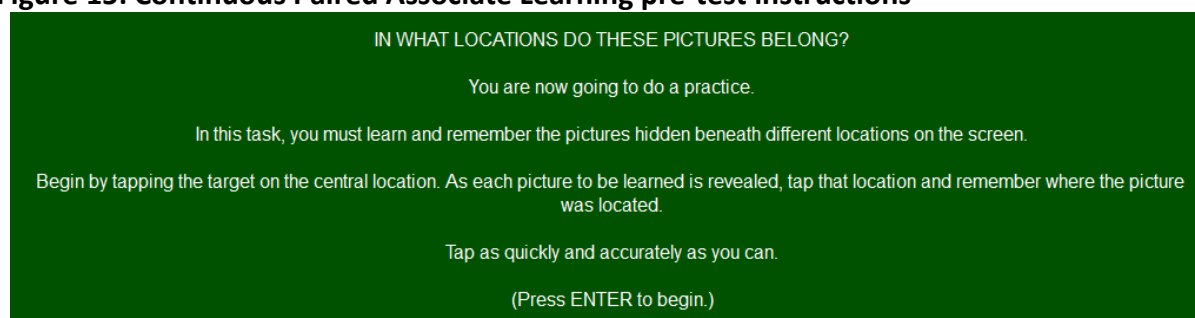
During cognitive testing, the participants were seated at a computer in a private room at the study site, with verbal instructions read out from a CogState™-produced script by the test supervisor (J.A.) to minimise bias. The tests took between 35 to 50 minutes to complete, depending on the individual. A practice session was performed at the study screening visit, followed by the actual assessment conducted once the participant was enrolled into the study.

2.3.2 Task descriptions

2.3.2.1 *Continuous Paired Associate Learning Task* (Administration time: 7 minutes)

In this task, the subject must learn and remember the different shapes hidden beneath different locations on the screen. In the first stage of the test, the subject is shown the pre-test on-screen instruction below (Figure 15).

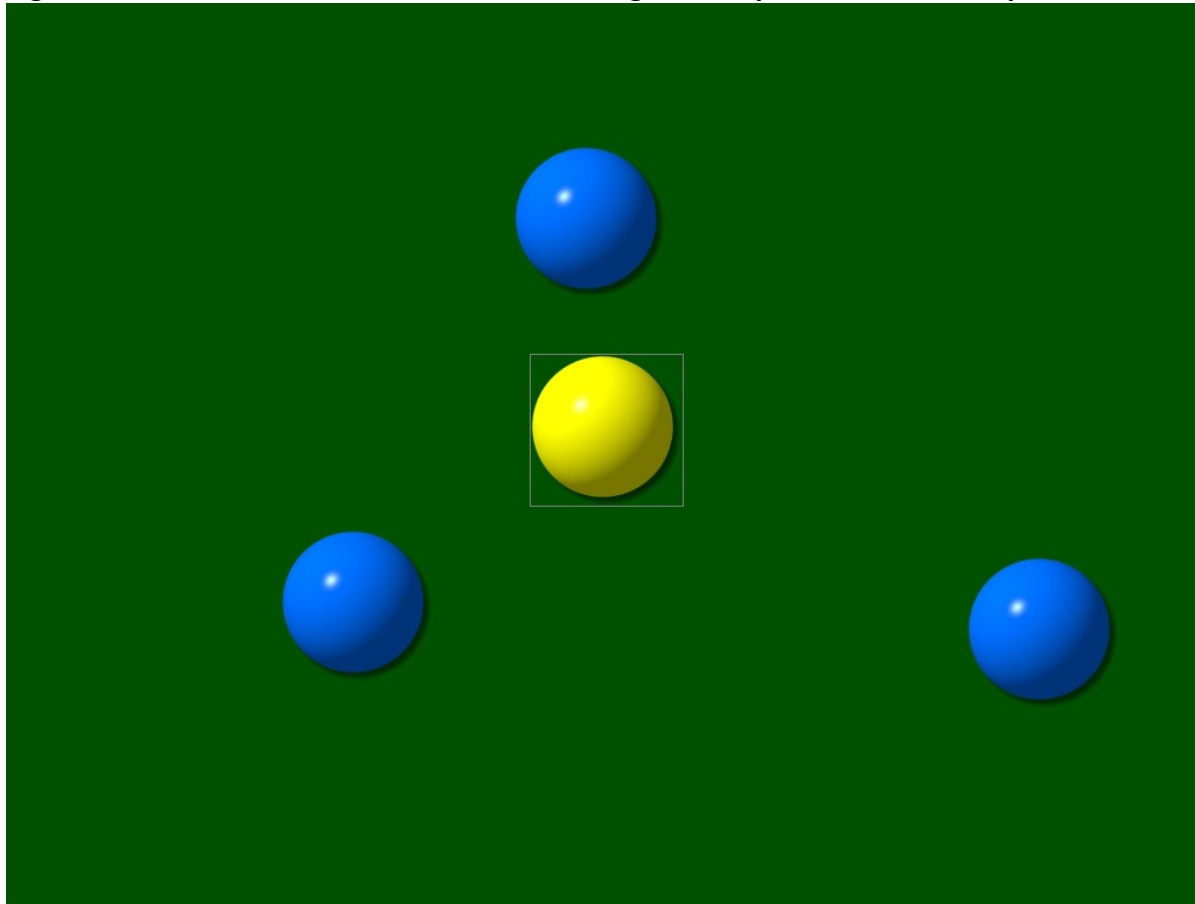
Figure 15: Continuous Paired Associate Learning pre-test instructions



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A picture is presented in the centre of the screen and the subject clicks on the peripheral location of the picture and must remember its location (Figure 16).

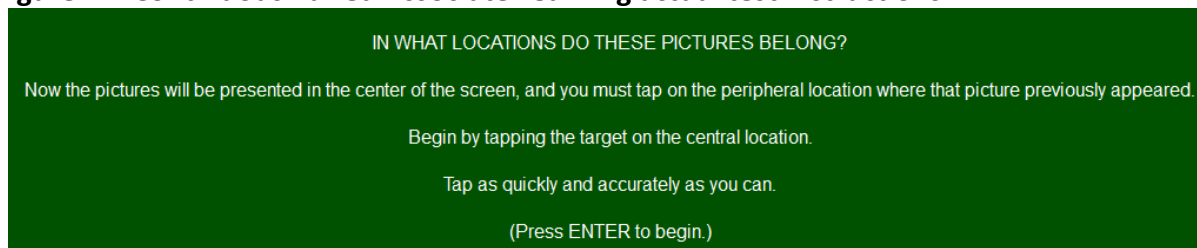
Figure 16: Continuous Paired Associate Learning test shapes hidden under spheres



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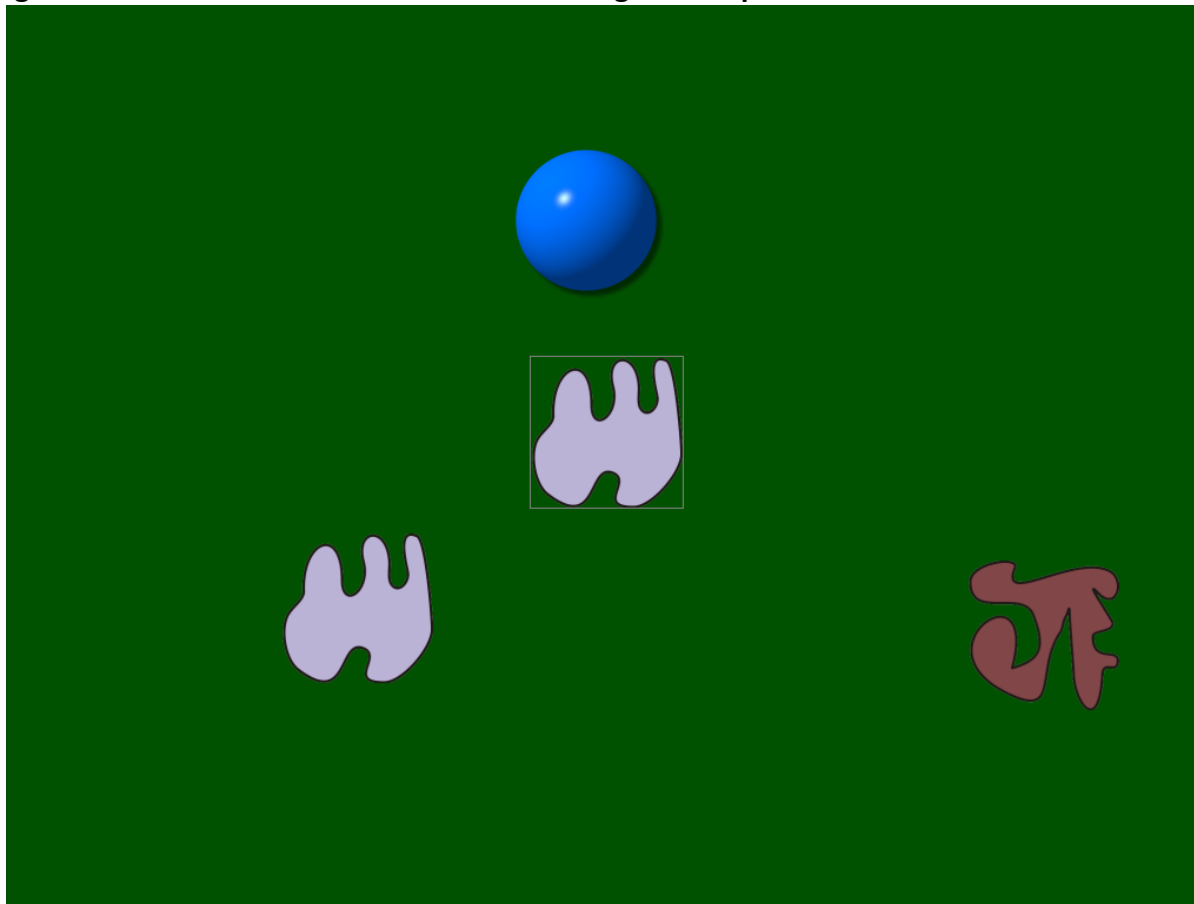
In the second stage of the test, the following on-screen instructions are presented to the subject (Figure 17). The same pictures are presented in the centre of the screen however, the peripheral location of each picture is hidden. The subject will need to click on the peripheral location where the shape previously appeared (Figure 18).

Figure 17: Continuous Paired Associate Learning actual test instructions



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Figure 18: Continuous Paired Associate Learning test shapes uncovered

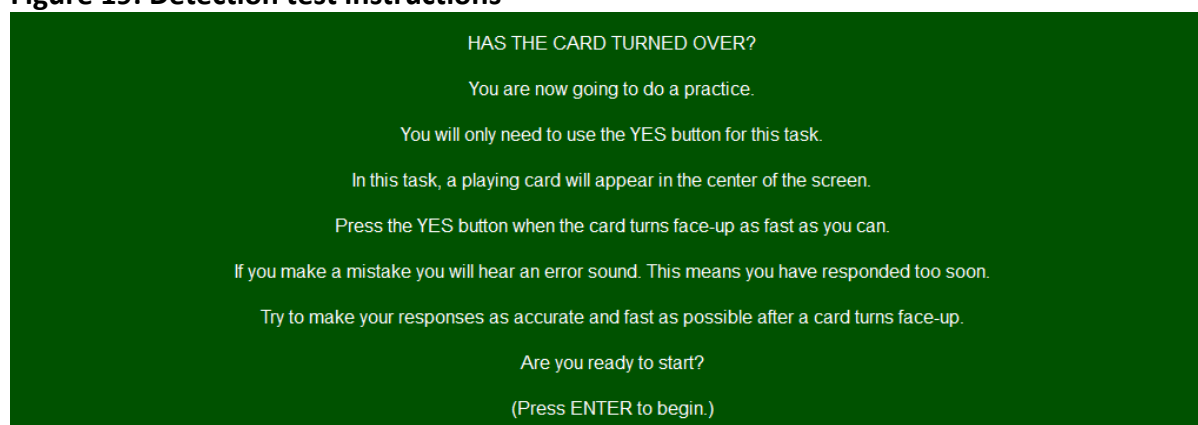


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2.3.2.2 Detection task (Administration time: 3 minutes)

This test measures processing speed using a simple reaction time paradigm. The on-screen instructions ask: "Has the card turned over?" (Figure 19).

Figure 19: Detection test instructions



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A playing card is displayed face down in the centre of the screen. As soon as the card flips over so it is face up (Figure 20), the subject must click the YES button for this task. The card will go to the back of the pack and the subject must then click the YES button again as soon as the next card flips over. The subject is encouraged to work as quickly and as accurately as they can.

Figure 20: Detection test card presentation

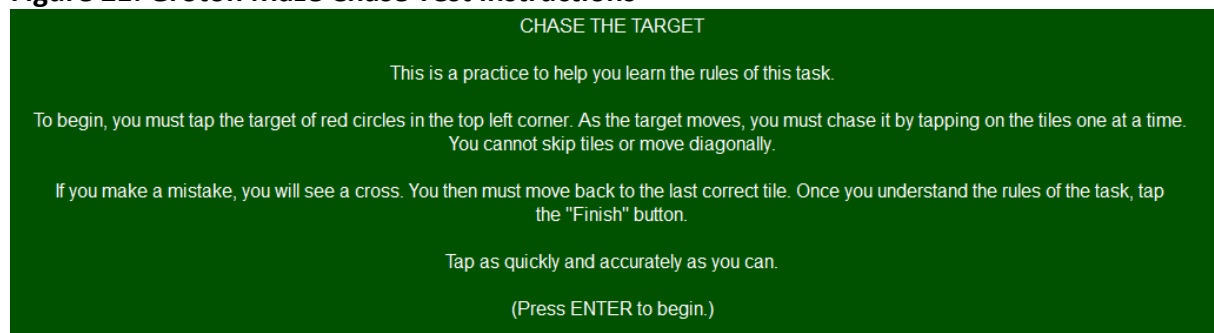


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2.3.2.3 Groton Maze Chase Test (Administration time: 7 minutes)

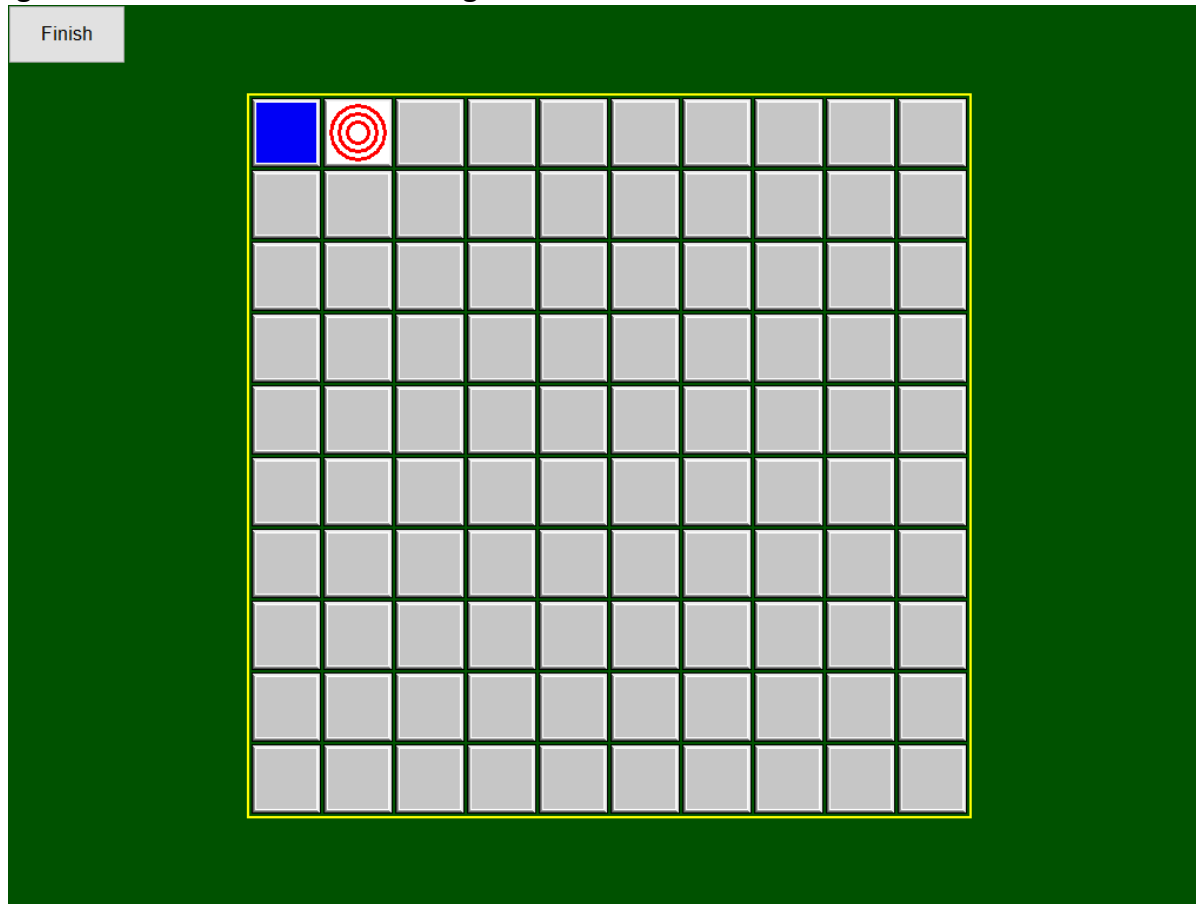
As per the instructions in Figure 21, the aim of this test is to chase a target by clicking on the tiles one at a time using the computer mouse (see Figure 22). If the subject makes a mistake, they must go back to the last correct tile and try again. The participants will complete a pre-test practice session prior to completing the actual test.

Figure 21: Groton Maze Chase Test instructions



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Figure 22: Groton Maze Chase Test grid

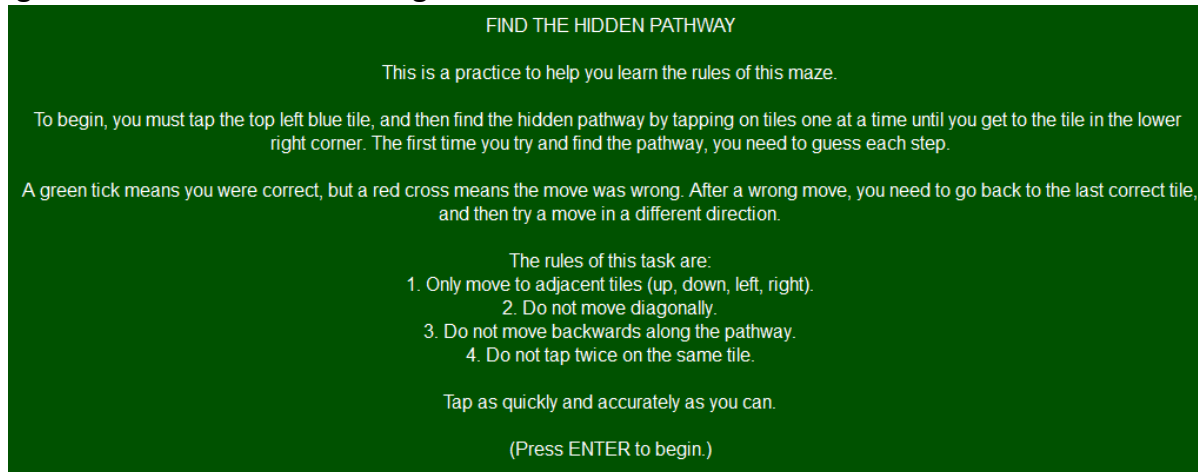


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2.3.2.4 Groton Maze Learning Test (Administration time: 7 minutes)

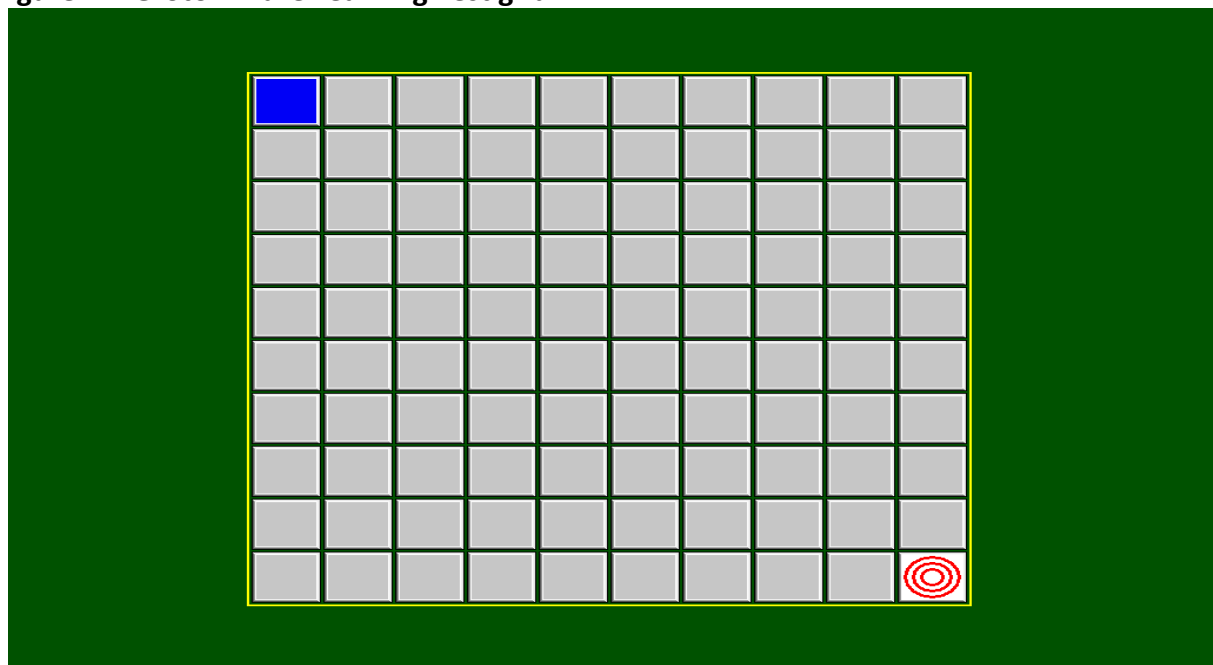
Instructions as per Figure 23 are displayed on the screen where the subject is instructed to find a 28-step pathway hidden among the 100 possible locations, within a 10 x 10 grid as per Figure 24. The subject is directed to move from the start location (top left), and to continue, by clicking on a tile next to their current location, one tile at a time, towards the end (bottom right), with the computer mouse. The computer indicates whether the choice is correct by revealing a green tick (i.e., this is the next step in the pathway) or incorrect by revealing a red cross (i.e., this is not the next step in the pathway). If a choice is incorrect, the subject must click on the last correct tile (i.e., where the last green tick was revealed). Once completed, they return to the start location and repeat the task four more times, trying to remember the pathway they have just completed.

Figure 23: Groton Maze Learning Test instructions



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Figure 24: Groton Maze Learning Test grid

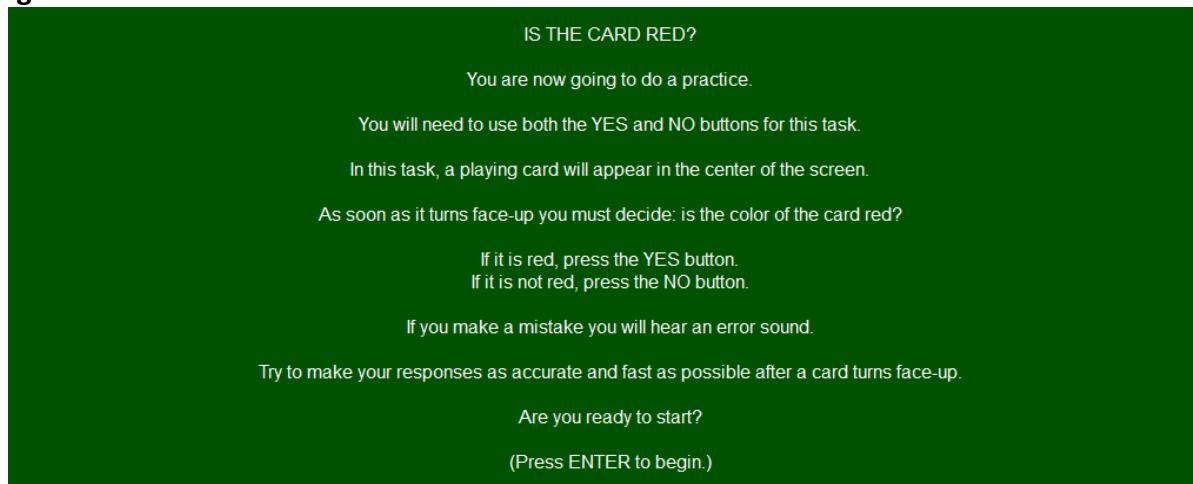


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2.3.2.5 Identification task (Administration time: 3 minutes)

This task measures attention using a choice reaction time paradigm. The subject is presented with on-screen instructions, as per Figure 25.

Figure 25: Identification task instructions



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A playing card is presented in the middle of the screen, face down. As soon as the card flips over so it is face up (Figure 26), the subject must decide whether the card is red or not. If the card is red, they must press click the YES button for this task, if it is not red, they should click the NO button for this task. The subject is instructed to work as quickly and as accurately as they can.

Figure 26: Identification test card presentation

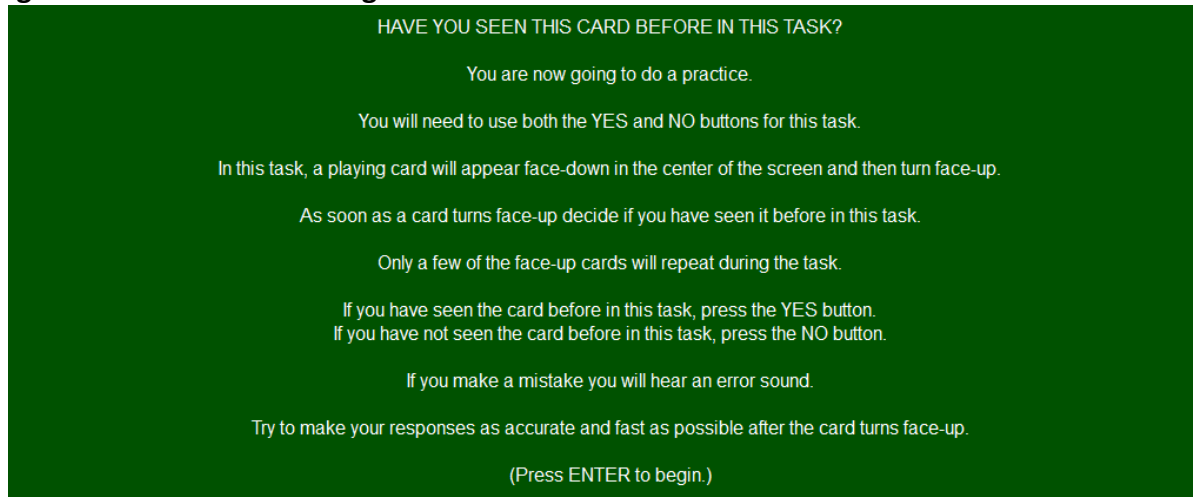


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2.3.2.6 One Card Learning task (Administration time: 6 minutes)

The instructions for this task are set out in Figure 27.

Figure 27: One Card Learning task instructions



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A playing card is displayed in the centre of the screen. The card will flip over so that it faces upwards (Figure 28). As soon as the card faces upwards, the subject needs to decide whether they have been shown that card before in this task and respond by clicking the YES button for this task if they have, or by clicking on the NO button for this task, if they have not been shown this card before in this task.

Figure 28: One Card Learning task card presentation

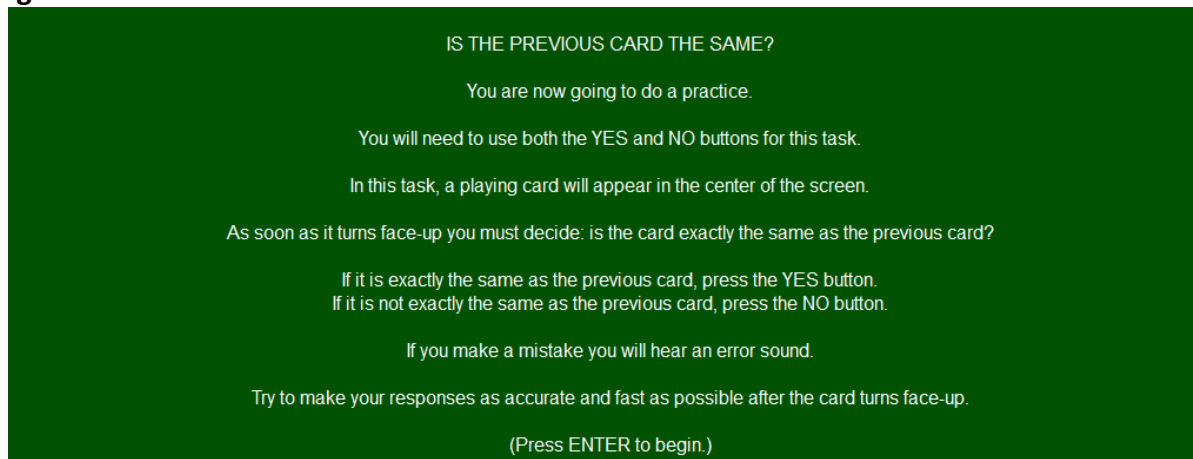


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2.3.2.7 One Back task (Administration time: 4 minutes)

The one back test measures working memory and the on-screen instructions are shown below in Figure 29.

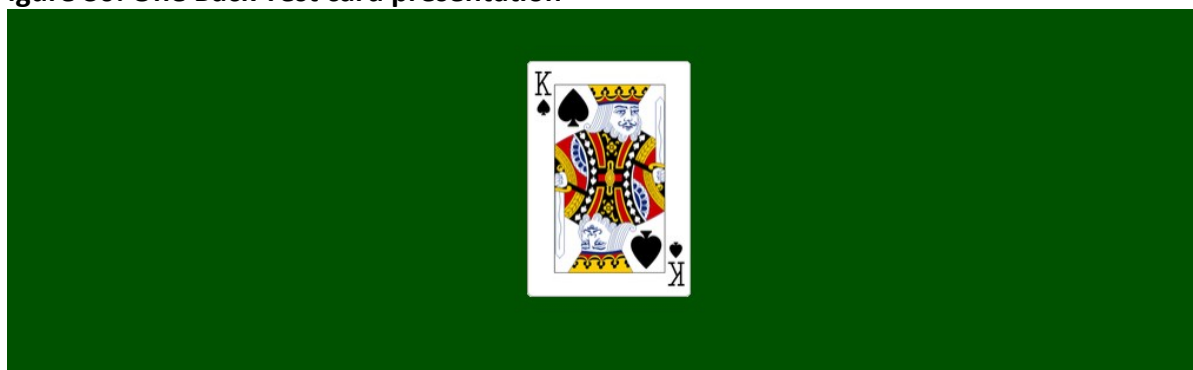
Figure 29: One Back task instructions



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A playing card is presented face-up in the centre of the screen (Figure 30). The subject must decide as each card is presented whether it is identical to the card just before it. If the card face up is identical to the one presented immediately before, the subject should click the YES button for this task and if it is not the same, they should click the NO button for this task.

Figure 30: One Back Test card presentation



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2.3.3 Data analysis

Due to the non-normality of the raw data, raw test scores were log-transformed or arcsine root-transformed where necessary, as detailed by the CogState™ guidelines for data analysis (see Table 8 below for details) and converted into z-scores, using the participants from both PET in AHI and PET in CHI studies as the reference population. This method was chosen because the CogState norms do not have adequate normative data for two of the domains tested: Groton Maze Chase Test and Continuous Paired Associate Learning.

$$z\ Score\ (z_{it}) = \frac{((x_{it} - \bar{x}_t) \times (Multiplicand))}{\sigma_t}$$

Where:

i = indexes the subject,

t = indexes the CogState™ test,

x_{it} = the subject's score,

$\bar{x}$ = mean performance score of the study sample for test t ,

σ_t = standard deviation of the study sample for test t

The multiplicand equals 1 for tests for which a higher score is indicative of better cognitive performance and -1 for tests where a lower score is indicative of better cognitive performance.

Table 8: Cognitive tests administered in the PET in AHI and PET in CHI studies by cognitive domain with data transformations

CogState™ neuropsychological test	Cognitive domain	Primary outcome measure	Interpretation
Continuous Paired Associate Learning Test (CPAL)	Visual memory: - Finding the correct location of the object	Accuracy of performance; total number of errors across seven rounds.	Lower score = better performance
Detection Test (DET)	Psychomotor function: - Has the card turned over?	Speed of performance; mean of the log ₁₀ transformed reaction times for the correct responses	Lower score = better performance
Groton Maze Chase Test (GMCT)	Speed of visual motor processing: - Chase the target	Total number of correct moves per second, chasing the target	Higher score = better performance
Groton Maze Learning Test (GMLT)	Executive function: - Find the hidden pathway	Total number of errors made in attempting to learn the same hidden pathway on five consecutive trials	Lower score = better performance
Identification test (IDN)	Attention function: - Is the card red?	Speed of performance; mean of the log ₁₀ transformed reaction times for the correct responses	Lower score = better performance
One Card Learning Test (OCL)	Visual Learning: - Have you seen this card before in the test?	Accuracy of performance: arcsine transformation of the square root of the proportion of correct responses	Higher score = better performance
One Back Test (ONB)	Working Memory function: - Is the card the same as that shown one card ago?	Accuracy of performance: arcsine transformation of the square root of the proportion of correct responses	Higher score = better performance

The domains were also combined in the following manner, as per manufacturer's recommendations:

1. Psychomotor, attention and visual motor processing domains
 - a. Detection
 - b. Identification
 - c. Groton Maze Chase Test
2. Visual learning and visual memory domains
 - a. One Card Learning Test
 - b. Continuous Paired Associate Learning
3. Working memory and executive function domains
 - a. One Back Test
 - b. Groton Maze Learning Test

A composite cognition score was then calculated by taking the average of the z-scores across all tests:

$$\text{Composite cognition score (comp}_i\text{)} = \frac{\sum_{t=1}^N z \text{ Score}_{it}}{N}$$

Where:

i = indexes the subject,

t = indexes the CogState™ test,

N = number of tests

Finally, T-scores (with a mean of 50 and a standard deviation of 10) using the whole cohort as the reference population were generated. A global T-score was then obtained by calculating the average of the seven individual domain T-scores. For all T-scores, higher scores signify better cognitive function.

2.4 Blood and cerebrospinal fluid NfL analysis

Cerebrospinal fluid and plasma NfL measurements on all samples apart from the baseline, week 48 and week 60 plasma samples from the SPARTAC trial, were performed on the supernatant of centrifuged and stored frozen samples using the ultra-sensitive Simoa digital immunoassay on a HD-X analyser (Quanterix, Billerica, MA, USA)[342] according to the manufacturer's guidelines, at the Fluid Biomarkers for Neurodegenerative Diseases laboratory, UK Dementia Research Institute, University College London, UK.

In short, cerebrospinal fluid and plasma samples were thawed at room temperature, then vortexed and spun at 10,000G for five minutes at room temperature. Cerebrospinal fluid samples were diluted 1:100 with assay diluent on the bench and plasma samples were diluted with assay diluent 1:4 on board the HD-X analyser instrument. Next, the samples were bound to paramagnetic beads coated with a capture antibody specific for human NfL. NfL-bound beads were then incubated with a biotinylated NfL-detection antibody, in turn conjugated to streptavidin- β -galactosidase complex, that acted as a fluorescent tag. Subsequent hydrolysis reaction with a resorufin β -D-galactopyranoside substrate produced a fluorescent signal proportional to the concentration of NfL present. Each sample was measured in duplicate. Sample concentrations were then extrapolated from a standard curve, fitted using a four-parameter logistic algorithm. Intra-assay and inter-assay coefficients of variability were less than 15%, as determined by two quality controls (low and high concentration). The lower limit of detection and lower limit of quantification NfL measured in this way was 0.038 pg/mL and 0.174 pg/mL, respectively, for both cerebrospinal fluid and plasma samples, as verified by the manufacturer.

Plasma NfL was measured in the samples from the SPARTAC trial participants at baseline, week 48 and week 60 at the beginning of my PhD, prior to the availability of the HD-X Quanterix analyser at University College London, UK. These samples were analysed at the Clinical Neurochemistry Laboratory at Sahlgrenska University Hospital, Sweden using the NF-Light ELISA kit (UmanDiagnostics AB, Sweden), transferred onto the Simoa platform using a homebrew kit (Quanterix™, USA)[30].

2.5 Thesis statistical analysis plan

Statistical analyses were conducted using SPSS version 24 (IBM Corp, Armonk, NY, US), except where specified otherwise. Descriptive statistics summarised variables using total (percentage) and median (interquartile range). Continuous variables were $\log_{10}$ transformed, to normalise distribution for analysis purposes. *P*-values <0.05 were considered statistically significant throughout.

The specific statistical methods used in this thesis are described in detail below and also presented in each results chapter.

2.5.1.1 Chapter 3 statistical analysis plan

The pooled correlation coefficient estimate and 95% confidence interval (CI) in the overall analysis was calculated using a random-effects meta-analysis of correlations based on Fisher's Z-transformation, incorporating the heterogeneity between studies due to the different statistical correlation techniques used and the different blood NfL assays used. Heterogeneity and between-study variance were assessed using the I^2 statistic and τ^2 (Sidik-Jonkman estimator). The rank correlation test of funnel plot asymmetry was used to assess for publication bias.

In pre-specified sensitivity analyses, pooled correlation coefficient estimates were stratified according to: (1) blood NfL assay utilised (Simoa digital immunoassay, ECL or ELISA), (2) whether plasma or serum NfL was quantified, (3) whether the neurological condition being investigated purely affected the central nervous system, or affected both the central and peripheral nervous system and (4) the statistical correlation technique used (Spearman's rank or Pearson's correlation coefficient; where unspecified, Pearson's correlation was

imputed). Pooled correlation estimates and 95% CI in these subgroup analyses were calculated using a fixed-effects model.

2.5.1.2 Chapter 4 statistical analysis plan

Differences in demographics were assessed using Wilcoxon's rank-sum test for continuous variables and chi-square test for categorical variables. Differences between PLWH, HIV-negative participants and blood bank donors were first assessed using the Kruskal-Wallis then Wilcoxon's rank-sum test for age, and chi-square test for gender. Differences in cerebrospinal fluid and plasma NfL concentrations and cerebrospinal fluid: plasma NfL ratios between PLWH and HIV-negative participants were tested for significance using Wilcoxon's rank-sum test. In a sub-analysis, differences in plasma NfL concentrations between PLWH, HIV-negative participants and blood bank donors were assessed using Wilcoxon's rank-sum test. Non-parametric statistical tests were employed as these do not make distributional assumptions.

The correlation between cerebrospinal fluid and plasma NfL (after $\log_{10}$ -transformation to reduce data skewness) was assessed using Pearson's correlation coefficient (a parametric statistical test), and the difference between groups was tested for significance using the Z-test after Fisher's transformation.

$\log_{10}$ -transformed cerebrospinal fluid and plasma NfL Z-scores were calculated which reduced data skewness and enabled direct comparisons between cerebrospinal fluid and plasma NfL. A Bland-Altman graph[343] was plotted to describe the agreement between $\log_{10}$ -transformed cerebrospinal fluid and plasma NfL Z-scores. This represents a plot of the difference between the cerebrospinal fluid and plasma NfL Z-score against their average for each individual.

Linear regression models were used to identify factors independently associated with $\log_{10}$ -transformed cerebrospinal fluid and plasma NfL, standardised to Z-scores with a mean of 0 and standard deviation of 1, separately in PLWH and HIV-negative participants. Factors included in the univariable linear regression analysis were female gender, age, Black African ethnicity, men who have sex with men sexual orientation, weight, body mass index, current

tobacco smoker, ex-tobacco smoker, current alcohol consumption, past alcohol consumption, recent recreational drug use, ever used injected recreational drugs, on hypertensive medication, global cognitive function T-score, serum creatinine, serum albumin, cerebrospinal fluid protein, cerebrospinal fluid neopterin and plasma neopterin. HIV-specific parameters (current CD4⁺ and CD8⁺ T-cell count, current CD4⁺/8⁺ T-cell ratio, nadir CD4⁺ count, years with CD4⁺ count <200cells/ μ L, prior AIDS diagnosis, on a non-nucleoside reverse-transcriptase inhibitors (NNRTI), on a protease inhibitor (PI), on tenofovir-disoproxil fumarate (TDF) and on atazanavir) significantly associated in univariable analyses were added to an additional model fitted in PLWH only. Factors associated with either cerebrospinal fluid or plasma NfL in either PLWH or HIV-negative participants in univariable analyses ($p < 0.05$) were included in the multivariable models

2.5.1.3 *Chapter 5 statistical analysis plan*

Participant baseline demographics and clinical parameters were described using median (interquartile range) and total (percentage), as appropriate. Differences in variables between participants who initiated cART during acute HIV infection and participants who initiated cART during chronic HIV infection were analysed using the Mann-Whitney U test and Fisher's exact test, as appropriate, and differences in variables between participants who initiated cART during acute HIV infection, during chronic HIV infection and control participants were analysed using the Kruskal-Wallis and Fisher's exact test, as appropriate.

The statistical analyses performed in this chapter are exploratory and differences in cerebral [¹¹C] PBR binding (total volume of distribution (V_T) and V_T normalised to cortical grey matter ($V_{T(DVR)}$)) at regions of interest between participants who initiated cART during acute HIV infection, during chronic HIV infection and control participants were assessed using the Kruskal-Wallis test. The 30 regions of interest were chosen based on regions noted to display TSPO binding signals in previous published studies. Differences in laboratory parameters (cerebrospinal fluid NfL, plasma NfL, cerebrospinal fluid protein and cerebrospinal fluid white cell count) and cognitive function test scores (individual domain z-scores, average domain combination z-scores and global composite T-scores) between participants who initiated cART during acute HIV infection versus chronic HIV infection, were analysed using the Mann-Whitney U test.

2.5.1.4 Chapter 6 statistical analysis plan

Spearman's rank correlation was utilised to analyse associations between cerebral [^{11}C] PBR V_T binding and age. In the two HIV-positive cohorts combined ('PET in AHI' and 'PET in CHI'), Spearman's rank correlation was used to analyse the associations between cerebral [^{11}C] PBR V_T binding on PET-CT and:

- HIV-specific clinical parameters: duration since HIV diagnosis, duration since commencing cART, duration between HIV diagnosis and cART commencement (i.e., duration of known untreated HIV disease) and pre-treatment HIV RNA load
- HIV-specific immunological parameters: most recent CD4^+ count, most recent CD8^+ count, most recent $\text{CD4}/\text{CD8}$ ratio and nadir CD4^+ count)
- biomarkers of central nervous system injury (cerebrospinal fluid NfL, plasma NfL, cerebrospinal fluid white cell count and cerebrospinal fluid protein)
- cognitive function test scores

Differences in [^{11}C] PBR V_T binding on PET-CT at the 30 pre-selected regions of interest by antiretroviral agent class (NNRTI, PI and INSTI) were analysed using the Kruskal-Wallis test.

2.5.1.5 Chapter 7 statistical analysis plan

Where appropriate, plasma NfL, total HIV DNA, integrated HIV DNA, cell-associated unspliced HIV RNA and ultra-sensitive HIV RNA were $\log_{10}$ -transformed to reduce data skewness. Plasma NfL <13 pg/mL was considered normal[293]. Differences in laboratory parameters (plasma NfL, total HIV DNA, integrated HIV DNA, cell-associated unspliced HIV RNA, ultra-sensitive plasma HIV RNA, % $\text{CD154}^+ \text{IFN-}\gamma^+ \text{CD4}^+$ cells and % $\text{CD107a}^+ \text{IFN-}\gamma^+ \text{CD8}^+$ cells) by study arms at each time point were analysed using Student's t-test and longitudinal changes in plasma NfL were analysed using mixed models. Correlations between plasma NfL and total HIV DNA, integrated HIV DNA, cell-associated unspliced HIV RNA, ultra-sensitive plasma HIV RNA and HIV-specific CD4^+ and CD8^+ polyfunctional T-cell responses at the time points were analysed using Spearman's rank statistics. The following factors: age, body mass index, ethnicity, duration since primary HIV infection diagnosis,

estimated glomerular filtration rate (calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula)[344], CD4⁺ T-cell count, CD8⁺ T-cell count, total HIV DNA in peripheral CD4⁺ T-cells and ultra-sensitive plasma HIV RNA) were included in the multivariable linear regression model.

2.5.1.6 Chapter 8 statistical analysis plan

Sample size calculation for this sub-study was based on the comparison of plasma NfL in patients of the ART48 arm at weeks 48 and week 60, using a two-sample paired-means test with plasma NfL on a log₁₀-scale and a two-sided α of 0.05 for the null hypothesis that there is no difference between the two time-points. I assumed that after stopping ART, plasma NfL would increase by 17.5%. On a log₁₀-scale, this corresponds to a difference of 0.07. Further, I assumed a standard deviation of 0.25 for a single measurement at both time-points based on Gisslen et al[293], and a correlation coefficient of 0.6 between the week 48 and week 60 measurements. Then, a sample size of 82 individuals would provide 80% power to detect this difference.

Descriptive statistics were described using median (interquartile range), geometric mean (95% confidence interval) and total (percentage), as required. Baseline characteristics between participants in the ART48 arm included in this analysis versus those excluded and baseline characteristics between male and female participants in the ART48 arm included in this analysis were compared using the Mann-Whitney test, two-sample t-test with equal variances, chi-square and Fisher's exact test, as appropriate.

Plasma NfL, plasma HIV RNA, plasma D-dimer, plasma IL-6 and total HIV DNA in CD4⁺ T-cells isolated from peripheral blood were log₁₀-transformed, where appropriate, to reduce data skewness. Paired-samples T-test (a parametric statistical test) was used to assess changes in plasma NfL, plasma HIV RNA, plasma IL-6, plasma D-dimer and total HIV DNA from baseline to week 48, week 48 to week 60 and week 48 to week 84 (where available), given the Normal distribution of the data. Plasma NfL concentrations greater than 13pg/mL were considered abnormal, based on the publication by Gisslén *et al*[293]. The lower limit of quantification for plasma HIV RNA was <50 copies/mL, except in Africa, where it was <400 copies/mL. As per the publication by Williams *et al*[345], total HIV DNA $\geq 3.88 \log_{10}$ total HIV

DNA copies/mL was categorised as 'high' and total HIV DNA $<3.88 \log_{10}$ total HIV DNA copies/mL was categorised as 'low'.

Correlations between plasma NfL and laboratory parameters were assessed using Pearson's correlation. At weeks 48, 60 and 84, a two-sample T-test of equal variance was performed to investigate whether plasma NfL differed significantly in those with detectable versus undetectable plasma HIV RNA.

The following factors: gender, age, plasma HIV RNA, CD4⁺ count, CD8⁺ count, CD4⁺/CD8⁺ ratio, duration between seroconversion and randomisation, serum creatinine, site of recruitment, weight and body mass index, and in subgroups with plasma D-dimer, plasma IL-6 and total HIV DNA data were included in univariate regression analyses at the four time points; factors significantly associated with plasma NfL were included in multivariable linear regression models.

Chapter 3

Correlation between cerebrospinal fluid and blood neurofilament light chain protein: A systematic review and meta-analysis

Chapter 3: Correlation between cerebrospinal fluid and blood neurofilament light chain protein: A systematic review and meta-analysis

3.1 Introduction

Neurofilament light chain protein (NfL), along with neurofilament medium and heavy chain proteins are neuronal cytoplasmic proteins highly expressed in large calibre myelinated axons throughout the body. Together, they offer major structural support to myelinated axons, maintain axonal calibre and facilitate effective nerve conduction [279,281,285]. Cerebrospinal fluid neurofilament light chain protein (NfL) is a well-established sensitive and dynamic biomarker of active central nervous system neuro-axonal injury[279,281,346]. Concentrations of cerebrospinal fluid NfL rise proportionally to the degree of neuro-axonal injury in a variety of neurological conditions including neurodegenerative, inflammatory, vascular and traumatic diseases[279,281,347–350].

However, the invasive nature of cerebrospinal fluid collection limits the widespread use of cerebrospinal fluid NfL and a method to reliably measure NfL in an alternative compartment, as a surrogate measure of cerebrospinal fluid NfL, is actively being pursued. Data supporting blood NfL as a proxy for cerebrospinal fluid NfL is emerging, with different studies reporting moderate to strong correlations between cerebrospinal fluid NfL and blood NfL depending on the neurological condition under investigation. Hence, the overall correlation coefficient estimate between cerebrospinal fluid NfL and blood NfL remains unknown. If blood NfL is to be recognised as a reliable surrogate marker of cerebrospinal fluid NfL, then the overall estimated correlation between cerebrospinal fluid and blood NfL needs to be established.

In order to test my PhD hypotheses, I plan to use plasma NfL throughout my thesis as a key biomarker of neural activation.

To gain a better fundamental understanding of the overall association between cerebrospinal fluid and blood NfL and how the association varies under different circumstances, I performed a systematic review and meta-analysis to determine the pooled correlation coefficient estimate between cerebrospinal fluid and blood NfL in human studies. Secondly, I stratified the pooled correlation coefficient estimates according to blood NfL assay, plasma versus serum NfL, conditions which purely affect the central nervous system versus conditions which affect both the central nervous system and peripheral nervous system and statistical correlation technique used, to further elucidate the factors which may affect the way blood NfL is interpreted, as a surrogate marker of cerebrospinal fluid NfL. This piece of work is important to enable a more accurate understanding and interpretation of blood NfL as a surrogate biomarker of cerebrospinal fluid NfL, under differing conditions.

3.2 Methods

3.2.1 Protocol and registration

This systematic review and meta-analysis was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines[351–353] and reported according to the Meta-Analysis Of Observational Studies in Epidemiology (MOOSE) proposal. The systematic review protocol was registered and approved in the International Prospective Register of Systematic Reviews (PROSPERO) database[353,354] (registration number: CRD42019140469). We searched publicly accessible published articles and institutional research ethics board approval and patient consent was not required for this systematic review.

3.2.2 Information sources and search strategy

We systematically searched the following electronic databases for eligible published articles from their inception to July 9, 2019: Ovid MEDLINE, Ovid Embase and Web of Science. The following key word search terms were used: [(*cerebrospinal fluid* or *spinal fluid* or *CSF*) and (*neurofilament* light* or *neuro filament* light* or *NFL* or *NFLs*)] and [(*plasma* or *blood* or

peripheral or serum) and (*neurofilament* light or neuro filament* light or NFL or NFLs*)]].

Grey literature sources were not accessed.

3.2.3 Eligibility criteria

Articles published in English were included if a correlation coefficient between paired cerebrospinal fluid and blood (plasma or serum) NfL in human participants was specifically reported. Articles were excluded for any of the following reasons: (1) duplicate articles, (2) cerebrospinal fluid and blood samples were collected during autopsy, (3) non-original research articles (review articles, letters in response to previous articles) and (4) non-peer reviewed abstracts and conference proceedings.

3.2.4 Study selection

Eligible articles were imported into Covidence[®], an online primary screening and data extraction tool. Two assessors (myself (JA) and Sophia von Widekind (SvW), a 5th year medical student at Imperial College London) separately screened the titles and abstracts for all the articles retrieved from the electronic database search to identify potentially eligible articles. Where it could be determined that an article did not meet the inclusion criteria based on screening the title and abstract alone, then the article was immediately rejected. JA and SvW then separately appraised the potentially eligible full-text articles against the inclusion and exclusion criteria as set out in the protocol to decide on final inclusion into the systematic review and meta-analysis. Disagreements about whether to include an article were resolved by my PhD supervisor (Professor Sarah Fidler, SF). Where further information was required, the corresponding author of the article was contacted by e-mail to request the specific missing information.

3.2.5 Data collection process and data items

Data from the full-text articles were independently extracted and checked for accuracy by JA and SvW and imported into a standardised record form on Microsoft Excel[®]. The following information was recorded: first author's surname, year of article publication, name of journal the article was published in, article title, location(s) where the study took place, study design, study setting, clinical condition(s) being investigated, number of

participants included in the study, inclusion and exclusion criteria, number of paired cerebrospinal fluid and blood samples measured for NfL, blood NfL assay used, plasma or serum samples analysed for NfL, statistical correlation technique used and statistical correlation coefficient value (ρ). Data extraction is summarized in Table 7. Any disagreements during data extraction were resolved by my PhD supervisor (SF).

3.2.6 Assessment of methodological quality

Quality assessment was performed at the study level by JA and SvW using the National Institutes of Health (NIH): National Heart Lung and Blood Institute (NHLBI) study quality assessment tools (attached in the Appendix section at the end of this thesis)[355]. Each item was scored as ✓ = 1 point, ✗ or ? = 0 points. Articles that scored 7 points or above for the 14 criteria was considered 'good' quality or 'low risk of bias'. Studies scoring between 5 and 6 points were considered 'fair' quality or 'moderate risk of bias' and studies scoring 4 points or below were considered 'poor' quality or 'high risk of bias'.

3.2.7 Statistical approach

Meta-analyses were performed using the statistical software R v3.6 and the *meta* package by our collaborating biostatistician, Dr Davide De Francesco (DDF). I then performed and reconfirmed the meta-analyses results using MedCalc for Windows, version 19.1.5 (MedCalc Software, Ostend, Belgium). The pooled correlation coefficient estimate and 95% confidence interval (CI) in the overall analysis was calculated using a random-effects meta-analysis of correlations based on Fisher's Z-transformation, incorporating the heterogeneity between studies due to the different statistical correlation techniques used and the different blood NfL assays used. Heterogeneity and between-study variance were assessed using the I^2 statistic and τ^2 (Sidik-Jonkman estimator). The rank correlation test of funnel plot asymmetry was used to assess for publication bias. A p -value <0.05 was considered statistically significant throughout.

In pre-specified sensitivity analyses, pooled correlation coefficient estimates were stratified according to: (1) blood NfL assay utilised (Simoa digital immunoassay, ECL or ELISA), (2) whether plasma or serum NfL was quantified, (3) whether the neurological condition being

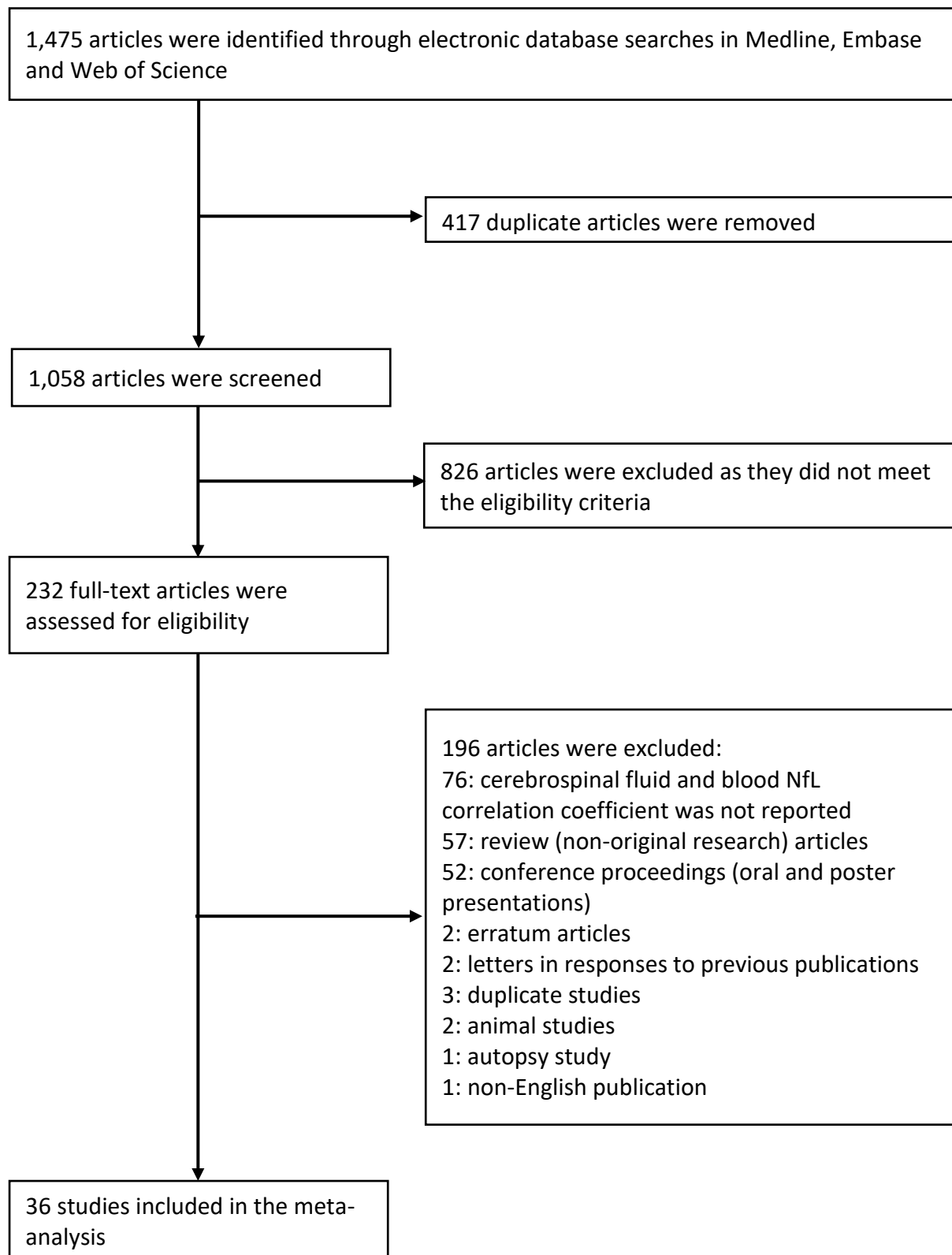
investigated purely affected the central nervous system, or affected both the central and peripheral nervous system and (4) the statistical correlation technique used (Spearman's rank or Pearson's correlation coefficient; where unspecified, Pearson's correlation was imputed). Pooled correlation estimates and 95% CI in these subgroup analyses were calculated using a fixed-effects model.

3.3 Results

3.3.1 Study selection

The electronic database searches returned 1,058 unique articles (Figure 31). Article titles and abstracts were screened against the inclusion and exclusion criteria and 826 articles were deemed ineligible (Figure 31). Full-text article review was undertaken in the remaining 232 articles and 36 articles[291,293–295,297,298,356–385] were judged to be eligible for inclusion into the final meta-analysis (Figure 31).

Figure 31: PRISMA flowchart of study selection



Legend: Search strategies generated 1,058 articles using the search terms as detailed in the Methods section. 36 studies were included in the meta-analysis of pooled correlation coefficient estimate.

3.3.2 Study characteristics

Data were extracted from the 36 eligible articles (Table 9) and included 3,961 paired cerebrospinal fluid and blood samples measured for NfL. Studies were performed in Europe (n=26), USA and Canada (n=5), China (n=1) and four studies enrolled participants in multiple sites globally. Table 9 describes the 36 individual articles included in this systematic review and meta-analysis.

Table 9: Characteristics of the studies included in the meta-analysis

First author's last name and year of publication	Clinical condition being investigated and number of paired CSF and blood NfL samples included	Clinical condition affecting the CNS only, CNS and PNS or PNS only	Plasma or serum NfL measured	Blood NfL assay used	Statistical correlation technique used	Correlation coefficient value	Quality assessment score
Abdellhak <i>et al</i> [382], 2018	n=80 MS, n=20 controls	CNS only	Serum	Simoa	Spearman's	MS: r=0.7 controls: r= 0.6	7
Abdellhak <i>et al</i> [383], 2019	n=71 PPMS	CNS only	Serum	Simoa	Spearman's	r=0.6	7
Benatar <i>et al</i> [384], 2017	n=154 (n=18 controls, n=110 at risk ALS, n=16 ALS, n=10 phenoconverters)	CNS+PNS	Serum	ECL	Spearman's	r=0.7	8
Bergman <i>et al</i> [295], 2016	n=60 PMS	CNS only	Serum	Simoa	Pearson's	r=0.775	7
Byrne <i>et al</i> [385], 2017	n=29 (n=12 controls, n=2 premanifest HD, n=15 manifest HD)	CNS+PNS	Plasma	Simoa	Pearson's	r=0.868	7
Byrne <i>et al</i> [356], 2018	n=80 (n=20 controls, n=20 pre-HD, n=40 manifest HD)	CNS+PNS	Plasma	Simoa	Pearson's	r=0.914	8

deFlon <i>et al</i> [357], 2019	n=281 RRMS	CNS only	Plasma	Simoa	Spearman's	r=0.445	7
Disanto <i>et al</i> [294], 2017	n=132 MS	CNS only	Serum	Simoa	Pearson's	r=0.77	7
Fortea <i>et al</i> [358], 2018	n=94 (n=54 asymptomatic, n=18 prodromal AD, n = 22 AD)	CNS only	Plasma	Simoa	not reported	r=0.80	8
Gaiottino <i>et al</i> [359], 2013	n=20 AD, n=19 GBS, n=45 ALS, n=66 controls	AD: CNS only, GBS: PNS only, ALS: CNS+PNS	Serum	ECL	Spearman's	AD: r=0.48 GBS: r=0.79 ALS: r=0.70 controls r=0.11	6
Gisslen <i>et al</i> [293], 2016	n=140 (n=121 with HIV, n=19 controls)	CNS+PNS	Plasma	Simoa	Pearson's	r=0.89	7
Gong <i>et al</i> [360], 2018	N=40 ALS, n=40 controls	CNS+PNS	Serum	ELISA	Spearman's	ALS r=0.5997 controls r=0.0226	7
Hakansson <i>et al</i> [361], 2018	n=63 (n=41 MS, n=22 controls)	CNS only	Serum	Simoa	Pearson's	r=0.74	7
Halaas <i>et al</i> [362], 2019	n=130 with hip fracture and delirium, n=123 with hip fracture and no delirium	CNS+PNS	Serum	Simoa	Spearman's	Cases: r= 0.56 controls r= 0.69	6
Hansson <i>et al</i> [297], 2017	Lund: n=245 (n=147 PD, n=28 MSA, n=15 PSP, n=5 CBS, n=50 controls) London: n=97 (n=5 PD, n=29 MSA, n=26 PSP, n=11 CBS, n=26 controls)	CNS+PNS	Plasma	Simoa	Spearman's	Lund cohort: r=0.730 London cohort: r=0.840	7
Kalm <i>et al</i> [363], 2017	n=43 (n=20 CSF:serum albumin <10, n=15 CSF:serum albumin 10–20, n=8 CSF:serum albumin > 20)	CNS+PNS	Serum	Simoa	Pearson's	r=0.6303	5

Kuhle <i>et al</i> [291], 2016	n=33 (a variety of neurological disorders)	CNS+PNS	Serum	ELISA, ECL, Simoa	Spearman's	Simoa: r = 0.88 ECL: r = 0.78 ELISA: r = 0.38	7
Kuhle <i>et al</i> [364], 2016	n=31 RRMS	CNS only	Serum	ECL	Pearson's	r=0.62	8
Lu <i>et al</i> [365], 2015	n=38 ALS, n = 20 controls	CNS+PNS	Serum	ECL	Spearman's	ALS: r=0.78 controls: r=0.57	8
Mariotto <i>et al</i> [366], 2018	n=12 acquired peripheral neuropathy	PNS only	Serum	Simoa	Pearson's	r=0.71	8
Mariotto <i>et al</i> [367], 2019	n = 23 CNS syndromes+autoantibodies to neuronal cell surface antigens	CNS only	Serum	Simoa	Spearman's	r=0.461	8
Marques <i>et al</i> [368], 2019	n=137 (n=55 PD, n=29 APD, n=53 controls)	CNS+PNS	Serum	Simoa	Spearman's	r=0.72	9
Mattsson <i>et al</i> [369], 2017	n=391 (n=112 controls, n=189 MCI, n=90 AD)	CNS only	Plasma	Simoa	Spearman's	r=0.59	8
Meeter <i>et al</i> [298], 2016	n=55 genetic FTD	CNS only	Serum	ECL	Spearman's	r=0.87	9
Menke <i>et al</i> [370], 2015	n=25 ALS, n=17 controls	CNS+PNS	Serum	ECL	Spearman's	ALS: r=0.642 controls: r=0.677	7
Mielke <i>et al</i> [371], 2019	n=79 without dementia (n=64 cognitively unimpaired, n=15 MCI)	CNS only	Plasma	ELISA	Spearman's	r=0.568	6
Novakova <i>et al</i> [372], 2017	n=434 MS, n=45 OND, n=42 controls	CNS only	Serum	Simoa	Spearman's	MS: r=0.620 controls: r=0.385 OND: r=0.740	8
Piehl <i>et al</i> [373], 2018	n=66 MS	CNS only	Serum	Simoa	Spearman's	r=0.672	7

Rojas <i>et al</i> [374], 2018	n=22 PSP	CNS only	Plasma	Simoa	Pearson's	r=0.64	8
Rojas <i>et al</i> [375], 2016	n=15 PSP	CNS only	Plasma	Simoa	Pearson's	r=0.74	7
Sanchez-Valle <i>et al</i> [376], 2018	n=35 AD	CNS only	Serum	Simoa	Spearman's	r=0.72	7
Sehr <i>et al</i> [377], 2019	n = 15 RRMS	CNS only	Plasma	Simoa	Spearman's	r=0.74	8
Staffaroni <i>et al</i> [378], 2019	n=49 sporadic CJD	CNS+PNS	Plasma	Simoa	Pearson's	r=0.8	7
Steinacker <i>et al</i> [379], 2018	n=37 bvFTD	CNS only	Serum	Simoa	Spearman's	r=0.706	8
Weydt <i>et al</i> [380], 2016	n=95 (n=19 controls, n=12 asymptomatic ALS, n=64 symptomatic ALS)	CNS+PNS	Serum and plasma	ECL	Spearman's	r=0.79	7
Wilke <i>et al</i> [381], 2016	n = 112 (n = 41 FTD, n = 25 ALS, n = 46 controls)	FTD: CNS only, ALS: CNS+PNS	Serum	ECL	Pearson's	FTD: r=0.72 ALS: r=0.58 controls: r=0.53	9

Abbreviations: CSF = cerebrospinal fluid, NfL = neurofilament light chain protein, CNS = central nervous system, PNS = peripheral nervous system, MS = multiple sclerosis, RRMS = relapsing-remitting multiple sclerosis, PMS = progressive multiple sclerosis, ECL = Electrochemiluminescence immunoassay, Simoa = single-molecule array digital immunoassay, ELISA = enzyme-linked immunosorbent assay, ALS = Amyotrophic lateral sclerosis, PPMS = Primary progressive multiple sclerosis, HD = Huntington's disease, AD = Alzheimer's disease, PD = Parkinson's disease, MSA = multiple system atrophy, PSP = progressive supranuclear palsy, CBS = corticobasal syndrome, GBS = Guillain-Barré syndrome, APD = atypical Parkinsonism disorders, MCI = mild cognitive impairment, FTD = frontotemporal dementia, bvFTD = behavioral variant frontotemporal dementia, CJD = Creutzfeldt–Jakob disease, OND = other neurological disorders

3.3.3 Summary of study characteristics

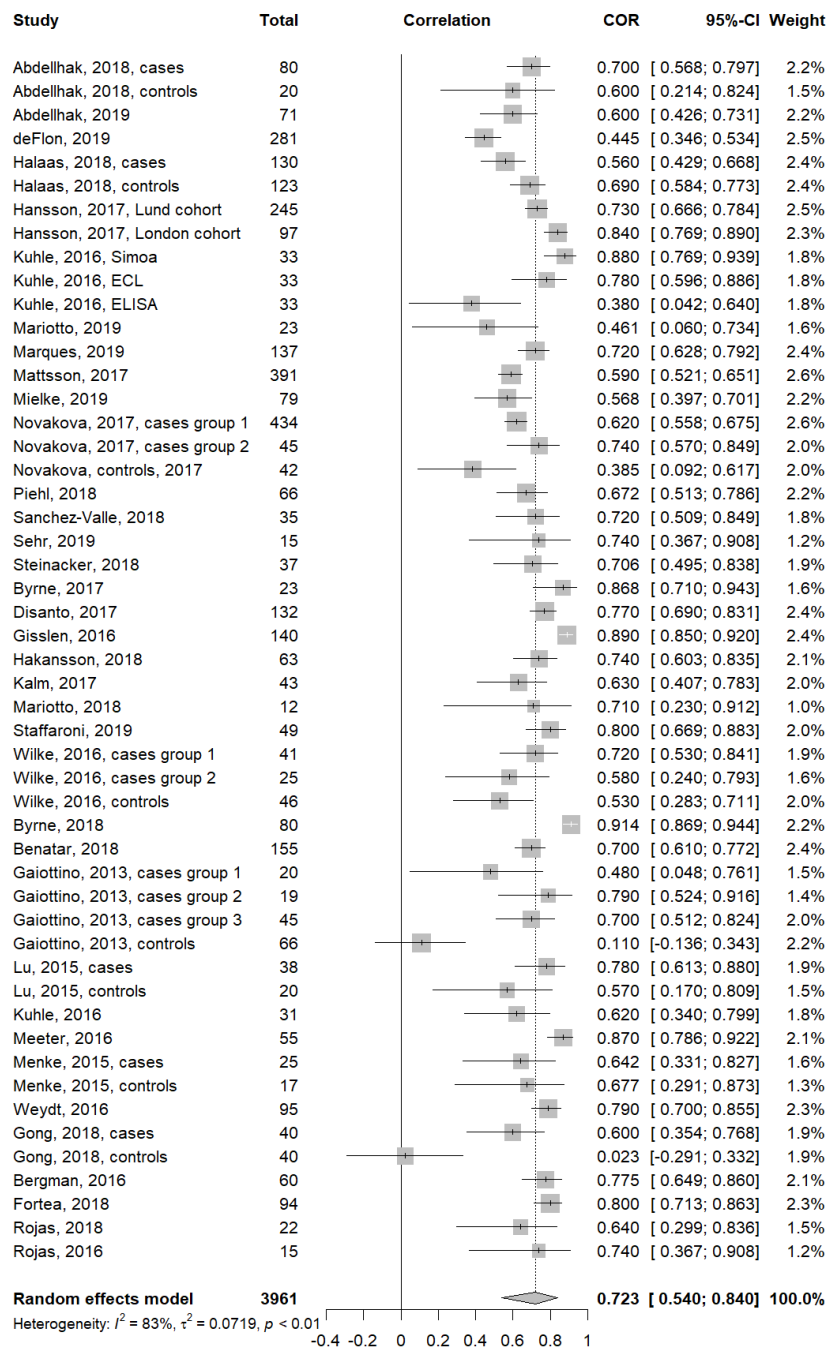
The Simoa digital immunoassay was used to measure blood NfL in 26/36 studies, ECL assay was used in 8/36 studies, ELISA was used in 1/36 study and one study reported blood NfL results separately using all three assays (Simoa, ECL and ELISA) for each blood NfL sample.

In total, 23/36 studies reported on the correlation coefficient between cerebrospinal fluid and serum NfL, 12/36 studies reported on the correlation coefficient between cerebrospinal fluid and plasma NfL, and one study combined the results from serum and plasma NfL measurements when reporting the correlation coefficient between cerebrospinal fluid and blood NfL. In total, 21 unique correlation coefficients between cerebrospinal fluid and blood NfL were reported in conditions affecting the central nervous system only and 17 unique correlation coefficients were reported in conditions affecting both the central nervous system and peripheral nervous system. Two correlation coefficients were reported for conditions affecting the peripheral nervous system only and seven correlation coefficients were reported in cohorts of control participants. In total, 22/36 studies reported a Spearman's rank correlation coefficient, 13/36 studies reported a Pearson's correlation coefficient, and the correlation technique was unspecified in one study. The corresponding author for this study did not respond to our correspondences requesting this missing information.

3.3.4 Meta-analyses results

Using a random-effects model, the overall pooled correlation coefficient estimate for cerebrospinal fluid and blood NfL across all 36 eligible articles was $r=0.723$ (95% confidence interval (CI) 0.540, 0.840) (Figure 32). Heterogeneity was significant with an I^2 result of 83% and τ^2 (Sidik-Jonkman estimator) of 0.072, $p<0.01$, thus stratified analyses were performed.

Figure 32: Forest plot of the pooled correlation coefficients for cerebrospinal fluid and blood neurofilament light protein

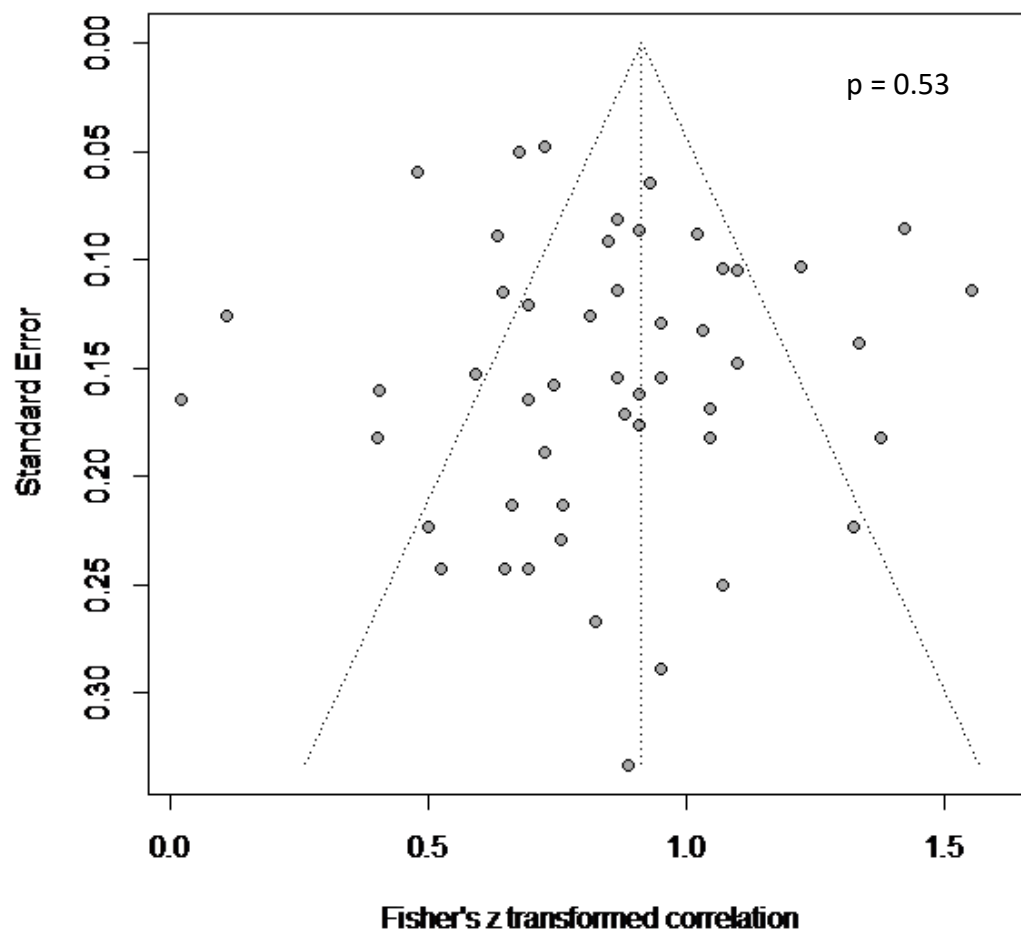


Legend: Forest plot of the summary correlation coefficients with corresponding 95% confidence intervals (CI) for the correlation between cerebrospinal fluid and blood neurofilament light protein from all eligible studies

3.3.5 Rank correlation test of funnel plot asymmetry

The rank correlation test of funnel plot asymmetry showed no asymmetry ($p=0.53$), indicating no obvious publication bias (Figure 33). The funnel plot usually presents effect sizes plotted against their standard errors[386], and in the event of publication bias, the funnel plot is expected to be skewed.

Figure 33: Funnel plot with 95% confidence limits

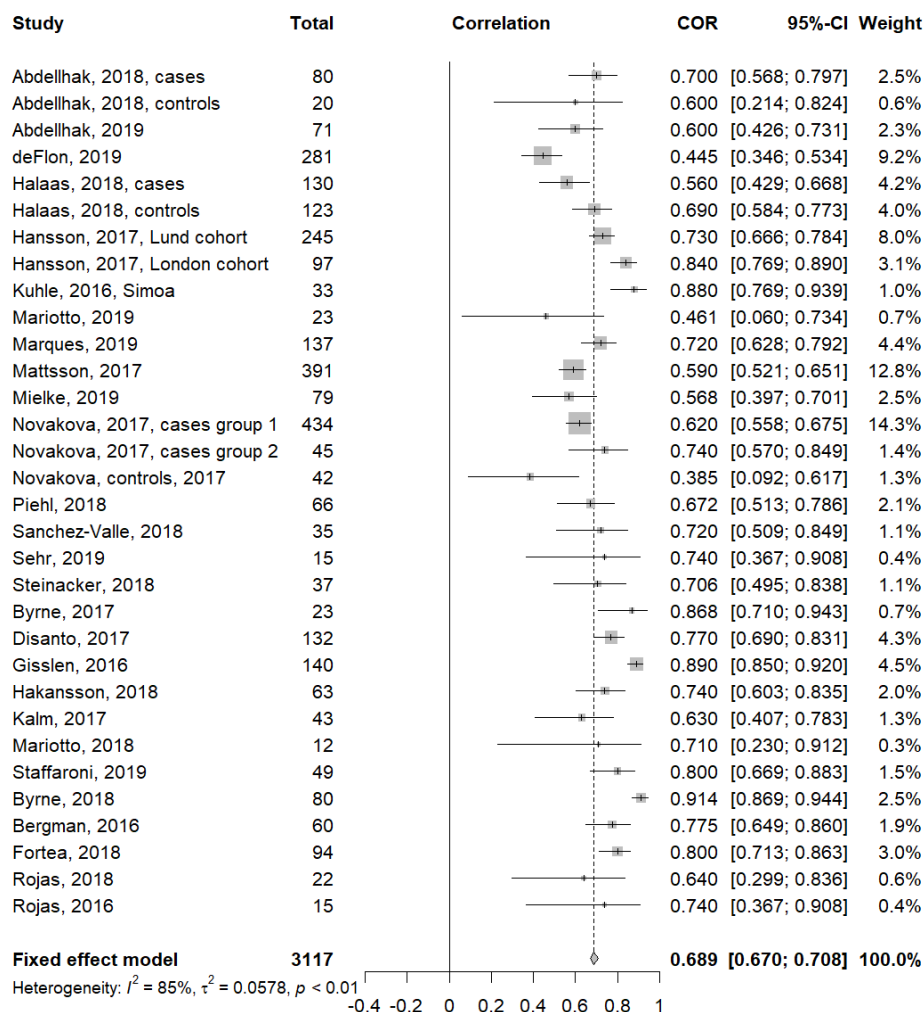


Legend: Fisher's Z-transformed correlation coefficient of the 36 individual studies (horizontal axis) against the standard error (vertical axis).

3.3.6 Blood NfL measurement assay

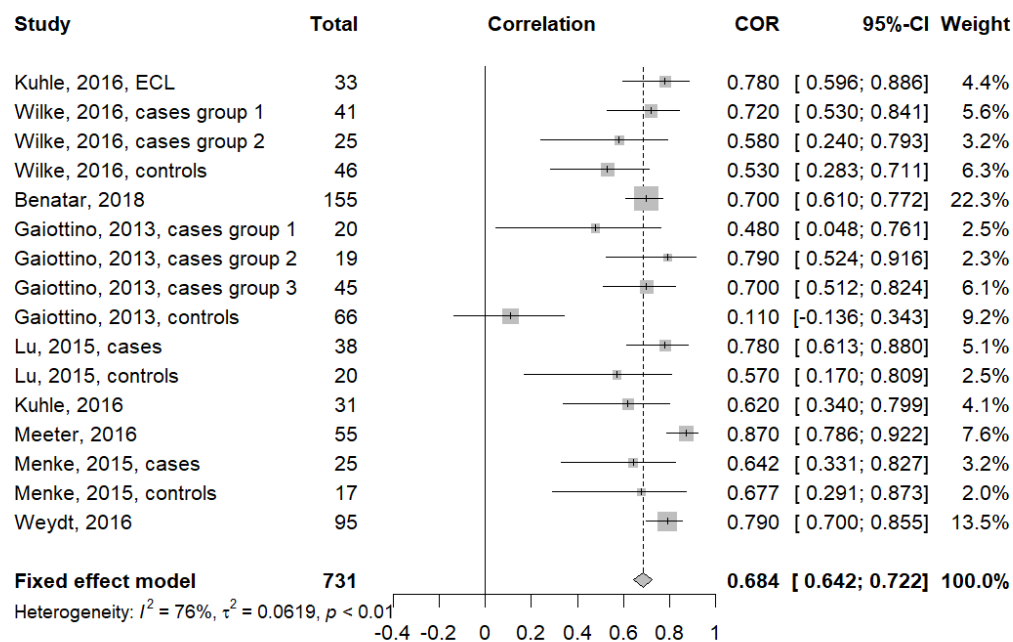
In sensitivity analyses, the pooled correlation coefficient was similar for studies which used the Simoa digital immunoassay (n=3,117) and ECL assay (n=731) to measure blood NfL (r=0.689 (95% CI: 0.670, 0.708) and r=0.684 (95% CI: 0.642, 0.722), respectively) (Figure 34 and 35), but the pooled correlation coefficient was weaker for studies which used ELISA (n=113) to measure blood NfL (r=0.354 (95% CI: 0.176, 0.510)) (Figure 36).

Figure 34: Forest plot of the pooled correlation coefficients for studies using the Simoa assay only



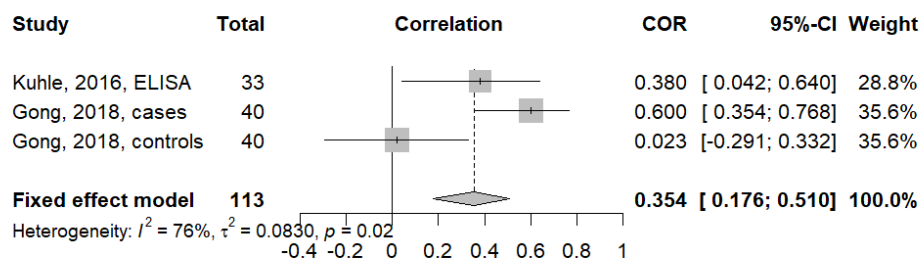
Legend: Forest plot of the summary correlation coefficients with corresponding 95% confidence intervals (CI) for the correlation between cerebrospinal fluid and blood neurofilament light protein from studies which used the Simoa digital immunoassay to measure blood NfL only.

Figure 35: Forest plot of the pooled correlation coefficients for studies using the ECL assay only



Legend: Forest plot of the summary correlation coefficients with corresponding 95% confidence intervals (CI) for the correlation between cerebrospinal fluid and blood neurofilament light protein from studies which used the electrochemiluminescence (ECL) assay to measure blood NfL only.

Figure 36: Forest plot of the pooled correlation coefficients for studies using the ELISA assay only

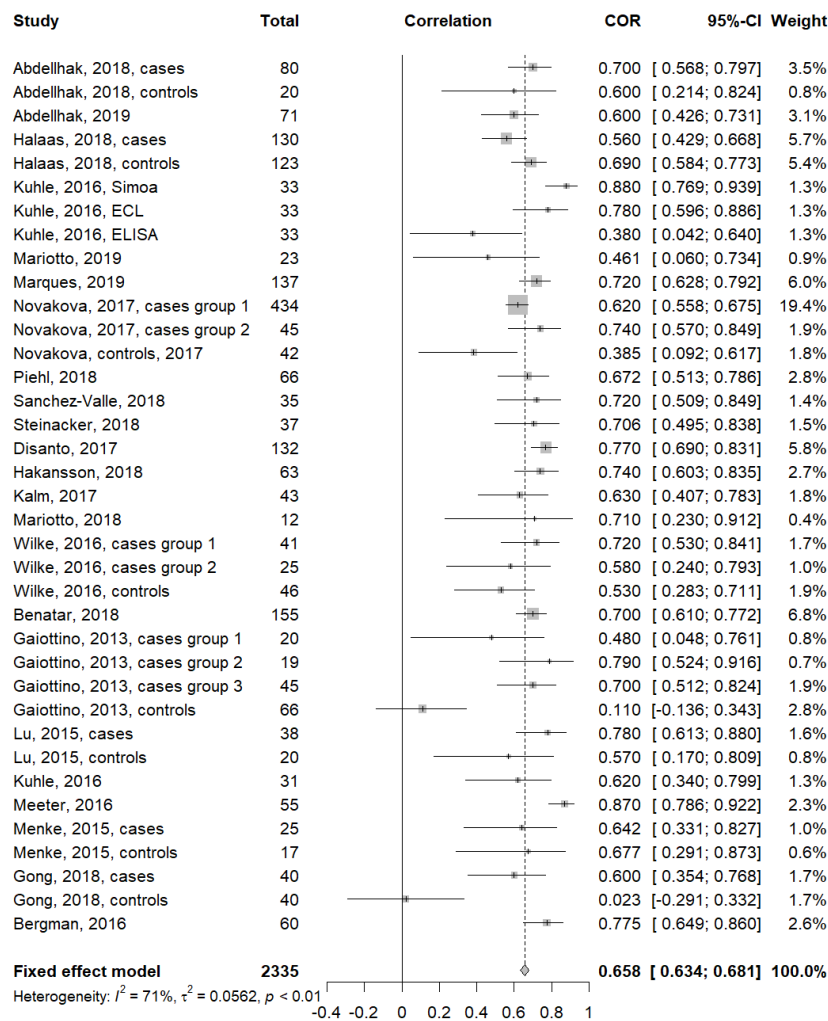


Legend: Forest plot of the summary correlation coefficients with corresponding 95% confidence intervals (CI) for the correlation between cerebrospinal fluid and blood neurofilament light protein from studies which used the enzyme-linked immunosorbent assay (ELISA) assay to measure blood NfL only.

3.3.7 Serum versus plasma NfL measurement

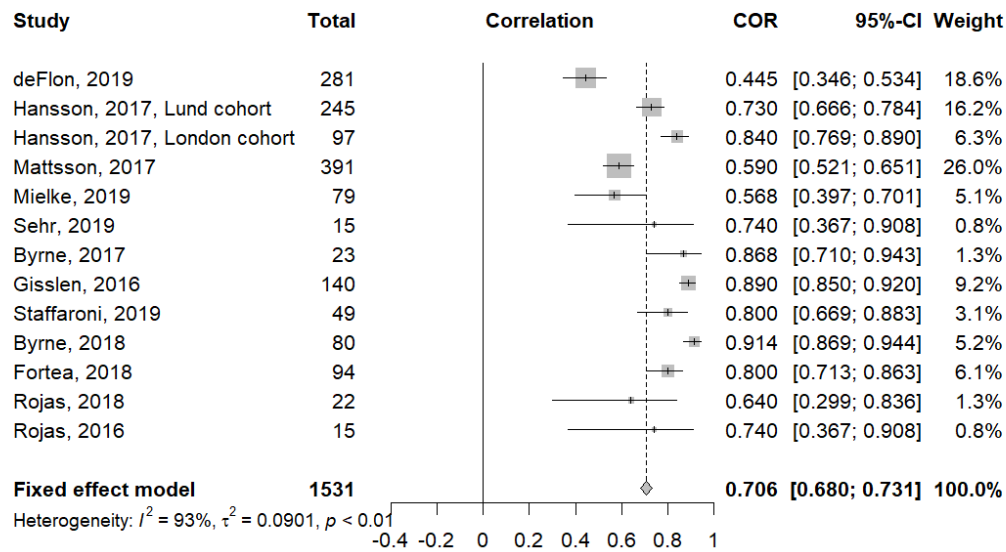
When the studies were stratified according to whether serum or plasma NfL was measured, studies measuring serum NfL (n=2,335) had a pooled correlation coefficient estimate of $r=0.658$ (95% CI: 0.634, 0.681) (Figure 37), and studies measuring plasma NfL (n=1,531) had a pooled estimate of $r=0.706$ (95% CI: 0.680, 0.731) (Figure 38).

Figure 37: Forest plot of the pooled correlation coefficients for studies that measured serum NfL



Legend: Forest plot of the summary correlation coefficients with corresponding 95% confidence intervals (CI) for the correlation between cerebrospinal fluid and blood neurofilament light protein from studies which measured serum NfL

Figure 38: Forest plot of the pooled correlation coefficients for studies that measured plasma NfL

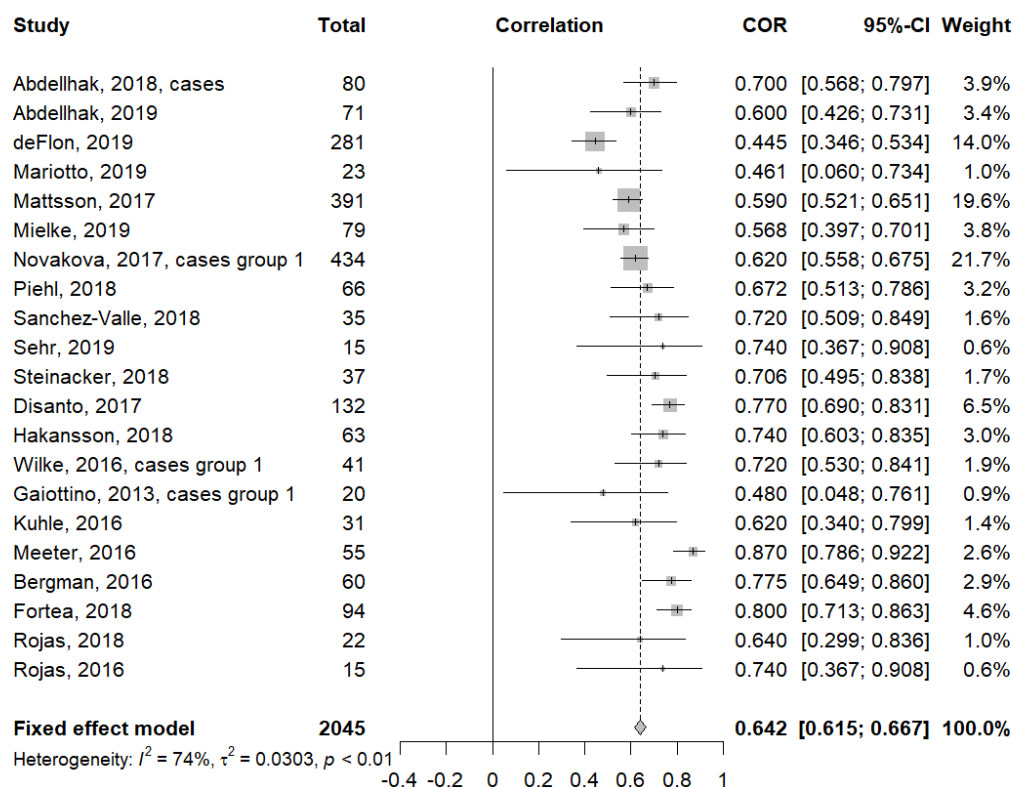


Legend: Forest plot of the summary correlation coefficients with corresponding 95% confidence intervals (CI) for the correlation between cerebrospinal fluid and blood neurofilament light protein from studies which measured plasma NfL

3.3.8 Conditions affecting the central and/or peripheral nervous system

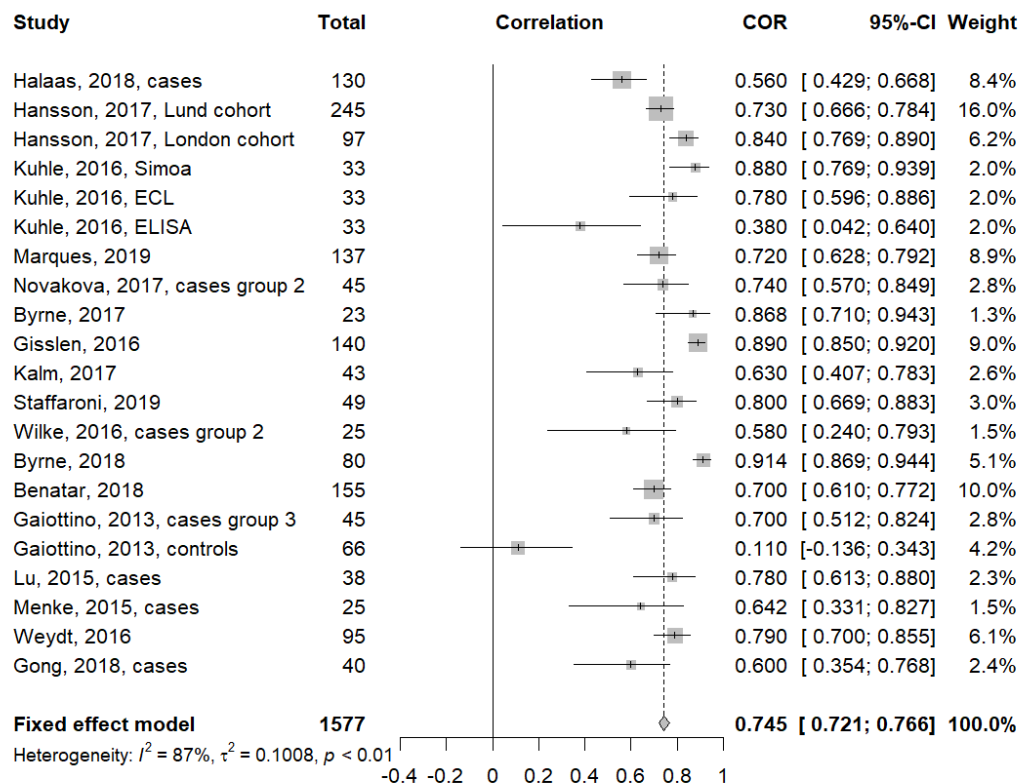
Studies including participants with clinical conditions that purely affected the central nervous system only (n=2,045) had an overall pooled correlation coefficient estimate of $r=0.642$ (95% CI: 0.615, 0.667) (Figure 39). Studies including participants with clinical conditions that affected both the central and peripheral nervous system (n= 1,577) had a higher overall pooled correlation coefficient estimate of $r=0.745$ (95% CI: 0.721, 0.766) (Figure 40). The pooled correlation coefficient estimate was lowest in the control participants (n=308), with $r=0.552$ (95% CI: 0.466, 0.627) (Figure 41).

Figure 39: Forest plot of the pooled correlation coefficients for studies involving conditions affecting the central nervous system only



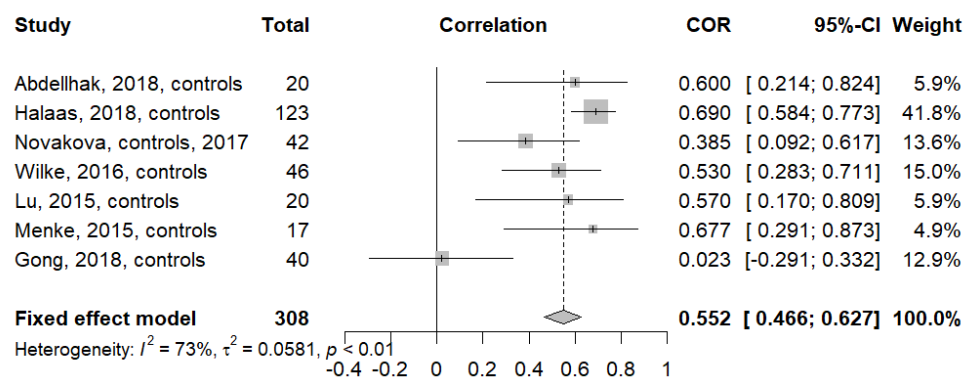
Legend: Forest plot of the summary correlation coefficients with corresponding 95% confidence intervals (CI) for the correlation between cerebrospinal fluid and blood neurofilament light protein from studies including participants with neurological disorders that only affected the central nervous system.

Figure 40: Forest plot of the pooled correlation coefficients for studies involving CNS and PNS disorders



Legend: Forest plot of the summary correlation coefficients with corresponding 95% confidence intervals (CI) for the correlation between cerebrospinal fluid and blood neurofilament light protein from studies including participants with neurological disorders that affected both the central nervous system and peripheral nervous system.

Figure 41: Forest plot of the pooled correlation coefficients from control participants

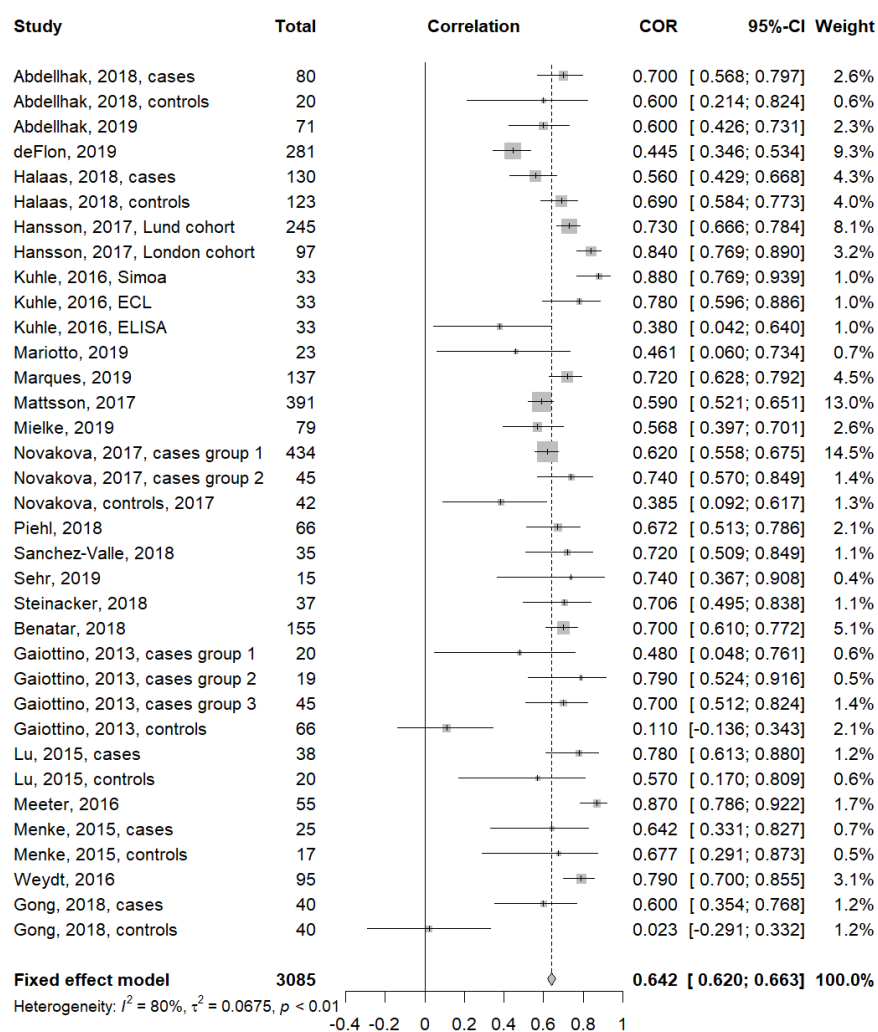


Legend: Forest plot of the summary correlation coefficients with corresponding 95% confidence intervals (CI) for the correlation between cerebrospinal fluid and blood neurofilament light protein from control participants.

3.3.9 Statistical correlation technique

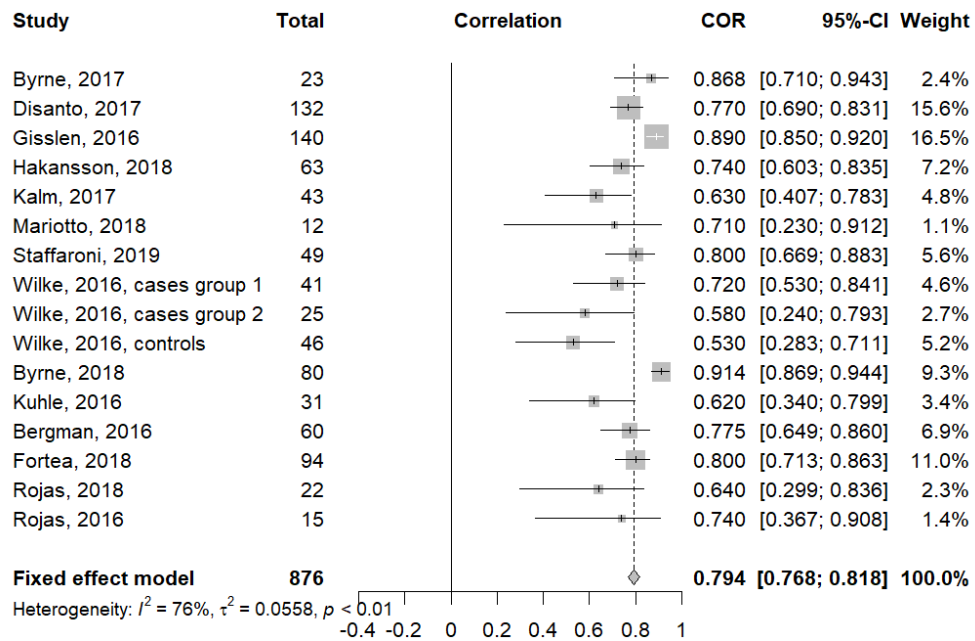
When stratified according to the statistical correlation technique utilised, studies reporting a Spearman's rank correlation coefficient (n=3,085) had a pooled correlation coefficient estimate of $r=0.642$ (95%CI: 0.620, 0.663) (Figure 42), whereas the pooled correlation coefficient estimate was higher for studies reporting a Pearson's correlation coefficient (n=876), with $r=0.794$ (95% CI: 0.768, 0.818) (Figure 43).

Figure 42: Forest plot of the pooled correlation coefficients from studies reporting Spearman's rank correlation coefficient



Legend: Forest plot of the summary correlation coefficients with corresponding 95% confidence intervals (CI) for the correlation between cerebrospinal fluid and blood neurofilament light protein from studies reporting Spearman's rank correlation coefficient.

Figure 43: Forest plot of the pooled correlation coefficients from studies reporting Pearson's correlation coefficient



Legend: Forest plot of the summary correlation coefficients with corresponding 95% confidence intervals (CI) for the correlation between cerebrospinal fluid and blood neurofilament light protein from studies reporting Pearson's correlation coefficient.

3.3.10 Quality assessment of included studies

The quality assessment outcomes of the included studies are summarised in Table 10. Overall, 32 studies were categorised as ‘good’ quality and four studies were categorised as ‘fair’ quality. The quality assessment tools used in this study are included in the Appendix section at the end of this thesis.

Table 10: Quality assessment of the studies included in the meta-analysis

First author's surname and year of publication	QA 1	QA 2	QA 3	QA 4	QA 5	QA 6	QA 7	QA 8	QA 9	QA 10	QA 11	QA 12	QA 13	QA 14	Quality assessment score	Outcome
Observational Cohort and Cross-Sectional Studies																
Abdellhak <i>et al</i> [382], 2018	Y	Y	NR	Y	N	Y	Y	N	Y	NA	Y	NR	NA	N	7	Good
Abdellhak <i>et al</i> [383], 2019	Y	Y	NR	Y	N	Y	Y	N	Y	NA	Y	NR	NA	N	7	Good
Benatar <i>et al</i> [384], 2017	Y	Y	NR	Y	N	Y	Y	N	Y	NA	Y	Y	NA	N	8	Good
Byrne <i>et al</i> [385], 2017	Y	Y	NR	Y	N	Y	Y	N	Y	NA	Y	NR	NA	N	7	Good
Byrne <i>et al</i> [356], 2018	Y	Y	NR	Y	Y	Y	Y	N	Y	NA	Y	NR	NA	N	8	Good
Disanto <i>et al</i> [294], 2017	Y	Y	NR	Y	NR	Y	Y	N	Y	NA	Y	NR	NA	N	7	Good
Fortea <i>et al</i> [358], 2018	Y	Y	Y	Y	N	Y	Y	N	N	NA	Y	NR	NA	N	7	Good
Gaiottino <i>et al</i> [359], 2013	Y	Y	NR	Y	N	Y	CD	N	Y	NA	Y	NR	NA	N	6	Fair
Gisslen <i>et al</i> [293], 2016	Y	Y	NR	Y	N	Y	Y	N	Y	NA	Y	NR	NA	N	7	Good

Gong <i>et al</i> [360], 2018	Y	Y	CD	Y	N	Y	Y	N	Y	NA	Y	NR	NA	N	7	Good
Hakansson <i>et al</i> [361], 2018	Y	Y	NR	Y	N	Y	Y	N	Y	NA	Y	NR	NA	N	7	Good
Halaas <i>et al</i> [362], 2019	Y	Y	NR	Y	N	Y	CD	N	Y	NA	Y	NR	NA	N	6	Fair
Hansson <i>et al</i> [297], 2017	Y	Y	NR	Y	N	Y	Y	N	Y	NA	Y	NR	NA	N	7	Good
Kalm <i>et al</i> [363], 2017	Y	Y	NR	CD	N	Y	CD	N	Y	NA	Y	NR	NA	N	5	Fair
Kuhle <i>et al</i> [291], 2016	Y	Y	NR	N	N	Y	Y	N	Y	NA	Y	Y	NA	N	7	Good
Kuhle <i>et al</i> [364], 2016	Y	Y	NR	Y	N	Y	Y	N	Y	NA	Y	Y	NA	N	8	Good
Lu <i>et al</i> [365], 2015	Y	Y	NR	Y	N	Y	Y	N	Y	NA	Y	Y	NA	N	8	Good
Mariotto <i>et al</i> [366], 2018	Y	Y	NR	Y	N	Y	Y	N	Y	NA	Y	Y	NA	N	8	Good
Mariotto <i>et al</i> [367], 2019	Y	Y	NR	Y	N	Y	Y	N	Y	NA	Y	Y	NA	N	8	Good
Marques <i>et al</i> [368], 2019	Y	Y	NR	Y	N	Y	Y	Y	Y	NA	Y	Y	NA	N	9	Good
Mattsson <i>et al</i> [369], 2017	Y	Y	NR	Y	N	Y	Y	Y	Y	NA	Y	NR	NA	N	8	Good
Meeter <i>et al</i> [298], 2016	Y	Y	N	Y	Y	Y	Y	Y	Y	NA	Y	NR	NA	N	9	Good
Menke <i>et al</i> [370], 2015	Y	Y	NR	Y	N	Y	Y	N	Y	NA	Y	NR	NA	N	7	Good
Mielke <i>et al</i> [371], 2019	Y	Y	NR	Y	N	Y	CD	N	Y	NA	Y	NR	NA	N	6	Fair
Novakova <i>et al</i> [372], 2017	Y	Y	NR	Y	N	Y	Y	Y	Y	NA	Y	NR	NA	N	8	Good
Piehl <i>et al</i> [373], 2018	Y	Y	NR	Y	N	Y	Y	NA	Y	NA	Y	NR	NA	N	7	Good
Rojas <i>et al</i> [374], 2018	Y	Y	NR	Y	N	Y	Y	N	Y	NA	Y	NR	NA	N	7	Good

Rojas <i>et al</i> [375], 2016	Y	Y	NR	Y	N	Y	Y	NA	Y	NA	Y	NR	NA	N	7	Good
Sanchez-Valle <i>et al</i> [376], 2018	Y	Y	NR	Y	N	Y	Y	NA	Y	NA	Y	NR	NA	N	7	Good
Sehr <i>et al</i> [377], 2019	Y	Y	Y	Y	N	Y	Y	NA	Y	NA	Y	NR	NA	N	8	Good
Staffaroni <i>et al</i> [378], 2019	Y	Y	NR	Y	N	Y	Y	N	Y	NA	Y	NR	NA	N	7	Good
Steinacker <i>et al</i> [379], 2018	Y	Y	NR	Y	N	Y	Y	Y	Y	NA	Y	NR	NA	N	8	Good
Weydt <i>et al</i> [380], 2016	Y	Y	NR	Y	N	Y	Y	NA	Y	NA	Y	NR	NA	N	7	Good
Wilke <i>et al</i> [381], 2016	Y	Y	Y	Y	N	Y	Y	Y	Y	NA	Y	NR	NA	N	9	Good
Before and after (Pre and Post) Studies with no control group																
Bergman <i>et al</i> [36], 2016	Y	Y	CD	Y	CD	Y	Y	NR	Y	NA	Y	NA	.	.	7	Good
deFlon <i>et al</i> [45], 2019	Y	Y	Y	N	CD	Y	Y	NR	Y	NA	Y	NA	.	.	7	Good
Abbreviations: QA = quality assessment question (see quality assessment tools in the Appendix section), Y = yes, N = no, CD = cannot be determined, NA = not applicable, NR = not reported																

3.4 Discussion

3.4.1 Summary of overall findings:

In this chapter, I have shown that the overall pooled correlation coefficient estimate between cerebrospinal fluid and blood NfL across different disease states and physiological conditions is moderate, as per the Chan definition[387]. While this finding is encouraging, it is vital to note that cerebrospinal fluid and blood NfL do not correlate perfectly in all conditions and additional factors may need to be considered when interpreting blood NfL measurements.

On stratification according to blood NfL measurement assay utilised, moderate correlations are demonstrated between cerebrospinal fluid and blood NfL in studies utilising Simoa and ECL blood NfL assays, but the correlations between cerebrospinal fluid and blood NfL in studies using the ELISA technique were much lower. Simoa is the current preferred blood NfL assay especially at low or physiological concentrations due to its high analytical sensitivity (low detection limit)[291,294] and the results presented in this chapter supports its use as the current most advanced surrogate measure of cerebrospinal fluid NfL.

The pooled correlation coefficient estimates were similarly moderate when stratified according to studies which measured plasma NfL versus serum NfL, in keeping with the literature confirming the lack of difference in NfL concentration when measured in these two matrices[388].

When stratified according to underlying disease process being investigated (conditions purely affecting the central nervous system versus conditions affecting both the central and peripheral nervous system versus control participants), the pooled correlation coefficient estimates were highest in participants with conditions affecting both the central and peripheral nervous systems and lowest in the control participants. Of note, the control participants included in the various publications comprised a wide spectrum of individuals ranging from neuro-asymptomatic control participants with no other diagnosed health conditions to neurologically symptomatic participants with condition(s) other than the index condition being examined in that study. Due to the heterogeneity of the control participant population, the pooled correlation coefficient of the control participants in this meta-

analysis is likely on over-estimation, due to some control participants possibly having elevated NfL concentrations. However, overall, the NfL concentration range in control participants was generally closer to the analytical sensitivity of the NfL assays utilized and more subjected to the effects of random variability, thus resulting in a lower correlation between cerebrospinal fluid and blood NfL, compared to participants with neurological conditions. This would suggest that blood NfL is a better surrogate measure of cerebrospinal fluid NfL in situations when cerebrospinal fluid NfL concentration ranges are higher. Additionally, participants with disorders affecting the central nervous system may have a more disrupted blood-brain barrier and thus leak more NfL from the cerebrospinal fluid space into the bloodstream, compared to control participants, who are more likely to have intact blood-brain barriers and thus leak less cerebrospinal fluid NfL into the bloodstream. Only two studies investigating diseases involving the peripheral nervous system reported a correlation between cerebrospinal fluid NfL and blood NfL[359,366], which may reflect the non-routine requirement for cerebrospinal fluid sampling in conditions affecting the peripheral nervous system only. The pooled correlation coefficient estimates between cerebrospinal fluid and blood NfL is higher in studies quoting a Pearson's correlation coefficient compared to those reporting Spearman's rank correlation coefficient, possibly due to the presence of outliers or to non-normality of NfL concentrations within the studies.

Strengths of this systematic review are the high methodological standards used to conduct the systematic review, and the inclusion of potential confounders in sensitivity analyses. Limitations include publication bias, which may result in an overestimation of the pooled correlation coefficient estimates. Most studies enrolled participants in Western Europe and North America, and it is unknown whether these results can be extrapolated to individuals globally. I only included articles published in English language as part of my search strategy and excluded one study that was not in English language, which may have affected the pooled correlation coefficient estimates. The number of blood samples measured for NfL using the ELISA technique was much smaller (n=113) compared to Simoa (n=3117) and ECL (n=731) techniques, as reflected by the much wider 95% confidence interval for the pooled correlation coefficient estimate between cerebrospinal fluid and blood NfL in samples measured using ELISA technique. However, the most likely explanation for the variable results is that blood NfL concentration measured by ELISA simply reflects noise, as the

analytical sensitivity of the ELISA is not sufficient to quantify NfL in blood reliably. I was unable to explore the impact of heterogeneous factors that may influence NfL measurement and interpretation such as lower body mass index[389], renal function[390], blood-brain barrier impairment[391], gender, unicentric versus multicentric studies, cross-sectional versus longitudinal studies and the duration between cerebrospinal fluid and blood sampling, due to the inconsistent availability of this data from the publications. Older age is associated with higher CSF and blood NfL concentrations[285] and some conditions included in this systematic review are predominantly associated with older age. Ideally, I would have adjusted for age in the meta-analyses, however, age was not consistently reported in the same manner in the studies included, and the lack of integration of age into the meta-analyses may impact the interpretation of the meta-analyses results.

3.5 Conclusion

Using rigorous systematic review and meta-analysis, I observed an overall moderate correlation between cerebrospinal fluid and blood NfL. To date, the strength of the correlation between cerebrospinal fluid and blood NfL has been uncertain due to the variations in reported correlation coefficients between studies performed under varying conditions.

The findings in this chapter support the use of blood NfL measurement as a surrogate marker of cerebrospinal fluid NfL and thus, neural activation, especially in situations when NfL concentrations are elevated and above concentrations seen under physiological conditions.

Chapter 4

Correlation between cerebrospinal fluid and plasma NfL in PLWH on suppressive cART, and in lifestyle- and age- similar HIV-negative controls: the COBRA cohort

Chapter 4: Correlation between cerebrospinal fluid and plasma NfL in PLWH on suppressive cART, and in lifestyle- and age-similar HIV-negative controls: the COBRA cohort

4.1 Introduction

In my previous chapter, I demonstrated that the overall pooled correlation between cerebrospinal fluid and blood NfL is moderate, with stronger correlations seen in conditions with higher cerebrospinal fluid and blood NfL concentrations.

To address my PhD hypotheses that concentrations of biomarkers of neural activation are associated with measures of HIV reservoir size and HIV viral transcription, I plan to utilise plasma NfL as a key biomarker of neuronal injury in various cohorts of PLWH undergoing different interventions.

Cerebrospinal fluid [269,275,285,288] and plasma NfL [293] have been shown to be highest in individuals with HIV-associated dementia and to correlate positively with HIV RNA[293]. Elevated cerebrospinal fluid NfL concentrations can also be consistently detected in neuro-asymptomatic PLWH not on cART, and is highest in those with CD4⁺ counts <50 cells/ μ L[276,288]. However, following the initiation of cART and HIV viral suppression, NfL concentrations in the cerebrospinal fluid and plasma frequently near-normalise[269,274,288].

To date, data on the correlation between cerebrospinal fluid and blood NfL in people living with HIV is limited. Early studies suggest that cerebrospinal fluid strongly correlates with blood NfL in a cohort of mainly untreated people with HIV[293]. Whether this strong correlation persists in PLWH on virologically suppressive cART, when cerebrospinal fluid and blood NfL concentrations are (near) normal, is unknown. Additionally, plasma NfL may perform differently as a surrogate marker of cerebrospinal fluid NfL in people with

untreated HIV, compared to people with HIV on suppressive cART, due to differences in the blood-brain barrier permeability. When NfL is released from central nervous system neuro-axonal units, it reaches the interstitial fluid which communicates freely with cerebrospinal fluid and blood. It could be postulated that an impaired blood-brain barrier from untreated HIV-disease or lifestyle factors such as alcohol and recreational drug use may be associated with higher transfer of NfL from the cerebrospinal fluid into the bloodstream.

Thus, it is crucial to understand how plasma NfL performs as a surrogate marker of cerebrospinal fluid in people with HIV on suppressive antiretroviral treatment, to enable a more accurate interpretation of plasma NfL in the following chapters of my PhD thesis.

In this chapter, I will firstly assess the degree of correlation between cerebrospinal fluid and plasma NfL in PLWH on virally suppressive antiretroviral treatment and in lifestyle-similar HIV-negative individuals and secondly, determine factors associated with cerebrospinal fluid and plasma NfL in PLWH on virally suppressive antiretroviral treatment and lifestyle-similar HIV-negative individuals. Finally, to explore the impact of lifestyle on blood NfL concentrations, I compared blood NfL concentrations between HIV-positive participants and HIV-negative control participants in the COBRA study and a cohort of blood bank donors.

4.2 Methods

4.2.1 Participants

HIV-positive and demographically similar HIV-negative participants were recruited into the 'COMorBidity in Relation to AIDS' (COBRA) study at sites in Amsterdam and London. All participants were aged ≥ 45 years, with no significant neurological conditions, intravenous recreational drug use in the previous six months, daily recreational drugs use (apart from cannabis), alcohol intake > 48 units per week or moderate/severe depression (see Chapter 2 Materials and Methods section for eligibility criteria and further details)[335]. All HIV-positive participants were required to be receiving cART and to have had plasma HIV RNA < 50 copies/mL for ≥ 12 months prior to enrolment[335].

4.2.2 Study procedures

At the baseline study visit, all participants underwent an assessment with detailed data collection (see Chapter 2: Materials and Methods for further details), along with blood and cerebrospinal fluid collection (see Chapter 2: Materials and Methods for further details).

4.2.3 Blood NfL measurement

I retrieved 1mL stored frozen plasma sample from each participant's baseline study visit and measured plasma NfL concentration, as described in Chapter 2: Materials and Methods.

4.2.4 Other biomarkers measured within the original COBRA study

Within the original study, the following biomarkers were measured in:

- i. **Blood:** CD4⁺ and CD8⁺ T-cell counts, albumin, creatinine, HIV RNA and neopterin
- ii. **Cerebrospinal fluid:** albumin, protein, leukocytes, glucose, HIV RNA, neopterin and NfL

Cerebrospinal fluid NfL was measured using the commercially-available sandwich ELISA technique (NF-light ELISA kit; UmanDiagnostics AB, Umeå, Sweden)[335].

Blood and cerebrospinal fluid HIV RNA were measured using the Abbott RealTime M2000 assay (Abbot, Chicago, USA) with a lower limit of detection of 40 HIV RNA copies/mL[335].

Estimated glomerular filtration rate was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula[344].

4.2.5 Plasma samples from blood bank donors

Blood-based materials from 35 age-matched unselected blood bank donors were obtained from the Dutch national blood bank in Amsterdam, The Netherlands (www.sanguin.nl), as part of a sub-study within the main COBRA study[154]. Age and gender information were available for the blood bank donors.

Blood bank donors from The Netherlands are regularly screened for blood-borne infections such as HIV, HTLV, Hepatitis B, Hepatitis C and syphilis. Individuals aged over 70 years or those with high-risk behaviour for acquiring blood-borne infections are excluded from blood donation. Overall, blood bank donors are at lower risk of acquiring blood borne infections than the general population but are often used as a control group for comparative studies.

I retrieved stored plasma samples from all 35 blood bank donors who were included in the previous COBRA sub-study, and measured plasma NfL using the same technique as described in Chapter 2: Materials and Methods section.

4.2.6 Statistical analyses

Differences in demographics were assessed using Wilcoxon's rank-sum test for continuous variables and chi-square test for categorical variables. Differences between PLWH, HIV-negative participants and blood bank donors were first assessed using the Kruskal-Wallis then Wilcoxon's rank-sum test for age, and chi-square test for gender. Differences in cerebrospinal fluid and plasma NfL concentrations and cerebrospinal fluid: plasma NfL ratios between PLWH and HIV-negative participants were tested for significance using Wilcoxon's rank-sum test. In a sub-analysis, differences in plasma NfL concentrations between PLWH, HIV-negative participants and blood bank donors were assessed using Wilcoxon's rank-sum test. Non-parametric statistical tests were employed as these do not make distributional assumptions.

The correlation between cerebrospinal fluid and plasma NfL (after $\log_{10}$ -transformation to reduce data skewness) was assessed using Pearson's correlation coefficient (a parametric statistical test), and the difference between groups was tested for significance using the Z-test after Fisher's transformation.

$\log_{10}$ -transformed cerebrospinal fluid and plasma NfL Z-scores were calculated which reduced data skewness and enabled direct comparisons between cerebrospinal fluid and plasma NfL. A Bland-Altman graph[343] was plotted to describe the agreement between $\log_{10}$ -transformed cerebrospinal fluid and plasma NfL Z-scores. This represents a plot of the difference between the cerebrospinal fluid and plasma NfL Z-score against their average for each individual.

Linear regression models were used to identify factors independently associated with $\log_{10}$ -transformed cerebrospinal fluid and plasma NfL, standardised to Z-scores with a mean of 0 and standard deviation of 1, separately in PLWH and HIV-negative participants. Factors included in the univariable linear regression analysis were female gender, age, Black African ethnicity, men who have sex with men sexual orientation, weight, body mass index, current tobacco smoker, ex-tobacco smoker, current alcohol consumption, past alcohol consumption, recent recreational drug use, ever used injected recreational drugs, on hypertensive medication, global cognitive function T-score, serum creatinine, serum albumin, cerebrospinal fluid protein, cerebrospinal fluid neopterin and plasma neopterin.

HIV-specific parameters (current CD4⁺ and CD8⁺ T-cell count, current CD4⁺/8⁺ T-cell ratio, nadir CD4⁺ count, years with CD4⁺ count <200cells/ μ L, prior AIDS diagnosis, on a non-nucleoside reverse-transcriptase inhibitors (NNRTI), on a protease inhibitor (PI), on tenofovir-disoproxil fumarate (TDF) and on atazanavir) significantly associated in univariable analyses were added to an additional model fitted in PLWH only.

The purpose of my regression analysis was to directly compare the impact of the various factors on change in cerebrospinal fluid and plasma NfL separately, rather than to identify the best fitting regression model. For this reason, I included all factors associated with either cerebrospinal fluid or plasma NfL in either PLWH or HIV-negative participants in univariable analyses ($p < 0.05$) in the multivariable models and did not perform a stepwise automatic selection approach to selecting factors to include in the models. The statistical analyses performed within this chapter were discussed with Professor Caroline Sabin, the lead biostatistician within the COBRA consortium, who approved the proposed statistical plans.

Formal sample size calculation was not performed. The proposal for this project was reviewed by the COBRA study consortium, members of whom included experienced biostatisticians, and it was agreed that this was a valuable piece of work to perform, and the number of stored paired samples were likely to provide a viable readout. Pragmatically, it would not have been possible to increase the sample size without including samples from other cohorts, which may have introduced biases.

All statistical analyses were conducted using SPSS version 26 (IBM Corp, Armonk, NY, US), apart from the Bland-Altman graph, which was performed using SAS version 9.4 (SAS Institute, Cary, NC, USA) by our collaborating biostatistician who was working on the COBRA study, DDF. *P*-values <0.05 were considered statistically significant throughout.

4.3 Results

In total, 132/134 PLWH and 79/79 HIV-negative participants from the COBRA cohort and all 35 blood bank donors who were included in the previous COBRA sub-study had sufficient stored plasma available and were included in this analysis.

4.3.1 Participant characteristics

For the participants included in the main COBRA study, socio-demographic data, lifestyle characteristics and anthropometrics at study baseline were described previously[335]. Apart from age and gender, this data is not available for the blood bank donors.

PLWH and HIV-negative participants in this analysis were similar in terms of age, gender, sexual orientation, anthropometric measurements, years of education and lifestyle behaviour in terms of smoking and recreational drug (Table 11). PLWH were less likely to be of white ethnicity ($p=0.02$) and current alcohol drinkers ($p=0.04$) than HIV-negative participants (Table 11).

Cerebrospinal fluid albumin data was not available in 30/79 HIV-negative participants. In participants with cerebrospinal fluid albumin data available, cerebrospinal fluid: serum albumin ratio (a marker of blood-brain barrier dysfunction) was similar between the groups, $p = 0.38$. Serum creatinine was higher in PLWH ($p = 0.01$) and correspondingly, estimated glomerular filtration rate was lower in PLWH ($p = 0.03$) (Table 11).

PLWH had been diagnosed for a median (IQR) of 15.0 (9.0, 19.6) years with a median (IQR) current CD4⁺ T-cell count of 618 (472, 809) cells/ μ L, current CD4⁺/CD8⁺ T-cell count ratio of 0.83 (0.60, 1.11) and nadir CD4⁺ T-cell count of 175 (85, 240) cells/ μ L. PLWH had been on cART for a median (IQR) of 12.8 (7.8, 16.9) years; all PLWH had undetectable plasma HIV RNA and 130/132 had undetectable cerebrospinal fluid HIV RNA (Table 11).

Table 11: Baseline characteristics of the COBRA cohort included in this sub-study

Variables, median (interquartile range) or n (%)	People with HIV (n=132)	HIV-negative participants (n=79)	p-value ¹
Age, years	56 (51, 62)	57 (52, 64)	0.30
Gender			0.66
Female	8 (6.1)	6 (7.6)	
Male	124 (93.9)	73 (92.4)	
Ethnicity			0.02
Black-African	16 (12.2)	2 (2.6)	
White	115 (87.8)	76 (97.4)	
Sexual orientation			0.39
Men who have sex with men	103 (78.0)	59 (74.7)	
Bisexual	10 (7.6)	4 (5.1)	
Heterosexual	18 (13.6)	16 (20.2)	
Years of education	14 (13, 16)	16 (14, 17)	0.21
Global T-score	51.2 (46.0, 54.9)	54.2 (50.2, 57.6)	<0.001
Weight, kg	79.6 (70.0, 87.7)	81.2 (72.5, 91.0)	0.14
Body mass index, kg/m ²	24.7 (22.7, 27.5)	24.6 (23.2, 28.4)	0.35
On hypertensive medication	29 (22.0)	14 (17.7)	0.46
Smoking status			0.22
Current smoker	39 (29.6)	20 (25.3)	
Ex-smoker	58 (43.9)	29 (36.7)	
Never smoked	35 (26.5)	30 (37.8)	
Years of smoking (current/ex-smokers)	29 (19, 37)	26 (15, 39)	0.40
Alcohol consumption			0.04
Current drinker	103 (78.0)	71 (91.0)	
Ex-drinker	17 (12.9)	3 (3.9)	
Never drank	12 (9.1)	4 (5.1)	
Years of drinking (current/ex drinkers)	29 (19, 37)	41 (35, 47)	0.01
Alcohol units/week (current drinkers)	5.5 (1.5, 15.0)	7.5 (1.5, 17.5)	0.39
Use of recreational drugs in past 6 months	43 (32.6)	18 (22.8)	0.13
Cerebrospinal fluid albumin, mg/L	299 (210, 368)	267 (214, 326)	0.43
Serum albumin, g/L	43 (41, 46)	43 (40, 45)	0.08
Cerebrospinal fluid/ serum albumin ratio	6.9 (4.9, 8.5)	6.2 (5.0, 7.5)	0.38
Creatinine, mmol/L	85.5 (76, 100)	79 (72, 92)	0.01
Estimated glomerular filtration rate, mL/min/1.73 m ²	85.5 (71.0, 97.3)	92.0 (77.4, 102.3)	0.03
Years since HIV diagnosis	15.0 (9.0, 19.6)	N/A	N/A
Duration of antiretroviral treatment, years	12.8 (7.8, 16.9)	N/A	N/A
CSF HIV RNA viral load <40 copies/mL	130 (98.5)	N/A	N/A
CD4 ⁺ T-cell count, cells/ μ L	618 (472, 809)	900 (692, 1174)	<0.001
CD8 ⁺ T-cell count, cells/ μ L	767 (611, 968)	422 (313, 614)	<0.001
CD4 ⁺ /CD8 ⁺ T-cell count ratio	0.83 (0.60, 1.11)	2.00 (1.44, 2.65)	<0.001
Nadir CD4 ⁺ T-cell count, cells/ μ L	175 (85, 240)	N/A	N/A
Months with CD4 ⁺ T-cell count <200, cells/ μ L	1.6 (0.0, 9.8)	N/A	N/A
Prior AIDS event	42 (31.8 %)	N/A	N/A

¹ Differences were assessed using Wilcoxon's rank-sum test and chi-square test, as appropriate

4.3.2 Comparison of characteristics with the blood bank donors

Blood bank donors were similar in age to the COBRA participants, but a higher proportion of blood bank donors were female, compared to the COBRA cohort (Table 12).

Table 12: Comparison of demographics between people with HIV, HIV-negative participants and blood bank donors

Variable, n (%) or median (IQR)	People with HIV n = 132	HIV-negative participants n = 79	Blood bank donors n = 35	p-value ¹
Age, years	56 (51, 62)	57 (52, 64)	59 (52, 65)	0.41
Gender				<0.001
Female	8 (6.1%)	6 (7.6%)	17 (48.6%)	
Male	124 (93.9%)	73 (92.4%)	18 (51.4%)	

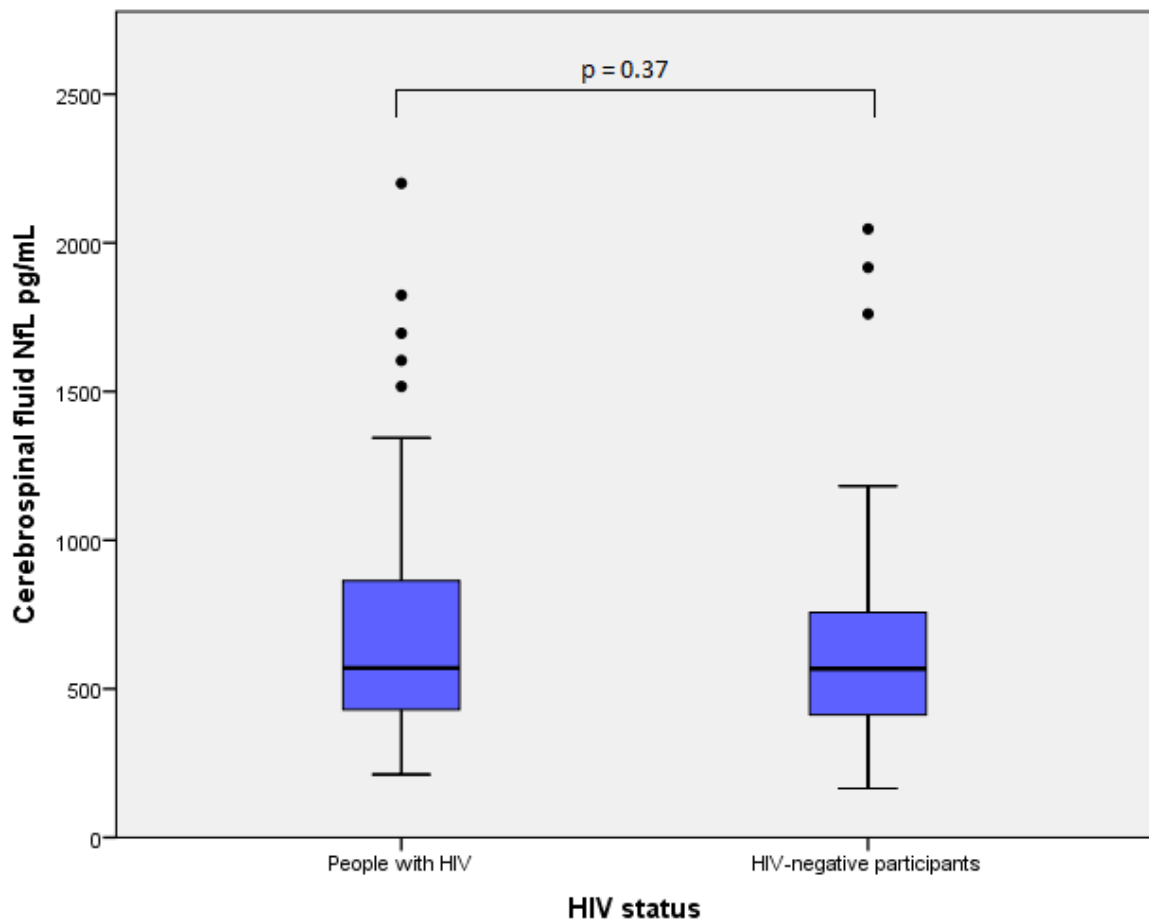
Abbreviations: IQR = interquartile range

¹ Differences were assessed using the Kruskal-Wallis then Wilcoxon's rank-sum test for age, and chi-square test for gender.

4.3.3 Comparison of cerebrospinal fluid and plasma NfL results in the COBRA cohort

Comparing PLWH and HIV-negative participants, neither cerebrospinal fluid NfL (median (IQR) 570 (430, 864) vs 568 (408, 767) pg/mL, $p=0.37$) (Figure 44), nor plasma NfL (median (IQR) 10.7 (7.9, 14.9) vs 9.9 (7.2, 12.5) pg/mL, $p=0.15$) (Figure 45) differed significantly between the groups (Figure 44 - 45 and Table 13). Furthermore, cerebrospinal fluid: plasma NfL ratios were similar between PLWH and HIV-negative participants (median (IQR) 54.7 (39.2, 75.6) vs 65.1 (45.4, 77.8), $p = 0.20$) (Figure 46 and Table 13).

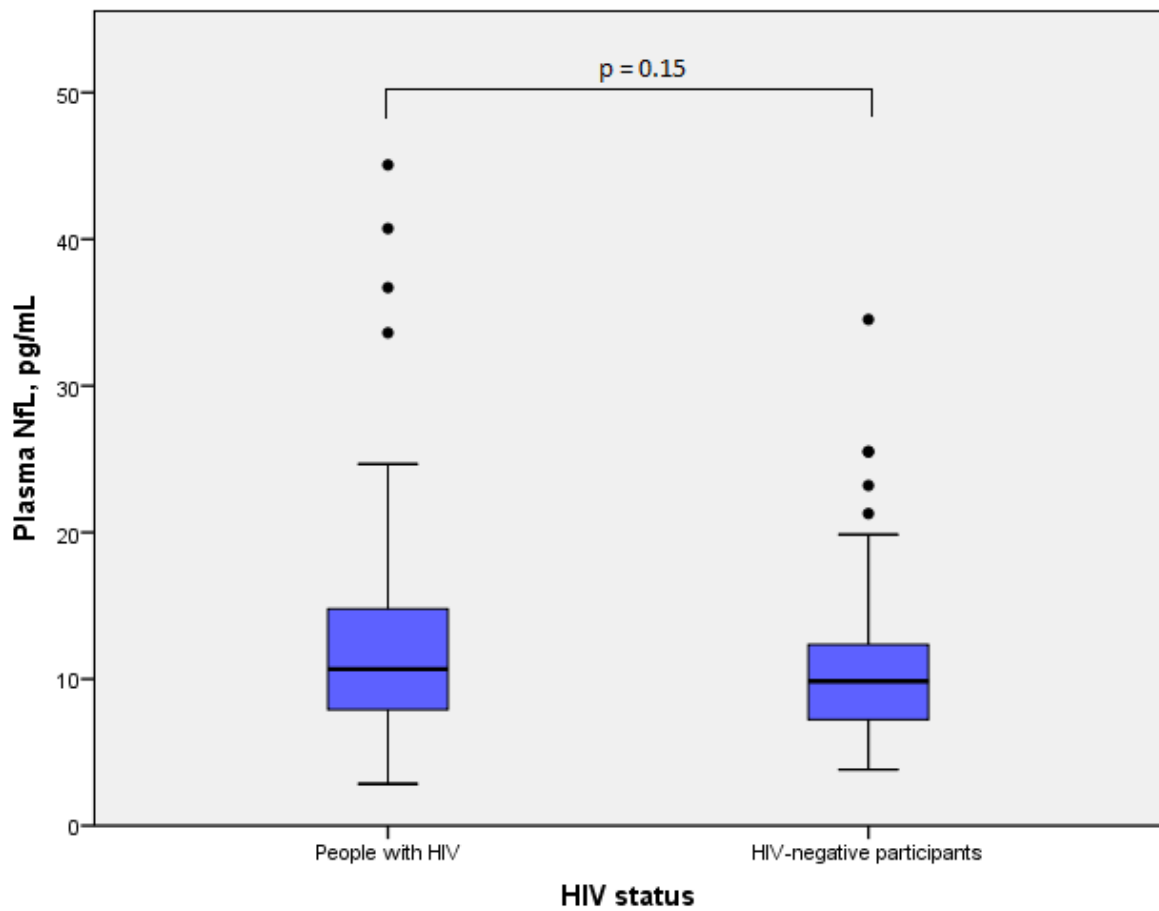
Figure 44: Boxplot comparing cerebrospinal fluid NfL by HIV status



Abbreviations: PLWH = people with HIV, NfL = neurofilament light chain protein

Legend: P-value calculated using the Wilcoxon rank-sum test. Number of participants in each group: PWH (n = 132) and HIV-negative individuals (n = 79). Error bars represent 95% confidence intervals.

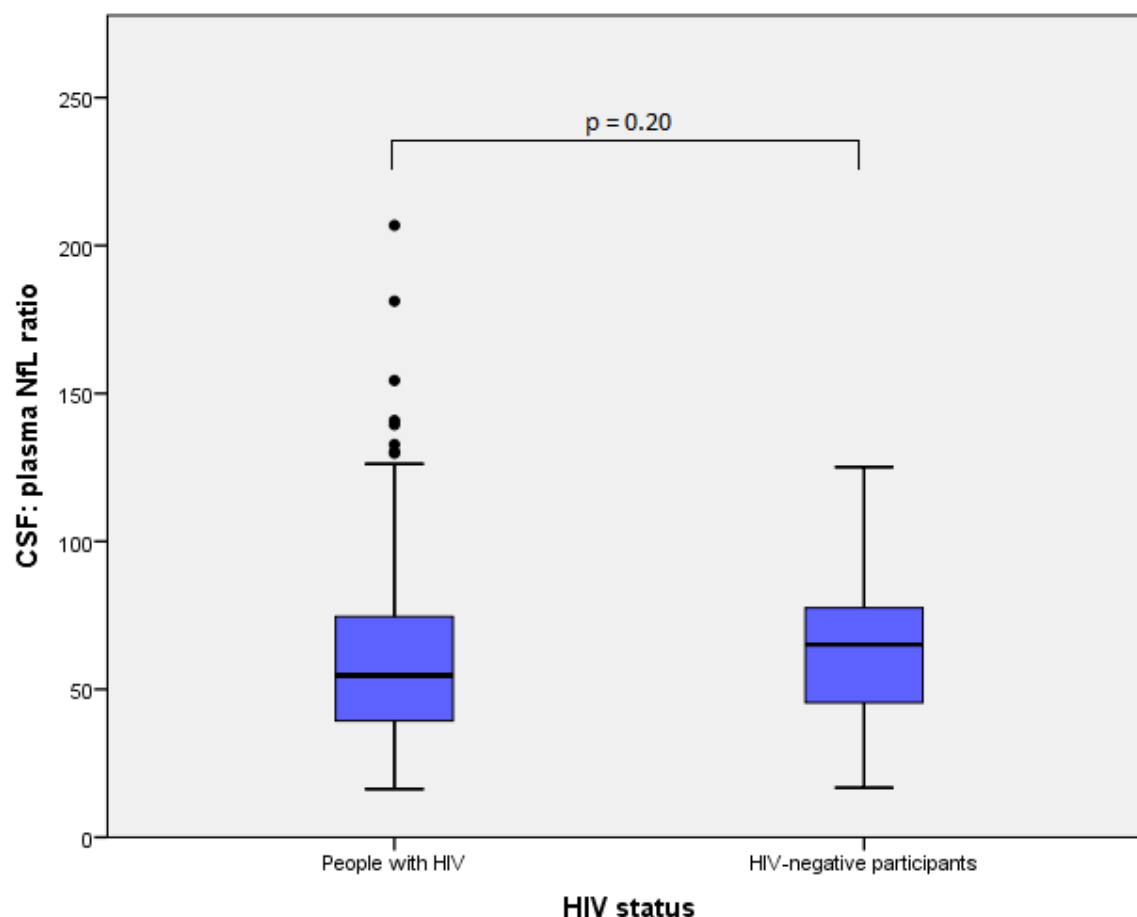
Figure 45: Boxplot comparing plasma NfL by HIV status



Abbreviations: PWH = people with HIV, NfL = neurofilament light chain protein

Legend: P-value calculated using the Wilcoxon rank-sum test. Number of participants in each group: PWH (n = 132) and HIV-negative individuals (n = 79). Error bars represent 95% confidence intervals.

Figure 46: Boxplot comparing cerebrospinal fluid: plasma NfL ratio by HIV status



Abbreviations: PWH = people with HIV, NfL = neurofilament light chain protein

Legend: P-value calculated using the Wilcoxon rank-sum test. Number of participants in each group: PWH (n = 132) and HIV-negative individuals (n = 79). Error bars represent 95% confidence intervals.

Table 13: Comparison between cerebrospinal fluid and plasma neurofilament light chain protein (NfL) in PWH and HIV-negative participants

Variable, median (IQR)	People with HIV n = 132	HIV-negative participants n = 79	p-value
CSF NfL	570 (430, 864)	568 (408, 767)	0.37
Plasma NfL	10.7 (7.9, 14.9)	9.9 (7.2, 12.5)	0.15
CSF: plasma NfL ratio	54.7 (39.2, 75.6)	65.1 (45.4, 77.8)	0.20

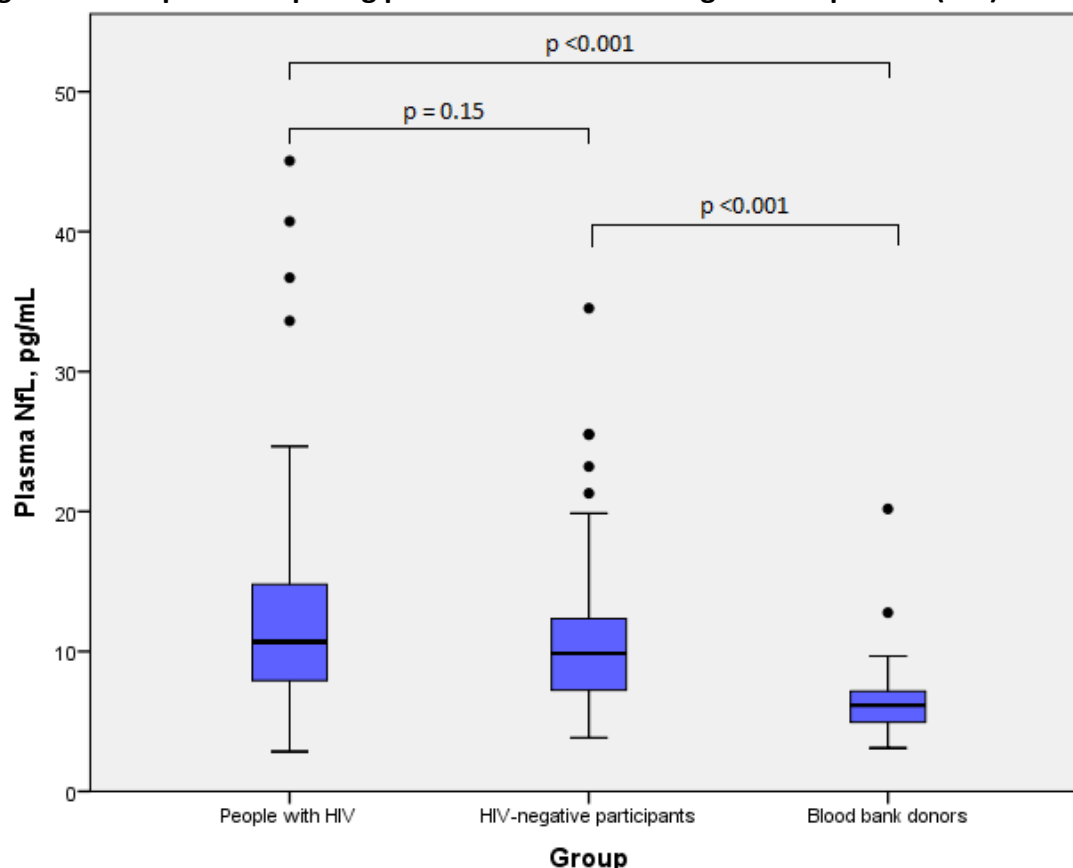
Abbreviation: IQR = interquartile range, NfL = neurofilament light chain protein, PLWH = people with HIV

Legend: P-values calculated using the Wilcoxon rank-sum test

4.3.4 Comparison of plasma NfL results in the COBRA cohort and blood bank donors

Plasma NfL was significantly lower in the blood bank donors cohort (median (IQR) 6.15 (4.94, 7.15)) compared to people with HIV (median (IQR) 10.7 (7.9, 14.9), $p < 0.001$) and HIV-negative participants (median (IQR) 9.9 (7.2, 12.5) $p < 0.001$) (Figure 47 and Table 14).

Figure 47: Boxplots comparing plasma neurofilament light chain protein (NfL)



Abbreviations: NfL = Neurofilament light chain protein

Legend: P-values calculated using the Wilcoxon rank-sum test. Number of participants in each group: people with HIV ($n = 132$), HIV-negative participants ($n = 79$) and blood bank donors ($n = 35$). Error bars represent 95% confidence intervals

Table 14: Comparison between plasma neurofilament light chain protein (NfL) in PLWH, HIV-negative participants and blood bank donors

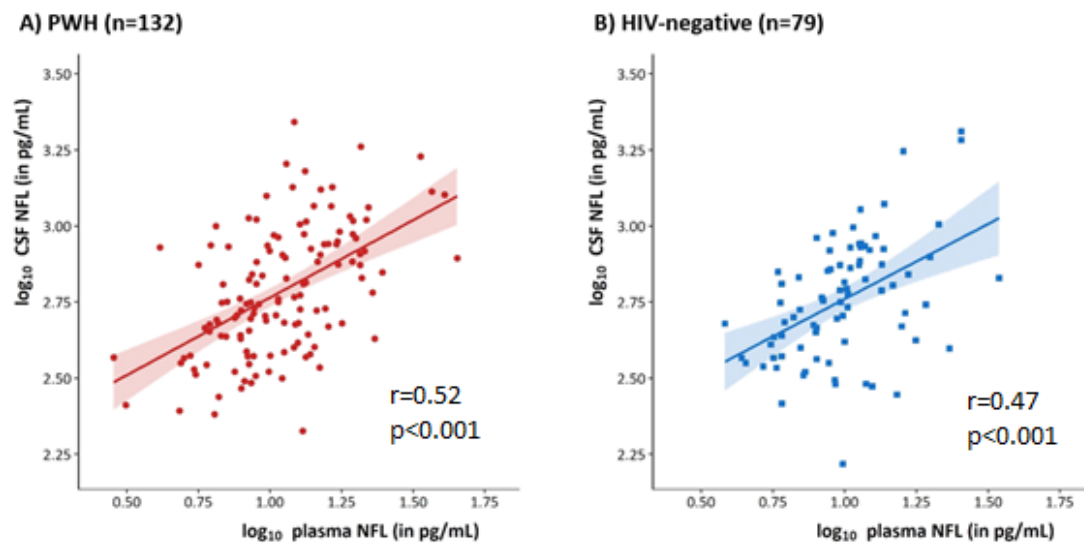
Variable, median (IQR)	People with HIV $n = 132$	HIV-negative participants $n = 79$	Blood bank donors $n = 35$	P-value
Plasma NfL	10.7 (7.9, 14.9)	9.9 (7.2, 12.5)	6.15 (4.94, 7.15)	<0.001

Abbreviation: IQR = interquartile range, NfL = neurofilament light chain protein, PLWH = people living with HIV.
Legend: P-value calculated using the Kruskal-Wallis test

4.3.5 Correlation between cerebrospinal fluid and plasma NfL

The correlation between cerebrospinal fluid and plasma NfL did not differ significantly by HIV status (PLWH: $r=0.52$ (95% confidence interval (CI) 0.38-0.63); HIV-negative participants: $r=0.47$ (95%CI 0.27-0.62), p -value testing the difference=0.63) (Figure 48).

Figure 48: Correlation between $\log_{10}$ -transformed plasma and cerebrospinal fluid neurofilament light chain protein (NfL) in A) PWH and B) HIV-negative participants



Legend: Correlations were assessed using Pearson's correlation coefficient. Abbreviations: PWH = people with HIV. P-values calculated using the Z-test after Fisher's transformation.

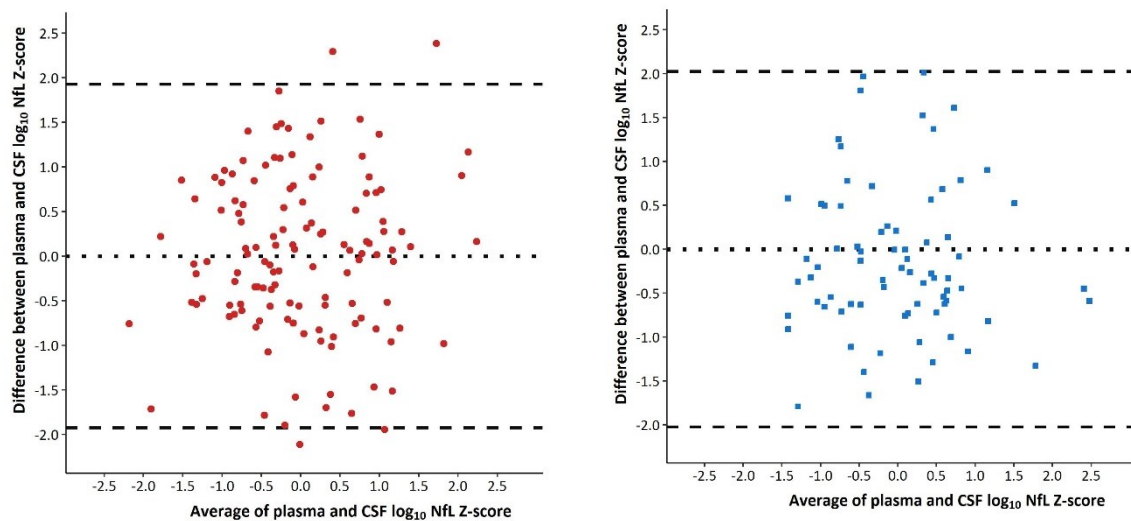
4.3.6 Bland-Altman plot

Bland-Altman plots are reported in Figure 49. Among PLWH, there was not a systematic over- or under-estimation of one score compared to the other. Mean (95%CI) difference between the scores was 0.00 (-0.17, 0.17), $p=0.99$ and the agreement between the two scores was not altered depending on the average cerebrospinal fluid and plasma NfL Z-score; the correlation (95%CI) between the difference and the mean of the two scores was -0.004 (-0.18, 0.17), $p=0.96$. The lower and upper limits of agreement (95%CI) between the two measurements were -1.97 (-2.25, 1.68) and 1.97 (1.68, 2.25), respectively, indicating substantial discrepancies between the NfL Z-scores obtained from the two compartments. A similar pattern was observed among HIV-negative individuals, with a mean (95%CI) difference of 0.00 (-0.23, 0.23) and no correlation between the difference and average [r (95%CI) = 0.00 (-0.22, 0.22), $p=0.99$].

Figure 49: Bland-Altman plot of plasma and cerebrospinal fluid NFL Z-scores in PWH (A) and HIV-negative participants (B)

A) PWH (n = 132)

B) HIV-negative participants (n=79)



Legend: Dashed lines signify upper and lower limits of agreement and dotted line signifies mean difference between the two Z-scores across all individuals. Abbreviations: PWH = people with HIV

4.3.7 Factors associated with cerebrospinal fluid and plasma NfL

In multivariable regression analysis, higher cerebrospinal fluid NfL Z-score was associated with older age and higher cerebrospinal fluid protein in both PLWH and HIV-negative participants. Lower cerebrospinal fluid NfL Z-score was significantly associated with female gender in HIV-negative participants (0.83 $\log_{10}$ cerebrospinal fluid NfL Z-score lower compared to men, $p=0.03$) and higher cerebrospinal fluid NfL Z-score approached a statistically significant association with lower bodyweight in PLWH (0.05 $\log_{10}$ cerebrospinal fluid NfL Z-score higher per 5kg decrease in bodyweight, $p=0.05$) (Table 15).

Higher plasma NfL Z-score was associated with older age, lower bodyweight and higher serum creatinine in both PLWH and HIV-negative participants, and higher cerebrospinal fluid NfL Z-score approached a statistically significant association with lower serum albumin in PLWH (0.31 $\log_{10}$ cerebrospinal fluid NfL Z-score higher per 10g/L decrease in serum albumin, $p=0.05$) (Table 15).

Table 15: Multivariable models to compare factors associated with cerebrospinal fluid and plasma log₁₀ NfL Z-scores between people living with HIV and HIV-negative participants, excluding HIV-specific parameters^{1,2}

Variable		People living with HIV n = 132		HIV-negative controls N = 79	
		Change (95% CI)	p-value	Change (95% CI)	p-value
log ₁₀ CSF NFL Z-score	Female vs male	-0.44 (-1.05, 0.17)	0.15	-0.83 (-1.56, -0.10)	0.03*
	Age (per 10 years)	0.69 (0.50, 0.88)	<0.001	0.74 (0.46, 1.02)	<0.001*
	Weight (per 5 Kg)	-0.05 (-0.10, 0.00)	0.05	0.02 (-0.04, 0.07)	0.55
	Hypertensive medication (yes vs no)	0.26 (-0.08, 0.61)	0.13	-0.05 (-0.64, 0.55)	0.87
	Serum creatinine (per mg/dL)	0.47 (-0.29, 1.24)	0.22	-0.71 (-2.00, 0.59)	0.28
	Serum albumin (per 10 g/L)	0.00 (-0.33, 0.34)	0.98	0.09 (-0.50, 0.67)	0.77
	CSF protein (per g/L)	1.46 (0.63, 2.29)	<0.001	1.81 (0.37, 3.25)	0.01*
	CSF neopterin	-0.01 (-0.04, 0.01)	0.33	0.04 (-0.06, 0.13)	0.45
	Plasma neopterin	0.02 (-0.01, 0.05)	0.18	-0.002 (-0.11, 0.11)	0.96
log ₁₀ plasma NFL Z-score	Female vs male	0.13 (-0.44, 0.69)	0.66	0.57 (-0.13, 1.27)	0.11
	Age (per 10 years)	0.62 (0.44, 0.80)	<0.001	0.63 (0.36, 0.90)	<0.001*
	Weight (per 5 Kg)	-0.13 (-0.18, -0.08)	<0.001	-0.06 (-0.11, 0.00)	0.05*
	Hypertensive medication (yes vs no)	0.28 (-0.03, 0.60)	0.08	0.18 (-0.39, 0.76)	0.53
	Serum creatinine (per mg/dL)	0.73 (0.02, 1.44)	0.04	1.64 (0.39, 2.89)	0.01*
	Serum albumin (per 10 g/L)	-0.31 (-0.63, 0.00)	0.05	-0.39 (-0.95, 0.18)	0.18
	CSF protein (per g/L)	-0.30 (-1.07, 0.47)	0.44	0.45 (-0.94, 1.84)	0.52
	CSF neopterin	0.01 (-0.02, 0.03)	0.69	0.08 (-0.01, 0.17)	0.07
	Plasma neopterin	0.03 (0.00, 0.05)	0.07	0.01 (-0.09, 0.11)	0.85

¹Parameter estimates reflect the associated impact (measured in standard deviations) of each independent variable in the model on the dependent variable. ²Variables included in the univariable linear regression analysis that were not statistically significant and not included in the multivariable model were Black African ethnicity, men who have sex with men sexual orientation, body mass index, current tobacco smoker, ex-tobacco smoker, current alcohol consumption, past alcohol consumption, recent recreational drug use, ever used injected recreational drugs, global cognitive function T-score, current CD4⁺ and CD8⁺ count and current CD4⁺/8⁺ ratio.

* signifies statistically significant results

Among PLWH, none of the HIV-specific parameters showed a significant association with either cerebrospinal fluid or plasma NFL Z-scores, after accounting for other factors (Table 16).

Table 16: Multivariable models to assess factors associated with cerebrospinal fluid and plasma log₁₀ NFL Z-scores in people with HIV only, and including HIV-related parameters^{1,2}

Variable		People with HIV N = 132	
		Change (95% CI)	p-value
log ₁₀ CSF NFL Z-score	Female vs male	-0.48 (-1.09, 0.14)	0.13
	Age (per 10 years)	0.69 (0.49, 0.89)	<0.001*
	Weight (per 5 Kg)	-0.06 (-0.11, -0.01)	0.03*
	Hypertensive medication (yes vs no)	0.28 (-0.06, 0.63)	0.11
	Serum creatinine (per mg/dL)	0.41 (-0.36, 1.18)	0.30
	Serum albumin (per 10 g/L)	-0.01 (-0.35, 0.33)	0.96
	CSF protein (per g/L)	1.37 (0.53, 2.21)	0.002*
	CSF neopterin	-0.01 (-0.04, 0.01)	0.34
	Plasma neopterin	0.02 (-0.01, 0.05)	0.22
	Time since HIV diagnosis (years)	0.18 (-0.22, 0.59)	0.38
	Time on cART (years)	-0.35 (-0.81, 0.11)	0.14
log ₁₀ plasma NFL Z-score	Female vs male	0.18 (-0.38, 0.75)	0.52
	Age (per 10 years)	0.58 (0.39, 0.76)	<0.001*
	Weight (per 5 Kg)	-0.12 (-0.17, -0.07)	<0.001*
	Hypertensive medication (yes vs no)	0.25 (-0.07, 0.57)	0.12
	Serum creatinine (per mg/dL)	0.79 (0.08, 1.50)	0.03*
	Serum albumin (per 10 g/L)	-0.29 (-0.60, 0.02)	0.07
	CSF protein (per g/L)	-0.23 (-1.00, 0.55)	0.56
	CSF neopterin	0.01 (-0.02, 0.03)	0.53
	Plasma neopterin	0.03 (0.00, 0.06)	0.05
	Time since HIV diagnosis (years)	0.30 (-0.08, 0.67)	0.12
	Time on cART (years)	-0.17 (-0.59, 0.26)	0.44

¹Parameter estimates reflect the associated impact (measured in standard deviations) of each independent variable in the model on the dependent variable. ²Variables included in the univariable linear regression analysis that were not statistically significant and not included in the multivariable model were Black African ethnicity, men who have sex with men sexual orientation, body mass index, current tobacco smoker, ex-tobacco smoker, current alcohol consumption, past alcohol consumption, recent recreational drug use, ever used injected recreational drugs, global cognitive function T-score, current CD4⁺ and CD8⁺ count, current CD4⁺/8⁺ ratio, nadir CD4⁺ count, years with CD4⁺ count <200cells/μL, prior AIDS diagnosis, on a non-nucleoside reverse transcriptase inhibitor (NNRTI), on a protease inhibitor (PI), on tenofovir-disoproxil fumarate (TDF) and on atazanavir. * signifies statistically significant results

4.4 Discussion

In this chapter, my primary aim was to investigate and compare the degree of correlation between cerebrospinal fluid and blood NfL in PLWH on virally suppressive antiretroviral treatment and in lifestyle-similar HIV-negative individuals, to gain a better understanding of how plasma NfL performs as a surrogate marker of cerebrospinal fluid NfL in people with HIV on suppressive cART. My results demonstrated that the correlation between cerebrospinal fluid and plasma NfL in PLWH on suppressive cART is moderate and similar to that observed in a cohort of lifestyle-similar HIV-negative controls.

The previous report suggesting a strong correlation between cerebrospinal fluid and plasma NfL in a cohort of mainly untreated PLWH[293] is likely a consequence of the much higher concentrations of cerebrospinal fluid and plasma NfL seen in that cohort, where the median cerebrospinal fluid and plasma NfL concentrations in individuals with HIV-associated dementia were 16,185 pg/mL and 114 pg/mL, respectively.

Interestingly, plasma NfL concentration was significantly lower in the blood bank donors compared to the PLWH and HIV-negative controls enrolled into the COBRA study. Given the lack of additional demographic factors apart from age and gender and detailed information about the lifestyles of the blood bank donors nor concurrent cerebrospinal fluid samples, I was unable to explore this further, and can only conclude that based on the limited information available, PLWH and HIV-negative controls in the COBRA study were observed to have evidence of increased neuro-axonal injury compared to blood bank donors. While I do not have sufficient data to definitively conclude that certain lifestyle behaviours may be associated with higher NfL concentrations, the association between neurotoxicity, neurodegeneration and neuronal injury with alcohol and recreational drug abuse has been well-described[392,393]. Given the strict eligibility criteria for blood donation in The Netherlands, certain lifestyle behaviours such as recreational drug use which plausibly increase cerebrospinal fluid and blood NfL may at least partly explain the elevated plasma NfL concentrations seen in the COBRA participants, compared to the blood bank donors. In order to explore the impact of lifestyle behaviours on NfL further, I would ideally like to recruit two further cohorts of participants who are age- and gender-matched with the COBRA cohort and fully abstain from alcohol, cigarette smoking and recreational drug use:

one HIV-positive cohort and one HIV-negative cohort. This way, I would be able to investigate the impact of lifestyle factors on cerebrospinal fluid and blood NfL measurements in PLWH and HIV-negative individuals.

Concentrations of cerebrospinal fluid and plasma NfL were similar in PLWH and HIV-negative participants, suggesting that PLWH on cART do not have increased biomarker evidence of neuro-axonal injury compared to lifestyle-similar HIV-negative individuals. Cerebrospinal fluid: plasma NfL ratios also did not differ significantly by HIV status, with plasma NfL being 55 – 65 times lower compared to cerebrospinal fluid NfL, consistent with published literature[293].

My results demonstrating that higher cerebrospinal fluid and plasma NfL were associated with older age echoes the findings in published studies[285,294]. The finding that higher cerebrospinal fluid NfL is associated with higher cerebrospinal fluid protein is not unexpected, given that higher cerebrospinal fluid protein may indicate the presence of central nervous system pathology, and hence higher neuronal damage. However, the finding that female gender is associated with lower cerebrospinal fluid NfL is not wholly unexpected, though the published data in this regard remains controversial with some studies demonstrating that cerebrospinal fluid NfL is higher in males[394,395], higher in females[365] or not significantly different by gender[396]. Studies investigating other neurological conditions which recruited larger numbers of female participants did not find a similar association between cerebrospinal fluid NfL and female gender. In order to study this in further detail, I would like to increase the enrolment of females into the cohort to a sufficient level where the study would be powered to determine whether gender differences in NfL persist, even when other demographic and clinical factors are controlled for.

My results suggest that consideration of renal function and body composition is an important requirement when interpreting plasma NfL. While renal excretion of NfL may explain my finding that higher plasma NfL is associated with poorer renal function, NfL has a molecular weight of about 68kDa and a high abundance of negatively charged amino acids in its sequence, both of which theoretically decrease its ability to cross the glomerular filtration barrier. Published data demonstrate that while plasma NfL decreased after

switching from tenofovir disoproxil fumarate to tenofovir alafenamide fumarate, no significant correlations between plasma NfL and serum creatinine pre- and post-switch, or between changes in plasma NfL and serum creatinine pre- and post-switch were observed[397]. Although lower bodyweight was independently associated with higher plasma NfL, body mass index was not an associated factor in this study, in contrast to a publication demonstrating a negative association between plasma NfL and body mass index[389]. I hypothesise that people with higher body weight, particularly in the people with HIV cohort, have 'returned to health', thus have lower levels of systemic inflammation, leading to lower levels of neuronal injury.

Strengths of this study include the comprehensively-phenotyped cohort and the lifestyle-similar HIV-negative individuals. The high analytical sensitivity of the Simoa NfL assay meant that all cerebrospinal fluid and plasma NfL results were above the limit of quantification (0.174 pg/mL, as verified by the manufacturer) and differences in NfL concentrations between the samples were detectable and measurable.

Limitations include the COBRA participants being predominantly white men who have sex with men from Western Europe, thus more data on the correlation between cerebrospinal fluid and plasma NFL are required from other populations. Another limitation was the lack of data on other factors that may increase plasma NfL (*e.g.*, peripheral neuropathy) and concomitant neurotoxic drug use. The number of female participants in this study was small and studies involving more female participants would be needed to verify the association between female gender and lower cerebrospinal fluid NfL. Cerebrospinal fluid/serum albumin ratio data was unavailable in 40 HIV-negative COBRA participants, and thus I could not include this factor (which is a surrogate measure of blood-brain barrier integrity), in the multivariate linear regression models. To date, evidence suggests that the association between blood NfL and markers of blood-brain impairment is poor[363,398] to fair[293,391], as per the Chan definition[387]. Ideally, this variable would be available, and I would include it as a factor in the linear regression analysis.

4.5 Conclusion

My findings corroborate the use of plasma NfL as a promising surrogate biomarker of cerebrospinal fluid NfL, in the context of PLWH on suppressive cART and supports the use of plasma NfL as a reliable marker of neuro-axonal injury, to address my hypotheses in the following chapters of my thesis.

My analysis suggests that while the degree of neuronal injury in PLWH on suppressive cART is comparable to lifestyle-similar HIV-negative individuals, there remains the suggestion that certain factors like renal function, body composition and lifestyle factors may impact peripheral NfL concentrations, and this postulation warrants further investigation.

Chapter 5

Central nervous system neural activation in people living with HIV on cART: a [^{11}C] PBR28 PET-CT study

Chapter 5: Central nervous system neural activation in people living with HIV on cART: a [¹¹C] PBR28 PET-CT study

5.1 Introduction

Since the introduction of modern cART, there has been a significant improvement in the prognosis and life expectancy for PLWH[399]. However, despite plasma HIV RNA load suppression, treated HIV disease remains associated with ongoing immune dysfunction and increased non-AIDS morbidity in PLWH[151]. Non-AIDS morbidity encompass conditions frequently associated with ageing, including neurological, metabolic and cardiovascular disease[151], and while the precise underlying mechanisms are unknown, it is thought to be associated with persistent immune activation, immune senescence and inflammation[400].

The mechanisms preventing full immune recovery during treated HIV disease are not fully understood. It is postulated that the HIV reservoir (consisting of replication-competent HIV DNA integrated into the host cell genome) may contribute to the persistence of immune dysfunction during treated HIV disease[401], due to low-level viral replication and intermittent antigenic stimulation[146].

The main HIV reservoir compartments include blood, lymph nodes, gut-associated lymphoid tissue, genital tract and the central nervous system [64,79,402,403]. The central nervous system is increasingly becoming recognised as a key HIV reservoir compartment to focus on especially in the context of HIV cure[79,404], however biomarkers to definitively identify central nervous system tissue reservoirs in individuals receiving suppressive cART are lacking. While routine brain biopsy is impractical, blood and cerebrospinal fluid samples are relatively easy to obtain but these fluid samples may not accurately reflect the HIV reservoir size or location within the central nervous system tissues.

To date, cerebrospinal fluid biomarkers and brain imaging have demonstrated evidence of central nervous system immune activation and neuroinflammation in otherwise well people

living with HIV on suppressive cART[150,168,210,405]. Separately, the presence of neuroinflammation has also been associated with cognitive dysfunction in individuals on suppressive cART[205]. However, data are limited with regards to the precise role of central nervous system HIV reservoirs in driving immune activation and neuroinflammation, and the longitudinal consequences of persistent immune activation and neuroinflammation on neurological sequelae such as cognitive dysfunction, in people with treated HIV disease.

The 18kDa TSPO, which is found on the outer mitochondrial membrane, has been shown to be highly expressed in activated microglia[406,407]. Synthetic radiolabelled ligands that selectively bind to TSPO have since been developed and can be utilised to image microglial activation in the human brain *in vivo*, using PET imaging[408]. This form of TSPO PET brain imaging has already been used extensively to investigate the role of neuroinflammation in neurodegenerative conditions, hepatic encephalopathy and stroke[409–411]. TSPO response to injury appears to correlate with the level of damage, with increased TSPO density seen with neurotoxicity[412,413] and demyelination[414]. More recently, radiolabelled TSPO binding on brain PET imaging has been used to demonstrate neuroinflammation in neuro-asymptomatic individuals who initiated cART during chronic HIV infection[210,211]. Data suggests that minimal TSPO ligand binding is expected in the healthy human brain, thus any significant change in TSPO radioligand binding could be presumed to be related to central nervous system pathology[415]. Given the theorised association between immune activation, inflammation and the HIV reservoir, radiolabelled TSPO binding on brain PET imaging may have a role in assessing the HIV reservoir in the central nervous system.

Strategies to reduce HIV reservoir size and inhibit persistent immune activation include the early initiation of cART, soon after acquiring HIV[251]. Studies have demonstrated a normalisation of peripheral blood markers of immune function[416] and a reduction in immune activation and HIV reservoir in the blood[112], when cART is initiated within 6 months after HIV acquisition. Early data suggests that a similar picture is seen in the central nervous system where acute untreated HIV is associated with increased central nervous system biomarkers of immune activation, inflammation and neuronal injury (as described in Chapter 1: Introduction) but the effect of early cART initiation soon after HIV acquisition on central nervous system HIV reservoir size remains uncertain.

This chapter will focus on addressing my first PhD hypothesis, that smaller HIV reservoir size, estimated by stratifying according to stage when cART was initiated, i.e. during acute HIV infection (when HIV reservoir size is presumed to be smaller) or chronic HIV infection (when HIV reservoir size is presumed to be larger), versus control participants (who, by definition, have no HIV reservoir) is associated with lower biomarker measures of neural activation, as measured by markers of microglial activation (radiolabelled TSPO [^{11}C] PBR28 binding on PET brain imaging), neuro-axonal injury and cognitive function.

5.2 Methods

The participants included in the analysis in this chapter underwent brain [^{11}C] PBR28 PET-CT imaging. HIV-positive participants were enrolled into the PET in AHI and PET in CHI studies:

1. [^{11}C] PBR28 PET in individuals treated during acute HIV infection (PET in AHI): study procedures conducted by myself
2. [^{11}C] PBR28 PET in individuals treated during chronic HIV (PET in CHI): study procedures conducted previously by a member of our research group (JV)

Both [^{11}C] PBR28 PET in HIV studies above were single-site, cross-sectional observational studies, investigating cerebral [^{11}C] PBR28 binding by PET-CT in individuals who commenced cART either during acute HIV infection (PET in AHI) or during chronic HIV infection (PET in CHI).

Historical brain [^{11}C] PBR28 PET-CT imaging data for the control participants were obtained from participants enrolled into either a human pharmacological blocking study to determine brain [^{11}C] PBR28 binding potential *in vivo*[309] (advertised to staff and students at Imperial College London using posters) or enrolled into a brain [^{11}C] PBR28 PET-CT imaging database for healthy volunteers supported by GlaxoSmithKline plc. All control participants were scanned at the same clinical imaging research facility as the participants in the PET in AHI and PET in CHI studies: the Imanova Centre for Imaging Sciences, which has since been acquired by Invicro, a Konica Minolta Company. Apart from females control participants being eligible to enrol, the eligibility criteria to enrol into the pharmacological blocking study

and the GlaxoSmithKline plc healthy volunteers' database were similar to the criteria for the PET and AHI and PET in CHI studies.

5.2.1 [¹¹C] PBR28 PET in treated acute HIV infection (PET in AHI)

5.2.1.1 *Participant selection*

Participants attending the HIV Clinical Trials Centre at St Mary's Hospital, Imperial College Healthcare NHS Trust, London, UK for their routine HIV Reservoir Targeting with Early Antiretroviral Therapy (HEATHER) study appointment were informed about the PET in AHI study. The HEATHER study is a prospective observational study of individuals who initiated cART within three months of HIV acquisition[334]. Interested participants were given the patient information sheet and invited to attend for a study screening visit.

Participants were required to be adult males >18 years of age, able to communicate in English, receiving cART for ≥3 months with plasma HIV RNA <50 copies/mL at screening, high affinity binders to [¹¹C] PBR28 on TSPO genotypic testing and in good health. Exclusion criteria included active opportunistic infection, significant co-morbidities, significant neurological conditions, alcohol or recreational drug dependency, contraindication to arterial cannulation, lumbar puncture or magnetic resonance (MR) imaging, body mass index >30 kg/m² and participation in another research study involving ionising radiation such that the subject would be exposed to a cumulative dose of >10 mSv in the previous 12 months.

5.2.1.2 *Study visits*

Screening visit: After the participant provided written informed consent, the following details were collected from the participants at the screening visit: demographics, medical history, medication list including cART, recreational drug and alcohol use and anthropometric assessment. HIV-specific information including current CD4⁺ and CD8⁺ T-cell counts, nadir CD4⁺ T-cell count, plasma HIV RNA load, duration since HIV diagnosis and duration participant had been on cART were obtained from the participants' electronic hospital records, with participant consent. Blood samples were collected and tested for

TSPO genotype, haematology and coagulation profile and the participant completed a cognitive function test practice session.

Imaging visit: If the participant was eligible to take part in the study following the screening visit study procedures and blood test results, the participant underwent structural cerebral MRI and cerebral PET-CT imaging with [^{11}C] PBR28 radioligand at the imaging visit.

Lumbar puncture visit: Following the imaging visit, the participants' cerebral magnetic resonance images were reviewed by an NHS consultant neuroradiologist to rule out any radiological contraindications to lumbar puncture. The participant was then invited to attend a third study visit for repeat cognitive function testing and blood and cerebrospinal fluid collection.

5.2.1.3 *Sample collection and analysis*

The following measurements were performed on the participants' fluid samples collected at the lumbar puncture visit (see above):

- iv. Plasma and cerebrospinal fluid NfL (see below)
- v. Cerebrospinal fluid protein, glucose, leukocytes and HIV RNA (as described in Chapter 2: Materials and Methods)

5.2.1.4 *Cerebral imaging*

Cerebral T1-weighted MRI and cerebral PET-CT imaging with [^{11}C] PBR28 ligand were performed as described in Chapter 2: Materials and Methods.

5.2.1.5 *Cerebral PET imaging analysis*

The cerebral [^{11}C] PBR28 PET-CT imaging data processing and analysis was performed by myself using the MIAKATTM pipeline, provided and supported by Invicro LLC, as described in Chapter 2: Materials and Methods.

5.2.1.5.1 Total volume of distribution (V_T)

A two-tissue compartment model using the metabolite-corrected plasma input function was applied to the dynamic PET data using a fixed blood volume correction of 5%. For each ROI

evaluated, the V_T was estimated from the rate constants as previously described[339]. Model fitting and parameter estimation was performed using software implemented in MATLAB R2019b (The MathWorks, Natick, MA, USA). The main outcome measured was the total V_T for each chosen ROI, for each participant. The V_T for a radioligand is defined as the ratio of radioligand concentration in tissue compared to that in plasma and is an alternative to the binding potential (density of the neuroreceptor) in the absence of a reference region, i.e., a region that does not contain the neuroreceptor.

5.2.1.5.2 Distribution volume ratio (DVR)

DVR was calculated for each region of interest by normalising V_T to cortical grey matter. Normalisation can control for unaccounted physiological factors that may impact cerebral radioligand signal in TSPO PET imaging studies and can also cancel out non-binding effects on the TSPO radioligand signals. Based on previous studies, cortical grey matter was chosen as the area for normalisation because of its relatively homogenous pattern of high binding in control participants[210]. The outcome measured was a normalised $V_{T(DVR)}$ for each chosen ROI, for each participant.

5.2.1.6 *Total HIV DNA in circulating CD4⁺ T cells and ultra-sensitive plasma HIV RNA*

I planned to measure total HIV DNA in circulating CD4⁺ T-cells isolated from PBMCs (as a proxy measure of the peripheral HIV reservoir size) and ultra-sensitive plasma HIV RNA using single-copy assay (as a measure of HIV viral transcription) in the Molecular Diagnostics Unit at Imperial College London, London, UK. Unfortunately, due to the COVID-19 pandemic, all non-essential laboratory research work here has been halted to enable the laboratory to focus on COVID-19 related research. Thus, I was unable to perform this laboratory work as planned, for this section of my PhD thesis.

5.2.1.7 *Blood and cerebrospinal fluid biomarker analysis*

5.2.1.7.1 Neurofilament light chain protein (NfL)

Cerebrospinal fluid and plasma NfL measurements were performed using the single-molecule array (Simoa) digital immunoassay on a HD-X analyser (Quanterix, Billerica, MA, USA)[342] as described in Chapter 2: Materials and Methods, at the Fluid Biomarkers for Neurodegenerative Diseases laboratory, UK Dementia Research Institute, University College London, UK.

5.2.1.8 *Cognitive function testing and test scores analysis*

Cognitive testing was performed using the CogState™ (CogState Ltd, Melbourne, Australia) battery of tests, as described in Chapter 2: Materials and Methods. All subjects completed a practice session at the screening visit and the actual cognitive function testing session was performed prior to the lumbar puncture procedure, at the third study visit.

Z-scores from the seven tasks were calculated for each participant using the combined results of the participants in the PET in AHI and PET in CHI studies as the reference. Each task related to a specific cognitive domain and included psychomotor speed (DET), attention (IDN), visual motor processing (GMCT), visual memory (CPAL), visual learning (OCL), executive function (GMLT) and working memory (ONB). Specific domains were combined (as specified by the manufacturer) in the following manner:

1. Psychomotor speed (DET), attention (IDN) and visual motor processing domains (GMCT)
2. Visual learning (OCL) and visual memory domains (CPAL)
3. Working memory (ONB) and executive function (GMLT) domains

A composite cognition z-score was then calculated by taking the average of the z-scores across all tests.

Finally, T-scores (with a mean of 50 and a standard deviation of 10) using the whole cohort as the reference population were generated and a global T-score was obtained by calculating the average of the seven individual domain T-scores. For all T-scores, higher scores signify better cognitive function.

5.2.2 [^{11}C] PBR28 PET in treated chronic HIV (PET in CHI)

Raw cerebral PET-CT imaging with [^{11}C] PBR28 data from the PET in CHI study were obtained from Invicro LLC, the research imaging facility where both PET in AHI and PET in CHI studies were carried out. The raw data from the PET in CHI study was run on the MIAKAT™ pipeline using the same process, by myself. Stored cerebrospinal fluid and plasma samples (where available) were retrieved from the freezer and processed alongside the samples from the PET in AHI study.

5.2.3 Statistical analysis

Participant baseline demographics and clinical parameters were described using median (interquartile range) and total (percentage), as appropriate. Differences in variables between participants who initiated cART during acute HIV infection and participants who initiated cART during chronic HIV infection were analysed using the Mann-Whitney U test and Fisher's exact test, as appropriate, and differences in variables between participants who initiated cART during acute HIV infection, during chronic HIV infection and control participants were analysed using the Kruskal-Wallis and Fisher's exact test, as appropriate.

The statistical analyses performed in this chapter are exploratory and differences in cerebral [^{11}C] PBR binding (total volume of distribution (V_T) and V_T normalised to cortical grey matter ($V_{T(DVR)}$)) at regions of interest between participants who initiated cART during acute HIV infection, during chronic HIV infection and control participants were assessed using the Kruskal-Wallis test. The 30 regions of interest were chosen based on regions noted to display TSPO binding signals in previous published studies. Due to the exploratory nature of the analysis at this stage to identify subtle signals, adjustments to multiple hypothesis testing were not performed. Based on the results of the initial exploratory analyses performed in this chapter, more detailed statistical analyses are planned to investigate differences in regional brain [^{11}C] PBR28 binding, prior to manuscript submission. Differences in laboratory parameters (cerebrospinal fluid NFL, plasma NFL, cerebrospinal fluid protein and cerebrospinal fluid white cell count) and cognitive function test scores (individual domain z-scores, average domain combination z-scores and global composite T-

scores) between participants who initiated cART during acute HIV infection versus chronic HIV infection, were analysed using the Mann-Whitney U test. Non-parametric statistical tests were utilised throughout this analysis as these do not make distributional assumptions.

Formal sample size calculation was not performed. This work was funded by the National Institute of Health Research (NIHR) Imperial Biomedical Research Centre (BRC) and the maximum number of [^{11}C] PBR28 PET-CT scans funded was twelve. This project was reviewed and approved by a research committee familiar with TSPO PET imaging research as the panel felt that this was an important avenue to explore and that a sample size of twelve in PWH who had initiated cART during acute HIV infection was sufficient as an exploratory analysis. TSPO PET neuroimaging studies are costly, which considerably limits the sample size.

5.3 Results

5.3.1 Demographics and clinical parameters

Participant demographics and clinical parameters are described in Table 17, stratified according to participants who initiated cART during acute HIV infection ($n = 9$), during chronic HIV infection ($n = 11$) and control participants ($n = 15$).

Ten of the control participants were enrolled into a previously completed brain [^{11}C] PBR28 study[309] and five of the control participants were enrolled into a healthy volunteers database for brain [^{11}C] PBR28 PET imaging, organised by the GlaxoSmithKline. Apart from age, gender, bodyweight and TSPO genotypic affinity binding, no other demographic or clinical data were available for the control participants. All participants included in my analysis in this chapter were high affinity binders on TSPO genotypic testing. For the PET in AHI study which I was responsible for, I screened 22 participants; 13 participants were ineligible due to being mixed- or low-affinity TSPO binders on genotypic testing.

All the HIV-positive participants were male, and 4/15 (27%) of control participants were female (Table 17). Overall, the control participants were younger but had a wider age range (median (IQR) 26 years (20, 59)) compared to the participants who initiated cART during acute HIV infection (median (IQR) 40 years (30, 46)), who were in turn, younger than the

participants who initiated cART during chronic HIV infection (median (IQR) 45 years (43, 52)) (Table 17). Of the three groups, participants who initiated cART during acute HIV infection had the lowest median bodyweight (median (IQR) 74.7 kg (69.1, 74.9)) compared to the control participants (median (IQR) 78.7 kg (69.2, 87.8)) and participants who initiated cART during chronic HIV infection, who had the highest median bodyweight (median (IQR) 83.9kg (71.5, 92.0)) (Table 17).

Participants from both HIV-positive groups were predominantly of white ethnicity (Table 17). Overall, participants who initiated cART during acute HIV infection versus chronic HIV infection had a shorter duration between HIV diagnosis and cART initiation (median (IQR) 3.6 weeks (3.3, 4.9) vs 16.0 weeks (7.1, 182.0), $p = 0.01$), shorter duration since HIV diagnosis (median (IQR) 3.3 years (2.6, 4.5) vs 15.0 years (3.0, 16.7), $p = 0.04$) and shorter duration on cART (median (IQR) 3.2 years (2.5, 4.4) vs 13.3 years (3.0, 15.0), $p = 0.05$) (Table 17).

Median pre-treatment HIV RNA load was numerically lower in participants who initiated cART during acute HIV infection compared to chronic HIV infection (median (IQR) 4.4 (3.2, 5.7) vs 5.1 (4.7, 5.5) $\log_{10}$ HIV RNA copies/mL, $p = 0.63$) but current CD4⁺ T-cell count was similar between the two HIV-positive groups (median (IQR) 687 (652, 1014) vs 671 (470, 810) cells/ μ L, $p = 0.34$) (Table 17). Participants who initiated cART during acute HIV infection compared to chronic HIV infection had higher nadir CD4⁺ T-cell count and higher current CD4⁺/CD8⁺ T-cell ratio (median (IQR) 586 (370, 687) vs 228 (155, 315) cells/ μ L, $p = 0.002$ and 1.37 (1.24, 1.42) vs 0.67 (0.63, 0.78), $p < 0.001$, respectively) and lower current CD8⁺ T-cell count (median (IQR) 553 (462, 779) vs 850 (767, 1310) cells/ μ L, $p = 0.02$) (Table 17).

The majority of participants who initiated cART during acute HIV infection were on INSTI-based cART regimens (67%), whereas most participants who initiated cART during chronic HIV infection were on either protease inhibitor (PI)-based (46%) or non-nucleoside reverse transcriptase inhibitor (NNRTI)-based cART regimens (36%) (Table 17).

Table 17: Demographics and clinical parameters (where available) of participants who underwent cerebral [¹¹C] PBR28 PET imaging

Variables	Participants who initiated cART during acute HIV infection n = 9	Participants who initiated cART during chronic HIV infection n = 11	Control participants n = 15	p-value
Male gender	9 (100)	11 (100)	11 (73)	0.049 ¹
Age, years	40 (30, 46)	45 (43, 52)	26 (20, 59)	0.11 ¹
Weight, kg	74.7 (69.1, 74.9)	83.9 (71.5, 92.0)	78.7 (69.2, 87.8)	0.25 ¹
Ethnicity			not available	0.01
White	6 (66.7)	8 (73)		
Mixed white and Asian	3 (33.3)	0		
Black African	0	2 (18)		
Hispanic	0	1 (9)		
Duration since HIV diagnosis, years	3.3 (2.6, 4.5)	15.0 (3.0, 16.7)	not applicable	0.04
Duration on cART, years	3.2 (2.5, 4.4)	13.3 (3.0, 15.0)	not applicable	0.05
Duration between HIV diagnosis and cART initiation, weeks	3.6 (3.3, 4.9)	16.0 (7.1, 182.0)	not applicable	0.01
Pre-treatment HIV RNA, log ₁₀ copies/mL	4.4 (3.2, 5.7)	5.1 (4.7, 5.5)	not applicable	0.63
Current CD4 ⁺ T-cell count, cells/μL	687 (652, 1014)	671 (470, 810)	not available	0.34
Nadir CD4 ⁺ T-cell count, cells/μL	586 (370, 687)	228 (155, 315)	not available	0.002
Current CD4 ⁺ :CD8 ⁺ T-cell ratio	1.37 (1.24, 1.42)	0.67 (0.63, 0.78)	not available	<0.001
Current CD8 ⁺ T-cell count, cells/μL	553 (462, 779)	850 (767, 1310)	not available	0.02
cART regimens			not applicable	0.04
PI-based	2 (22)	5 (46)		
NNRTI-based	1 (11)	4 (36)		
INSTI-based	6 (67)	2 (18)		

Abbreviations: cART = combination antiretroviral treatment; CSF = cerebrospinal fluid; PI = protease inhibitor; NNRTI = non-nucleoside reverse transcriptase inhibitor; INSTI = integrase strand transferase inhibitor

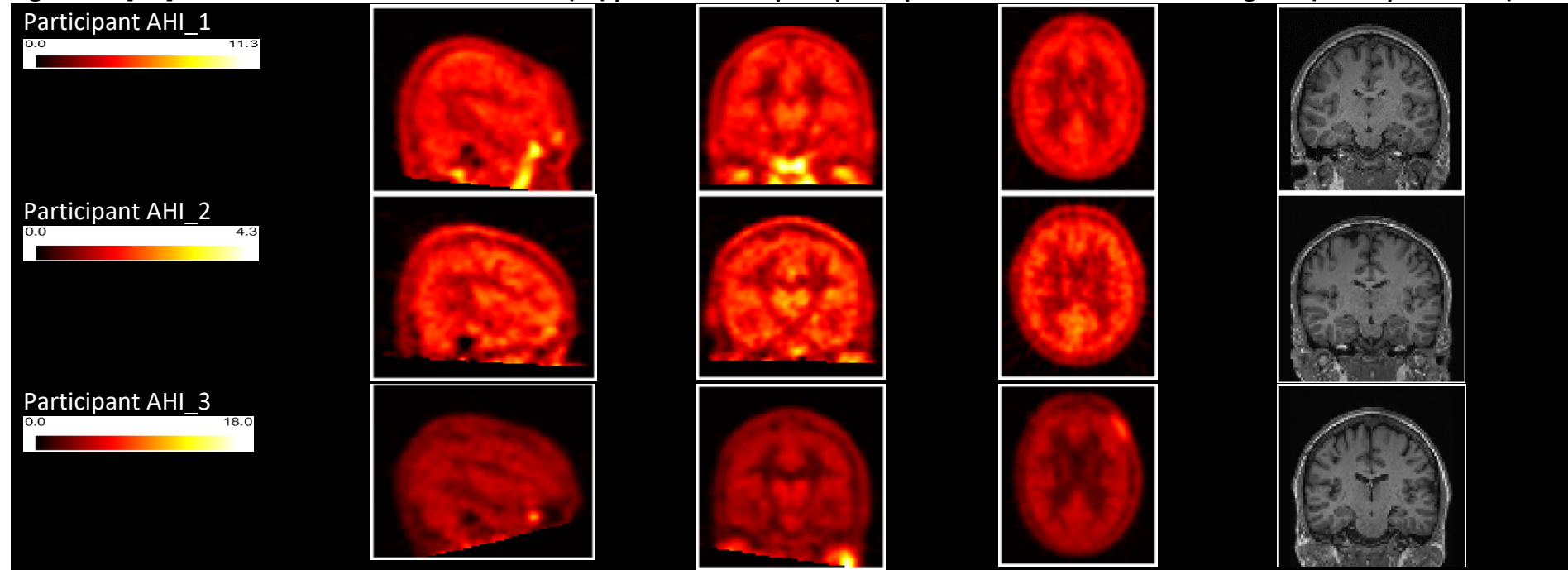
Legend: Values are median (interquartile range) or n (%). Differences in variables between participants who initiated cART during acute HIV infection versus chronic HIV infection were analysed using the Mann Whitney U test and Fisher's exact test, as appropriate, unless stated otherwise. ¹ Differences in age, gender and bodyweight were analysed between participants who initiated cART during acute HIV infection, participants who initiated cART during chronic HIV infection and control participants using the Kruskal-Wallis test and Fisher's exact test, as appropriate.

5.3.2 Parametric [^{11}C] PBR 28 V_T maps for all the participants included in this analysis

Parametric brain [^{11}C] PBR 28 V_T maps and age, clinical, cognitive and NfL (cerebrospinal fluid and plasma) data for the individual participants in the PET in AHI (participants AHI_1 to AHI_9) and PET in CHI studies (participants CHI_1 to CHI_11) are presented in Figures 50 – 56 and Tables 18 - 24.

Parametric brain [^{11}C] PBR 28 V_T maps and age, gender and body weight data for the individual control participants in this analysis (control participants 1 to 15) are presented in Figures 57 - 59; clinical, laboratory and cognitive data were not available in these participants.

Figure 50: [¹¹C] PBR28 total volume of distribution (V_T) parametric maps of participants who initiated cART during AHI (Participants 1 – 3)



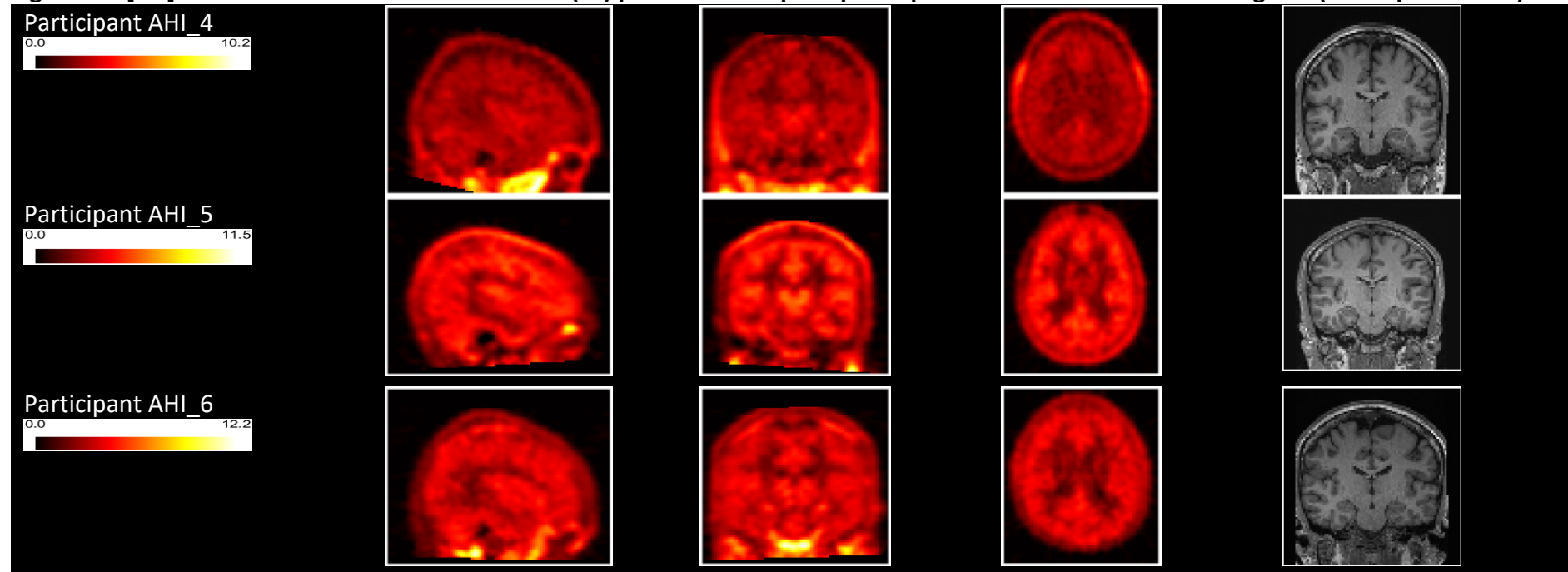
Legend: Images represent parametric maps of [¹¹C] PBR28 total volume of distribution (V_T) of HIV-positive participants who initiated cART during acute HIV infection: participants 1, 2 and 3. Abbreviations: cART = combination antiretroviral treatment, AHI = acute HIV infection

Table 18: Clinical parameters, cognitive function results and NfL results in participants 1, 2 and 3, enrolled into the PET in AHI study

Participant number	Age, years	Current CD4 ⁺ count (cells/μL)	CD8 ⁺ count (cells/μL)	CD4 ⁺ /CD8 ⁺ ratio	Nadir CD4 ⁺ count (cells/μL)	Pre-treatment HIV RNA, lg ₁₀ cp/mL	Duration on cART, years	Global composite cognitive function T-score	Plasma NfL, pg/mL	CSF NfL, pg/mL
AHI_1	24	451	324	1.39	443	3.23	1.16	56.2	4.5	353
AHI_2	40	1014	462	2.19	968	4.20	3.35	47.3	6.0	1117
AHI_3	47	685	553	1.24	370	2.67	5.86	49.6	4.0	n/a

Abbreviations: AHI = acute HIV infection; cART = combination antiretroviral treatment, CSF = cerebrospinal fluid, NfL = neurofilament light chain protein; n/a = not available

Figure 51: [¹¹C] PBR28 total volume of distribution (V_T) parametric maps of participants who initiated cART during AHI (Participants 4 – 6)



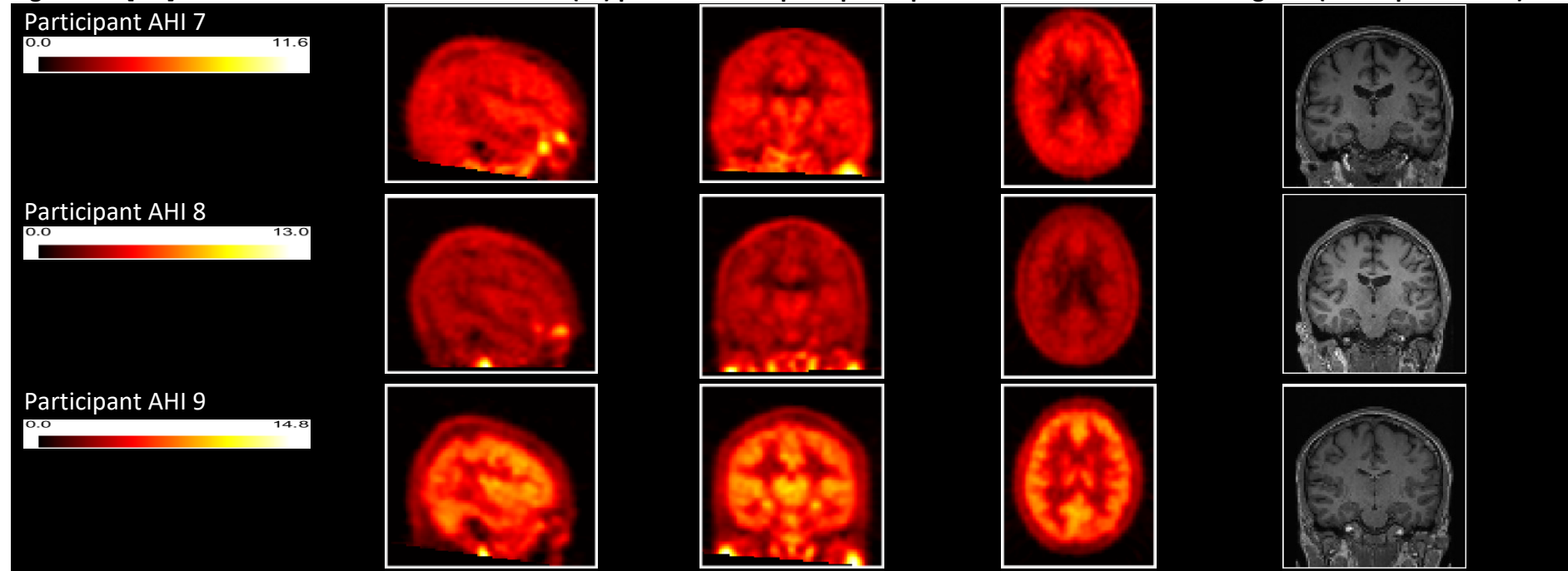
Legend: Images represent parametric maps of [¹¹C] PBR28 total volume of distribution (V_T) of HIV-positive participants who initiated cART during acute HIV infection: participants 4, 5 and 6. Abbreviations: cART = combination antiretroviral treatment, AHI = acute HIV infection

Table 19: Clinical parameters, cognitive function results and NfL results in participants 4, 5 and 6, enrolled into the PET in AHI study

Participant number	Age, years	Current CD4 ⁺ count (cells/μL)	CD8 ⁺ count (cells/μL)	CD4 ⁺ /CD8 ⁺ ratio	Nadir CD4 ⁺ count (cells/μL)	Pre-treatment HIV RNA, lg ₁₀ cp/mL	Duration on cART, years	Global composite cognitive function T-score	Plasma NfL, pg/mL	CSF NfL, pg/mL
AHI_4	34	652	460	1.42	652	7.00	2.95	56.0	5.3	395
AHI_5	30	529	624	0.85	152	6.69	2.32	59.4	6.0	403
AHI_6	46	881	902	0.98	586	2.65	3.24	47.3	3.8	397

Abbreviations: AHI = acute HIV infection; cART = combination antiretroviral treatment, CSF = cerebrospinal fluid, NfL = neurofilament light chain protein; n/a = not available

Figure 52: [¹¹C] PBR28 total volume of distribution (V_T) parametric maps of participants who initiated cART during AHI (Participants 7 – 9)



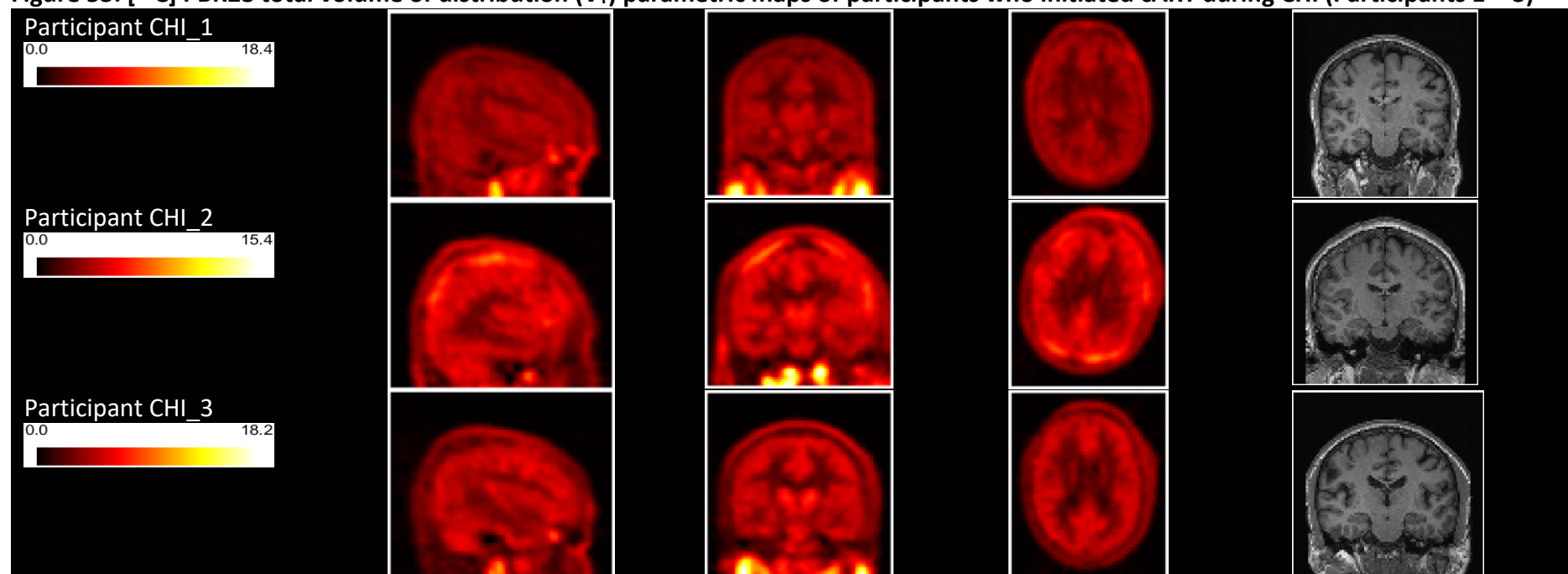
Legend: Images represent parametric maps of [¹¹C] PBR28 total volume of distribution (V_T) of HIV-positive participants who initiated cART during acute HIV infection: participants 7, 8 and 9. Abbreviations: cART = combination antiretroviral treatment, AHI = acute HIV infection

Table 20: Clinical parameters, cognitive function results and NfL results in participants 7, 8 and 9, enrolled into the PET in AHI study

Participant number	Age, years	Current CD4 ⁺ count (cells/μL)	CD8 ⁺ count (cells/μL)	CD4 ⁺ /CD8 ⁺ ratio	Nadir CD4 ⁺ count (cells/μL)	Pre-treatment HIV RNA, Ig ₁₀ cp/mL	Duration on cART, years	Global composite cognitive function T-score	Plasma NfL, pg/mL	CSF NfL, pg/mL
AHI_7	48	1474	779	1.89	902	5.70	8.61	45.7	8.3	n/a
AHI_8	26	1238	978	1.27	354	4.44	2.50	57.3	4.9	365
AHI_9	40	687	501	1.37	687	5.59	4.41	55.9	3.4	459

Abbreviations: AHI = acute HIV infection; cART = combination antiretroviral treatment, CSF = cerebrospinal fluid, NfL = neurofilament light chain protein; n/a = not available

Figure 53: [¹¹C] PBR28 total volume of distribution (V_T) parametric maps of participants who initiated cART during CHI (Participants 1 – 3)



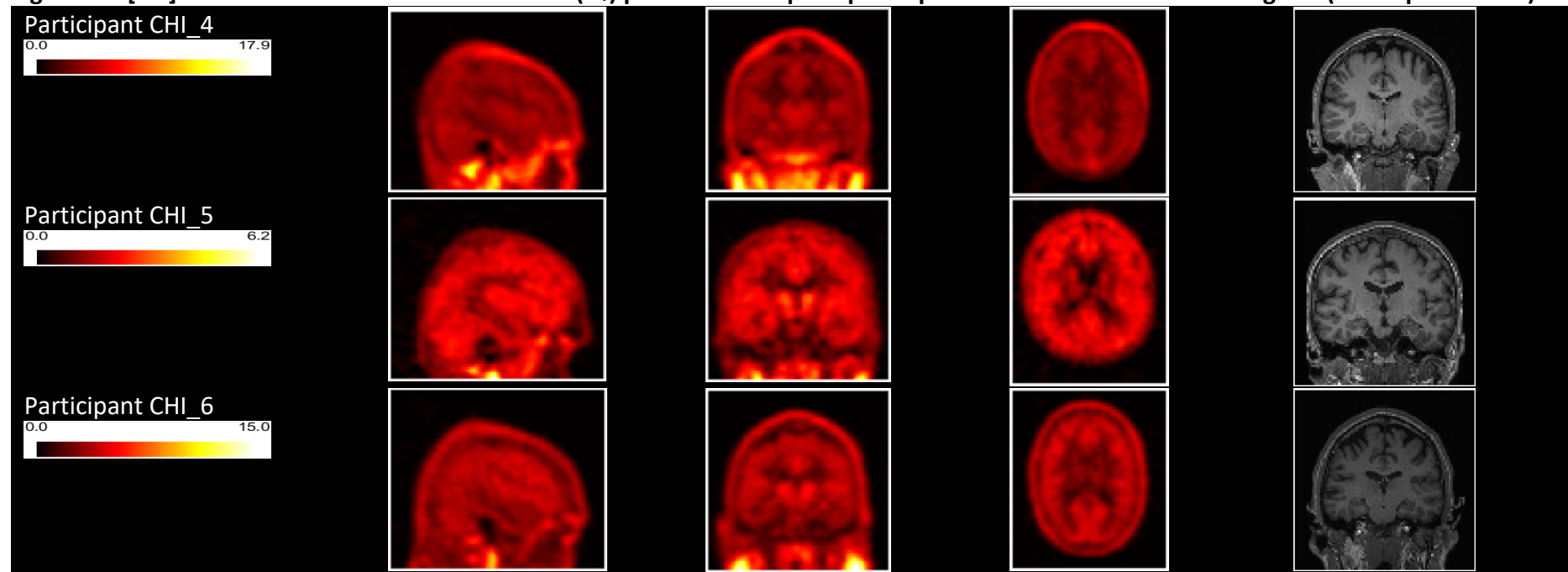
Legend: Images represent parametric maps of [¹¹C] PBR28 total volume of distribution (V_T) of HIV-positive participants who initiated cART during chronic HIV infection: participants 1, 2 and 3. Abbreviations: cART = combination antiretroviral treatment, CHI = chronic HIV infection

Table 21: Clinical parameters, cognitive function results and NfL results in participants 1, 2 and 3, enrolled into the PET in CHI study

Participant number	Age, years	Current CD4 ⁺ count (cells/μL)	CD8 ⁺ count (cells/μL)	CD4 ⁺ /CD8 ⁺ ratio	Nadir CD4 ⁺ count (cells/μL)	Pre-treatment HIV RNA, lg ₁₀ cp/mL	Duration on cART, years	Global composite cognitive function T-score	Plasma NfL, pg/mL	CSF NfL, pg/mL
CHI_1	39	900	1240	0.73	350	n/a	15.0	49.5	5.8	n/a
CHI_2	45	700	850	0.82	110	4.84	13.0	45.8	4.3	n/a
CHI_3	43	350	480	0.73	150	n/a	3.0	52.2	n/a	n/a

Abbreviations: CHI = chronic HIV infection; cART = combination antiretroviral treatment, CSF = cerebrospinal fluid, NfL = neurofilament light chain protein; n/a =not available

Figure 54: [¹¹C] PBR28 total volume of distribution (V_T) parametric maps of participants who initiated cART during CHI (Participants 4 – 6)



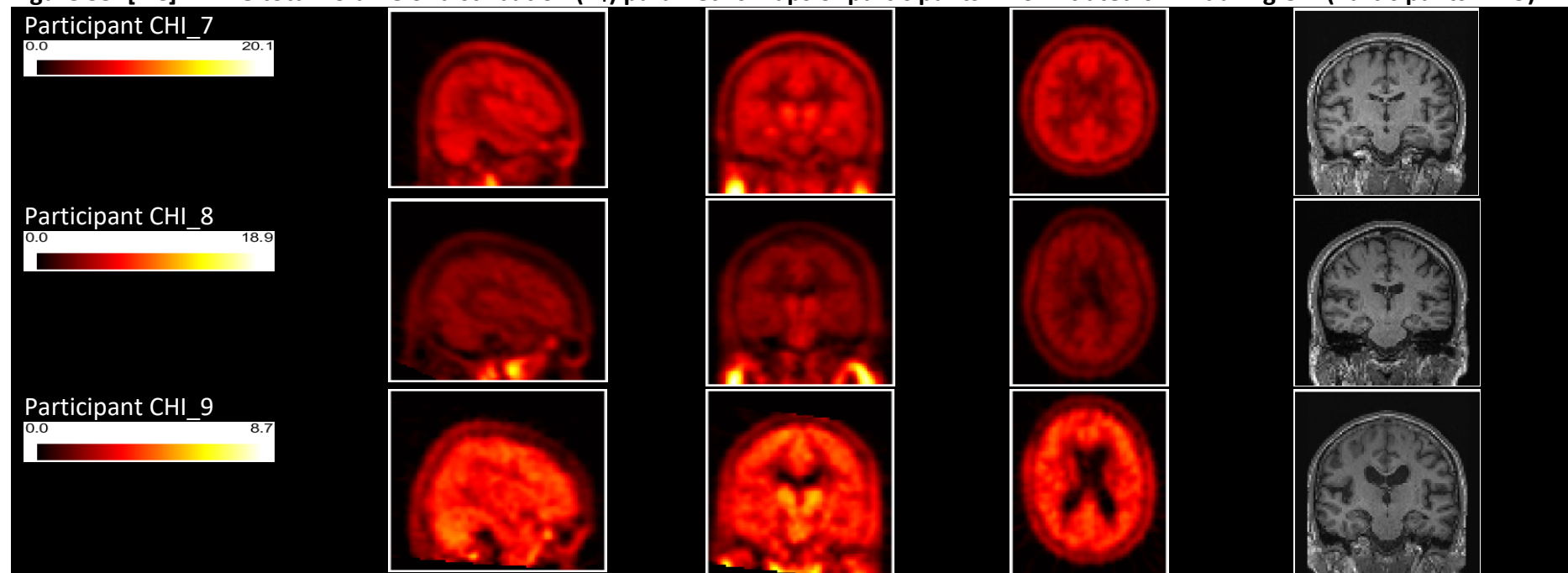
Legend: Images represent parametric maps of [¹¹C] PBR28 total volume of distribution (V_T) of HIV-positive participants who initiated cART during chronic HIV infection: participants 4, 5 and 6. Abbreviations: cART = combination antiretroviral treatment, CHI = chronic HIV infection

Table 22: Clinical parameters, cognitive function results and NfL results in participants 4, 5 and 6, enrolled into the PET in CHI study

Participant number	Age, years	Current CD4 ⁺ count (cells/μL)	CD8 ⁺ count (cells/μL)	CD4 ⁺ /CD8 ⁺ ratio	Nadir CD4 ⁺ count (cells/μL)	Pre-treatment HIV RNA, lg ₁₀ cp/mL	Duration on cART, years	Global composite cognitive function T-score	Plasma NfL, pg/mL	CSF NfL, pg/mL
CHI_4	26	500	1380	0.36	228	5.42	3.0	46.7	n/a	n/a
CHI_5	49	720	800	0.90	250	6.21	14.0	48.4	10.4	n/a
CHI_6	43	1240	1940	0.64	345	4.54	2.0	46.3	n/a	n/a

Abbreviations: CHI = chronic HIV infection; cART = combination antiretroviral treatment, CSF = cerebrospinal fluid, NfL = neurofilament light chain protein; n/a =not available

Figure 55: [¹¹C] PBR28 total volume of distribution (V_T) parametric maps of participants who initiated cART during CHI (Participants 7 – 9)



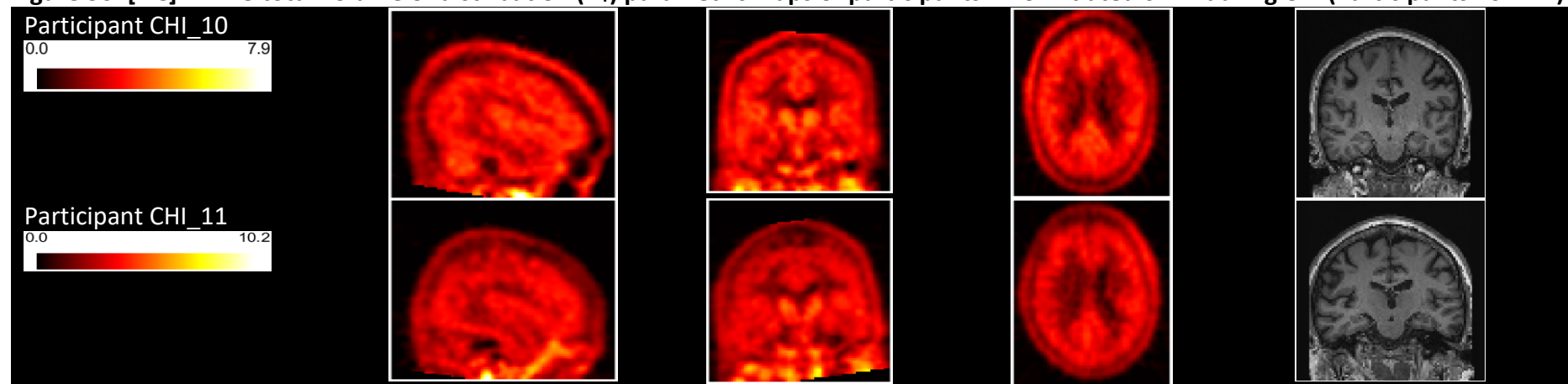
Legend: Images represent parametric maps of [¹¹C] PBR28 total volume of distribution (V_T) of HIV-positive participants who initiated cART during chronic HIV infection: participants 7, 8 and 9. Abbreviations: cART = combination antiretroviral treatment, CHI = chronic HIV infection

Table 23: Clinical parameters, cognitive function results and NfL results in participants 7, 8 and 9, enrolled into the PET in CHI study

Participant number	Age, years	Current CD4 ⁺ count (cells/μL)	CD8 ⁺ count (cells/μL)	CD4 ⁺ /CD8 ⁺ ratio	Nadir CD4 ⁺ count (cells/μL)	Pre-treatment HIV RNA, lg ₁₀ cp/mL	Duration on cART, years	Global composite cognitive function T-score	Plasma NfL, pg/mL	CSF NfL, pg/mL
CHI_7	44	440	660	0.67	160	5.27	4.0	43.9	n/a	n/a
CHI_8	49	160	850	0.19	100	n/a	n/a	42.9	n/a	n/a
CHI_9	62	1093	1772	0.62	310	3.52	22.55	46.6	15.9	1039

Abbreviations: CHI = chronic HIV infection; cART = combination antiretroviral treatment, CSF = cerebrospinal fluid, NfL = neurofilament light chain protein; n/a =not available

Figure 56: [¹¹C] PBR28 total volume of distribution (V_T) parametric maps of participants who initiated cART during CHI (Participants 10 – 11)



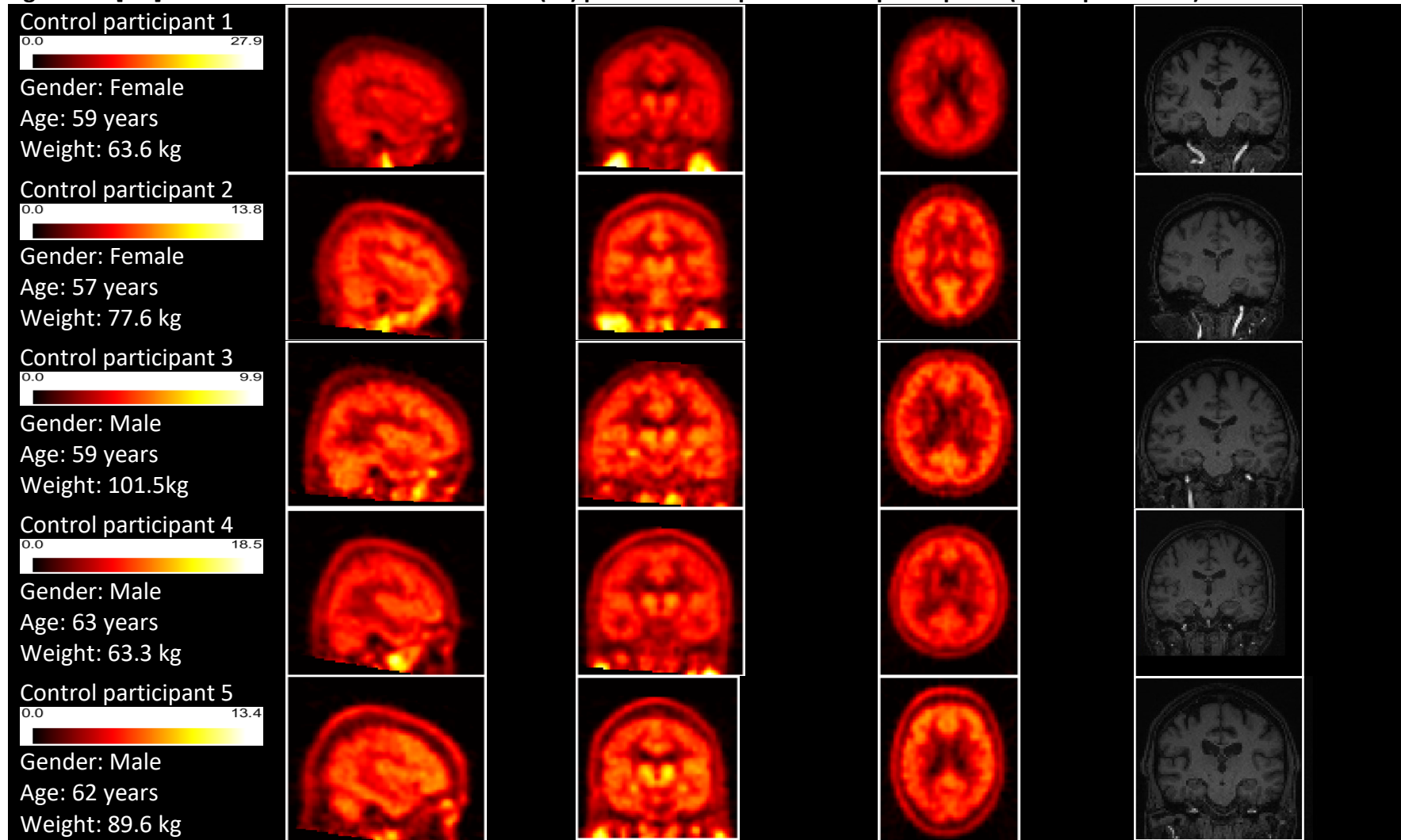
Legend: Images represent parametric maps of [¹¹C] PBR28 total volume of distribution (V_T) of HIV-positive participants who initiated cART during chronic HIV infection: participants 10 and 11. Abbreviations: cART = combination antiretroviral treatment, CHI = chronic HIV infection

Table 24: Clinical parameters, cognitive function results and NfL results in participants 10 and 11, enrolled into the PET in CHI study

Participant number	Age, years	Current CD4 ⁺ count (cells/μL)	CD8 ⁺ count (cells/μL)	CD4 ⁺ /CD8 ⁺ ratio	Nadir CD4 ⁺ count (cells/μL)	Pre-treatment HIV RNA, lg ₁₀ cp/mL	Duration on cART, years	Global composite cognitive function T-score	Plasma NfL, pg/mL	CSF NfL, pg/mL
CHI_10	55	606	733	0.83	320	4.97	15.85	54.1	6.5	1071
CHI_11	60	671	1025	0.65	200	5.63	13.54	48.9	9.6	938

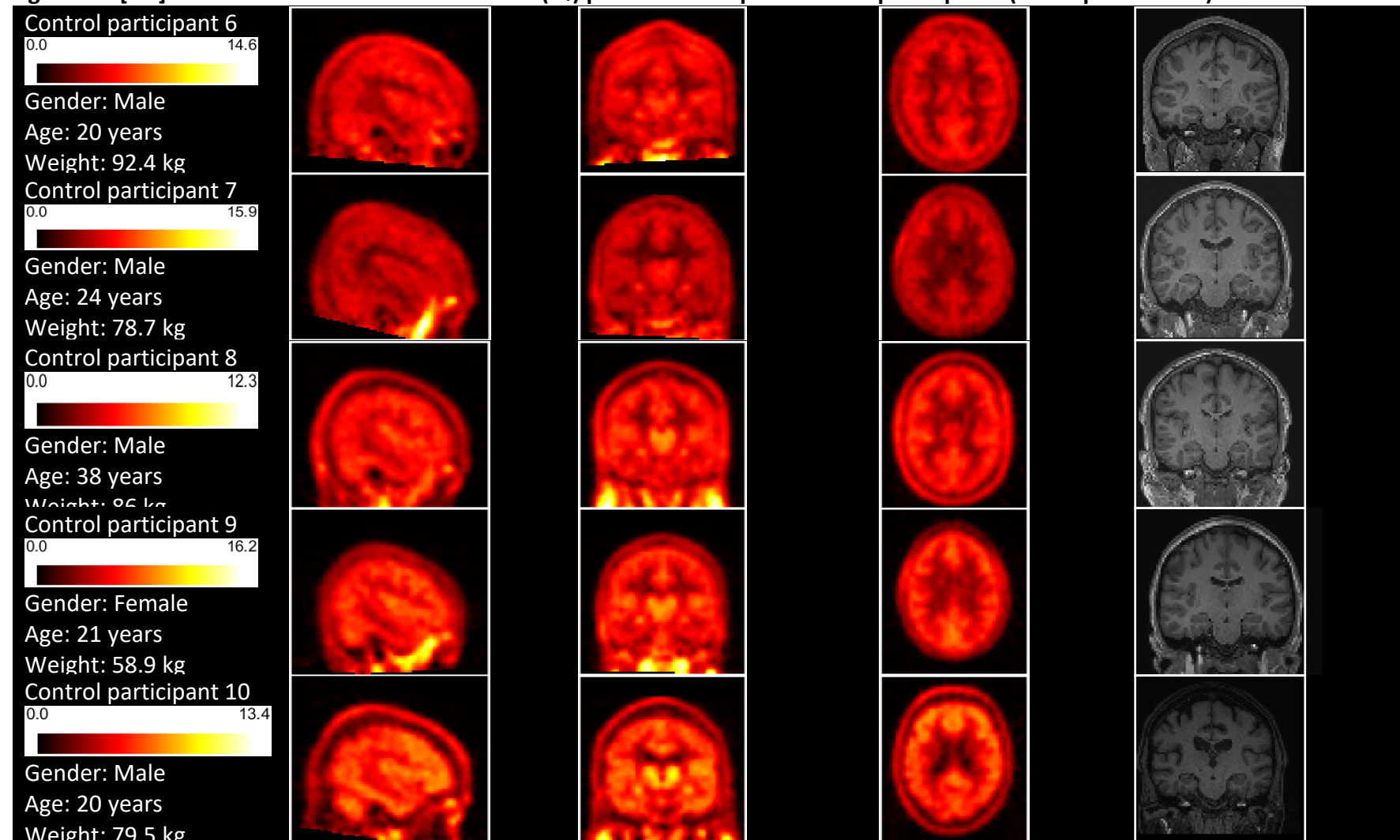
Abbreviations: CHI = chronic HIV infection; cART = combination antiretroviral treatment, CSF = cerebrospinal fluid, NfL = neurofilament light chain protein; n/a =not available

Figure 57: [^{11}C] PBR28 total volume of distribution (V_T) parametric maps of control participants (Participants 1 - 5)



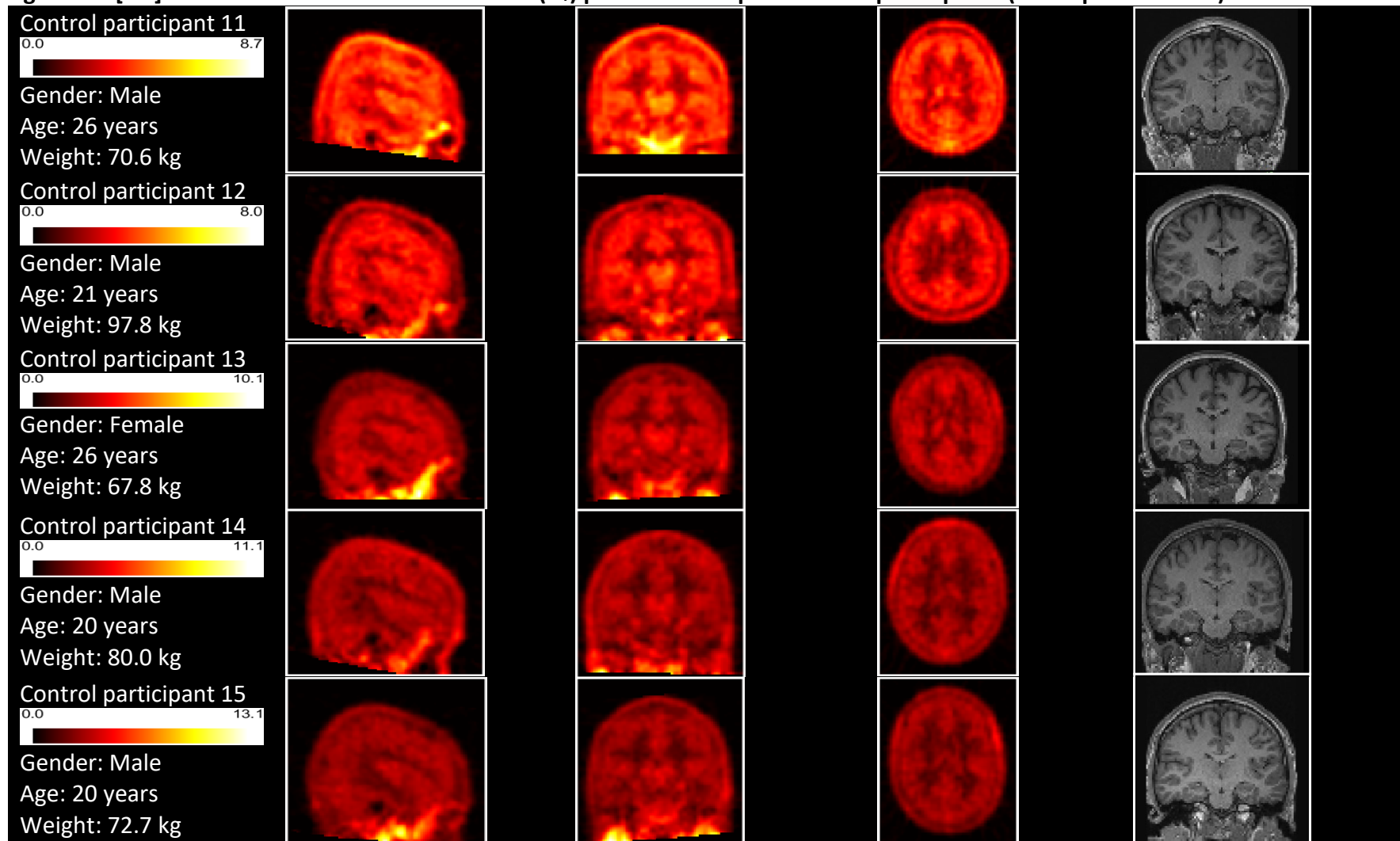
Legend: Images represent parametric maps of [^{11}C] PBR28 total volume of distribution (V_T) of control participants: participants 1, 2, 3, 4 and 5.

Figure 58: [^{11}C] PBR28 total volume of distribution (V_T) parametric maps of control participants (Participants 6 - 10)



Legend: Images represent parametric maps of [^{11}C] PBR28 total volume of distribution (V_T) of control participants: participants 6, 7, 8, 9 and 10.

Figure 59: [^{11}C] PBR28 total volume of distribution (V_T) parametric maps of control participants (Participants 11 - 15)



Legend: Images represent parametric maps of [^{11}C] PBR28 total volume of distribution (V_T) of control participants: participants 11, 12, 13, 14 and 15

5.3.3 Brain [^{11}C] PBR28 V_T results from selected brain regions for each participant

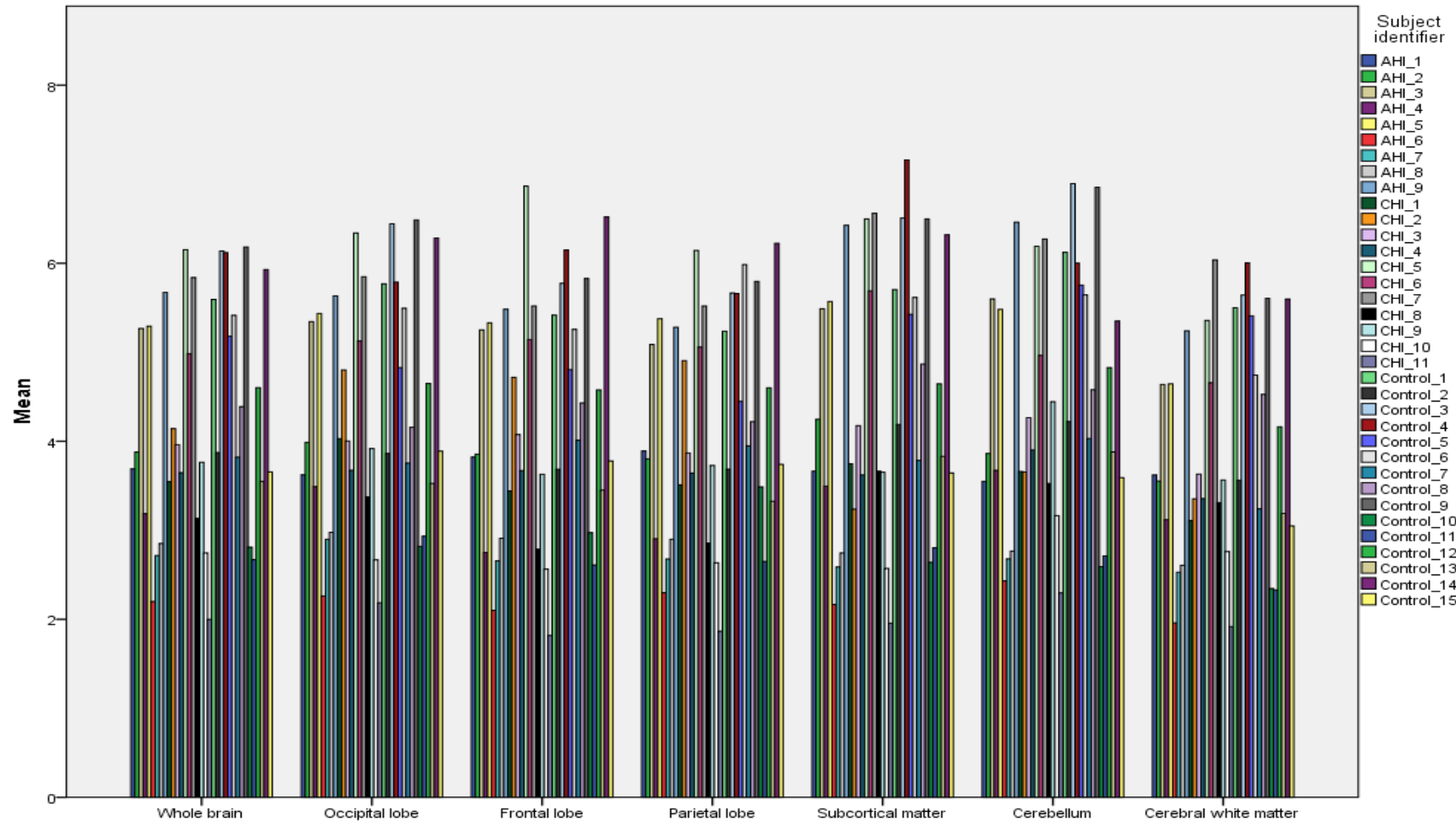
Figures 60 - 62 illustrate the [^{11}C] PBR28 binding by V_T in selected brain regions for all the participants included in the analyses in this chapter: participants who initiated cART during acute HIV infection (AHI_1 to AHI_9), participants who initiated cART during chronic HIV infection (CHI_1 to CHI_11) and control participants (control_1 to control_15).

[^{11}C] PBR28 V_T binding in the majority of brain regions were fairly homogenous amongst the participants. Of note, distinct outlying increased [^{11}C] PBR28 V_T binding in two separate regions of the selected regions of interest were noted in two unique participants, denoted with red arrows in Figures 61 and 62. The regions with increased [^{11}C] PBR28 V_T binding were:

1. Amygdala for control participant_3
2. Caudate for participant CHI_4

Neither control participant_3 nor participant CHI_4 had plasma or cerebrospinal fluid NFL measurements available.

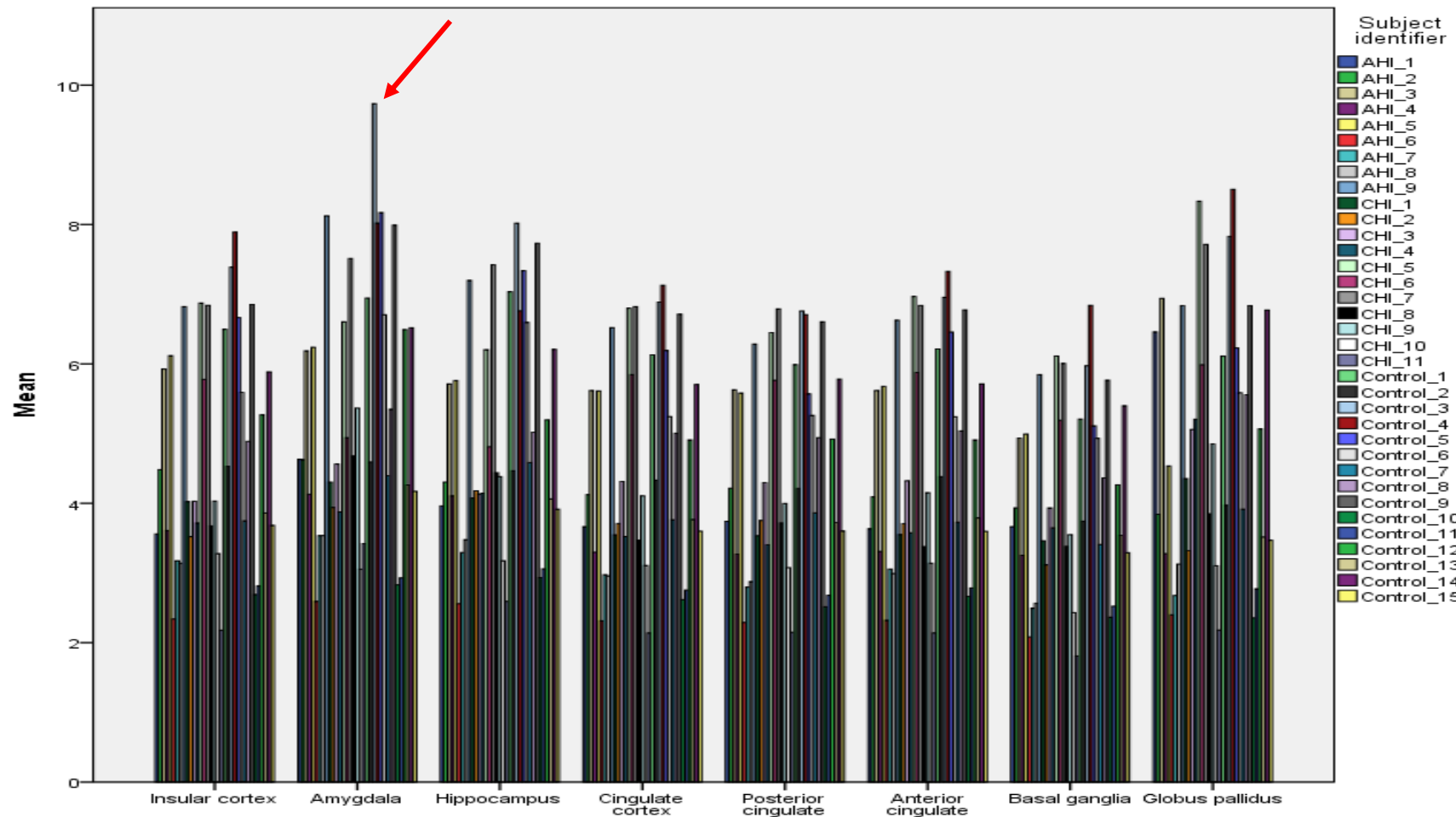
Figure 60: [^{11}C] PBR28 V_T in selected brain regions: whole brain, occipital lobe, frontal lobe, parietal lobe, subcortical matter, cerebellum and cerebral white matter, from participants in all three groups (n = 35)



[^{11}C] PBR28 V_T in selected brain regions from participants in all three groups: participants who initiated cART during acute HIV infection (AHI_1 to AHI_9), participants who initiated cART during chronic HIV infection (CHI_1 to CHI_11) and control participants (Control_1 to Control_15).

Abbreviations: cART = combination antiretroviral treatment.

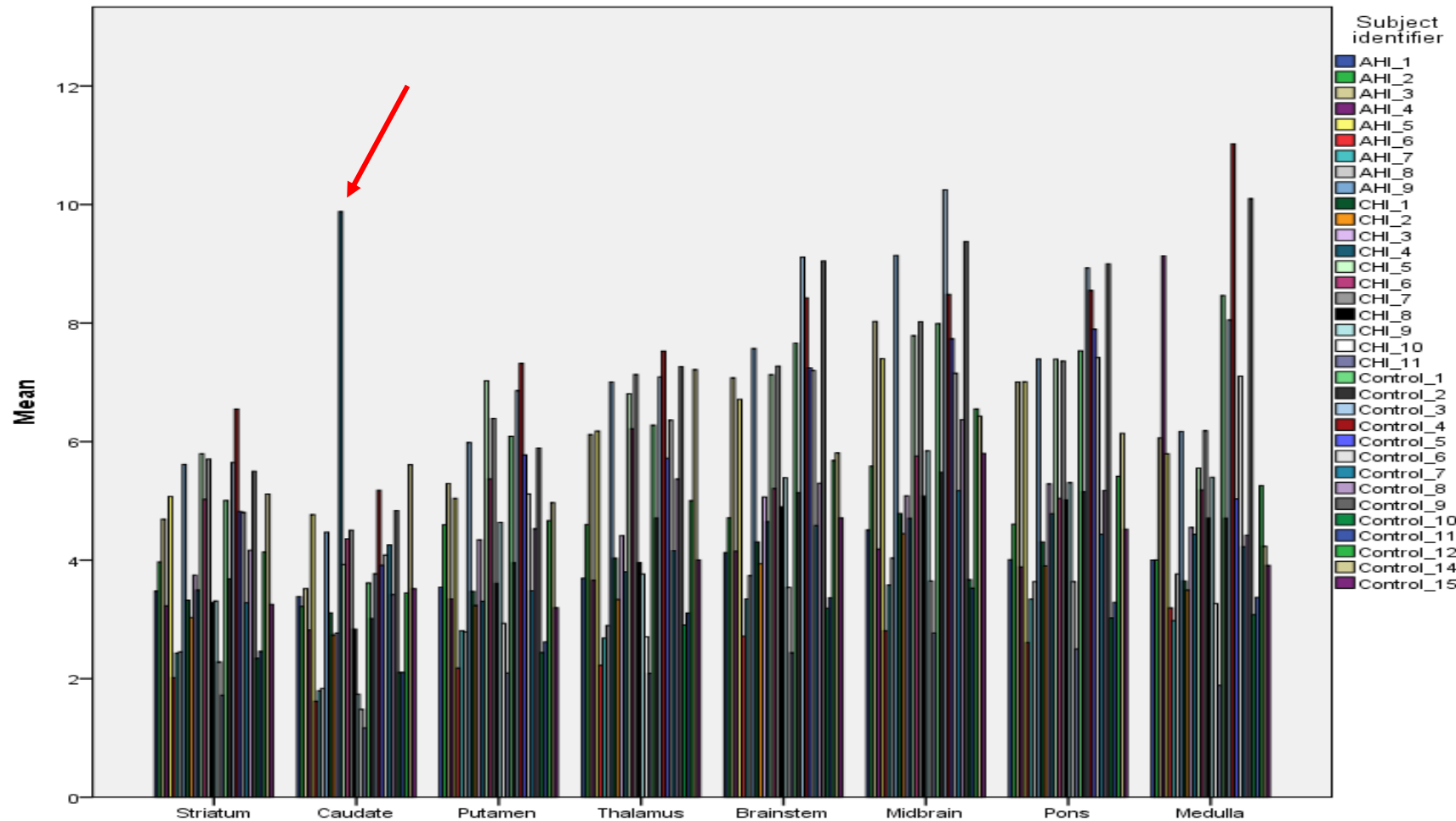
Figure 61: [^{11}C] PBR28 VT in selected brain regions: insular cortex, amygdala, hippocampus, cingulate cortex, posterior cingulate gyrus, anterior cingulate, basal ganglia and globus pallidus, from participants in all three groups (n = 35)



[^{11}C] PBR28 V_T in selected brain regions from participants in all three groups: participants who initiated cART during acute HIV infection (AHI_1 to AHI_9), participants who initiated cART during chronic HIV infection (CHI_1 to CHI_11) and control participants (Control_1 to Control_15).

Abbreviations: cART = combination antiretroviral treatment. Red arrows denote outlying increased [^{11}C] PBR28 V_T binding

Figure 62: [^{11}C] PBR28 V_T in selected brain regions: striatum, caudate, putamen, thalamus, brainstem, midbrain, pons and medulla, from participants in all three groups (n = 35)



[^{11}C] PBR28 V_T in selected brain regions from participants in all three groups: participants who initiated cART during acute HIV infection (AHI_1 to AHI_9), participants who initiated cART during chronic HIV infection (CHI_1 to CHI_11) and control participants (Control_1 to Control_15). Abbreviations: cART = combination antiretroviral treatment. Red arrows denote outlying increased [^{11}C] PBR28 V_T binding

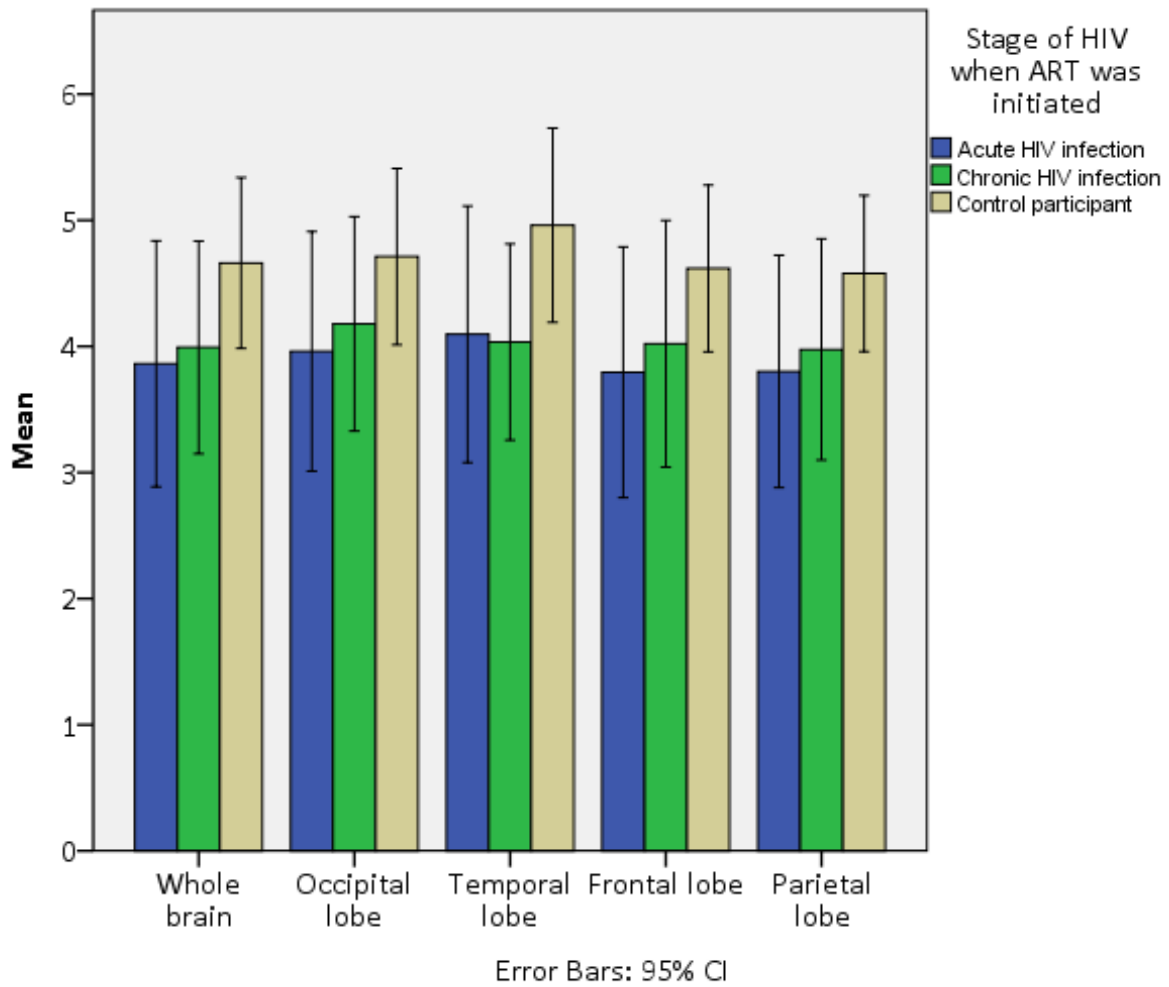
5.3.4 PBR28 binding results

5.3.4.1 *[¹¹C] PBR28 total volume of distribution (V_T) results*

When comparing regional brain [¹¹C] PBR V_T binding between the three groups, control participants consistently demonstrated higher mean [¹¹C] PBR28 (V_T) binding compared to both groups of participants with HIV at all the selected regions of interest (Figures 63 – 65). Between participants who initiated cART during acute HIV infection versus chronic HIV infection, regional brain [¹¹C] PBR V_T binding was broadly similar, especially at the temporal lobe, hippocampus, insular cortex, thalamus, striatum and brainstem (Figures 63 – 65). Participants who initiated cART during chronic HIV infection had a higher mean [¹¹C] PBR28 (V_T) binding in many of the selected regions of interest (whole brain, occipital lobe, frontal lobe, parietal lobe, cingulate cortex, posterior cingulate, anterior cingulate, subcortical matter, basal ganglia, globus pallidus, caudate, putamen, cerebellum, pons and cerebral white matter) (Figures 63 – 65), while mean [¹¹C] PBR28 (V_T) binding was higher in participants who initiated cART during acute HIV infection at the amygdala, midbrain and medulla (Figures 64 – 65).

Table 25 demonstrates the mean [¹¹C] PBR V_T binding at the selected brain regions, stratified according to participants who initiated cART during acute HIV infection, participants who initiated cART during chronic HIV infection and control participants. Overall, no statistically significant difference was seen in [¹¹C] PBR28 (V_T) binding between the three groups of participants at any of the selected regions of interest ($p > 0.05$ by the Kruskal-Wallis test for differences between the three groups at all 30 regions of interest) (Table 25).

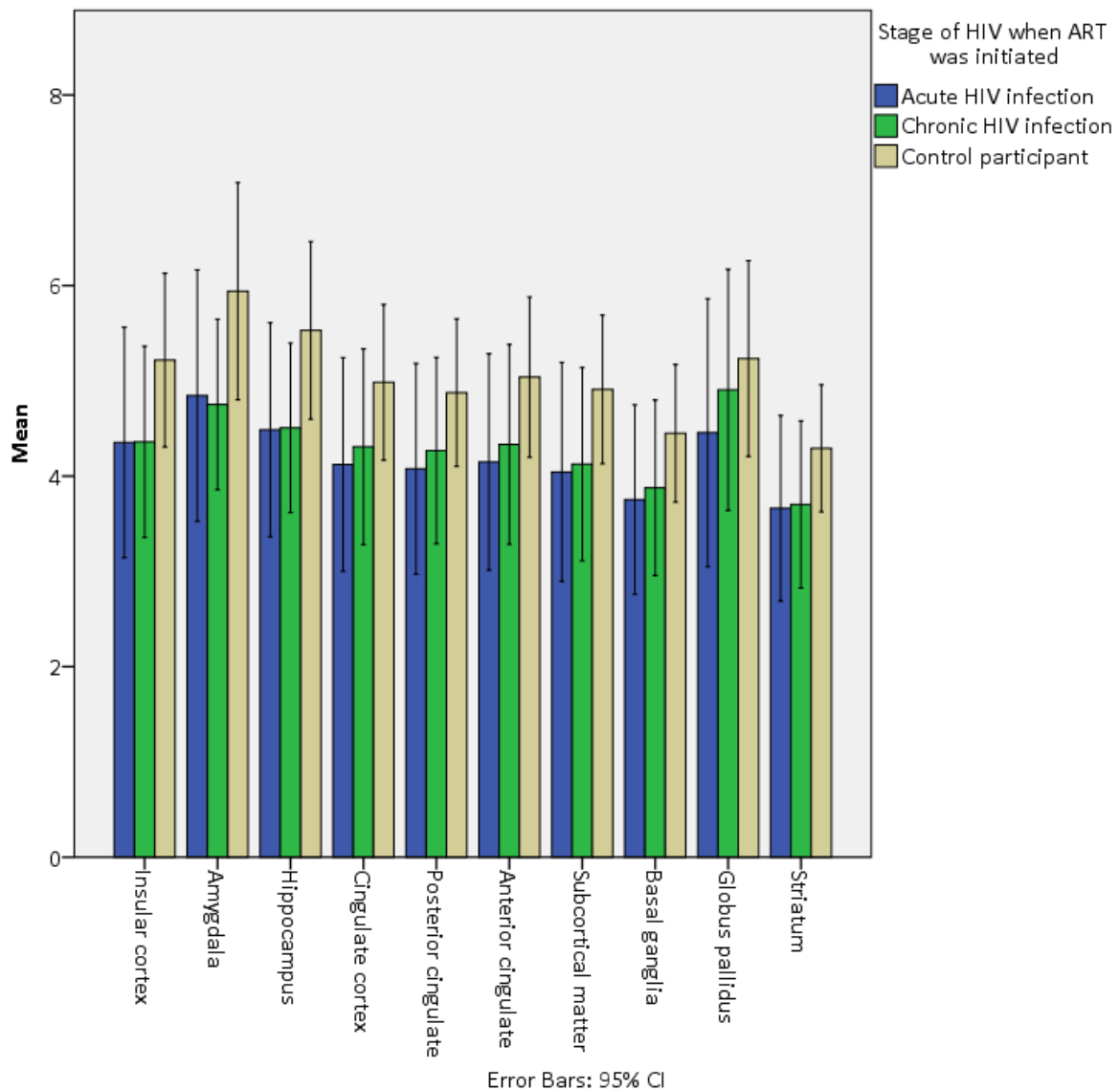
Figure 63: [^{11}C] PBR28 total V_T binding in selected brain regions: whole brain, occipital lobe, temporal lobe, frontal lobe and parietal lobe, stratified according to stage when cART was initiated and control participants



Abbreviations: CI = confidence intervals, cART = combination antiretroviral treatment

Legend: Bar chart representing [^{11}C] PBR28 total V_T from five selected brain regions. Number of participants according to stage when ART was initiated: acute HIV infection ($n = 9$), chronic HIV infection ($n = 11$), control participants ($n = 15$)

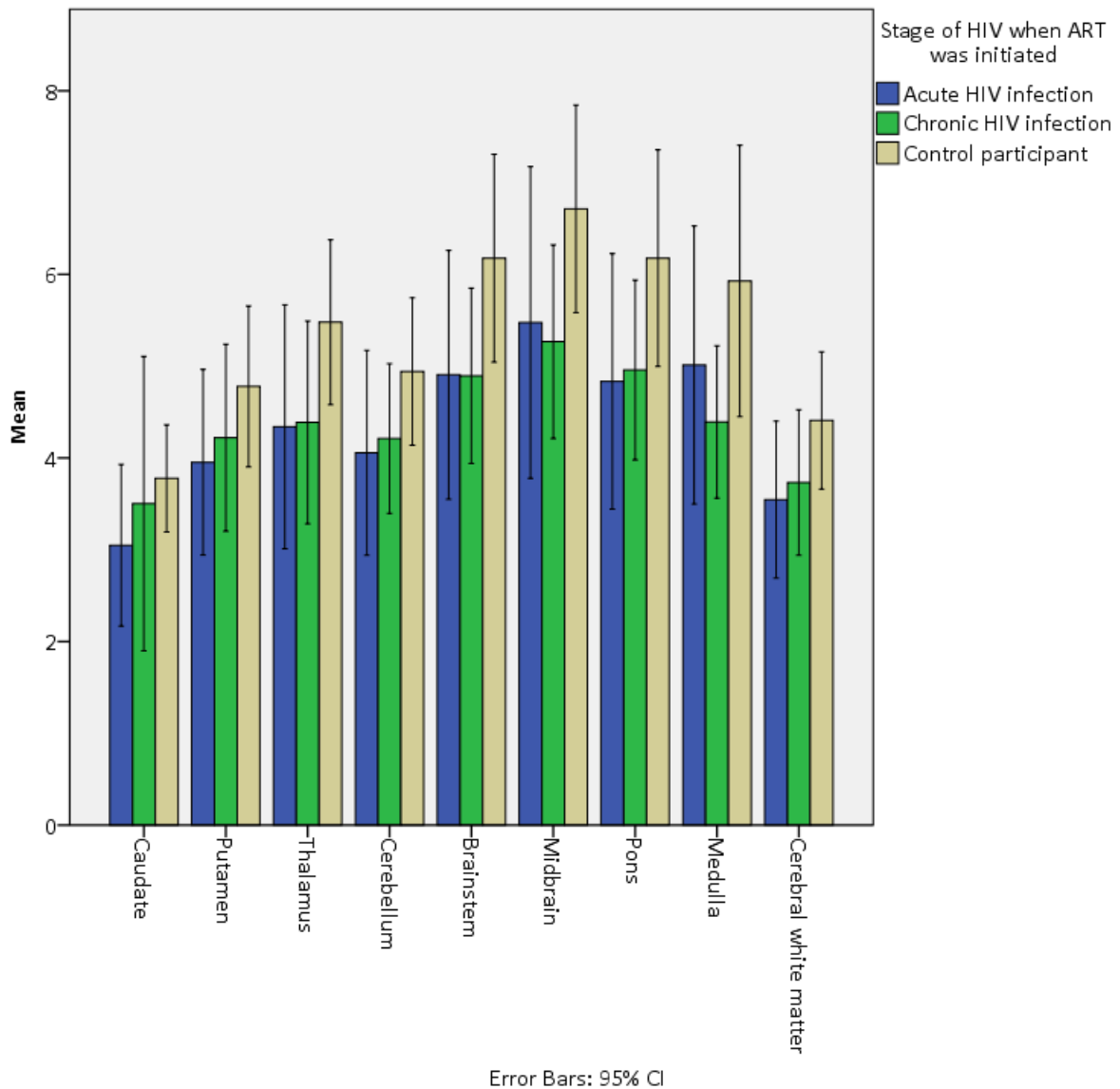
Figure 64: [^{11}C] PBR28 total V_T binding in selected brain regions: insular cortex, amygdala, hippocampus, cingulate cortex, posterior cingulate, anterior cingulate, subcortical matter, basal ganglia, globus pallidus and striatum, stratified according to stage when cART was initiated and control participants



Abbreviations: CI = confidence intervals, cART = combination antiretroviral treatment

Legend: Bar chart representing [^{11}C] PBR28 total V_T from ten selected brain regions. Number of participants according to stage when ART was initiated: acute HIV infection ($n = 9$), chronic HIV infection ($n = 11$), control participants ($n = 15$)

Figure 65: [^{11}C] PBR28 total V_T binding in nine selected brain regions: caudate, putamen, thalamus, cerebellum, brainstem, midbrain, pons, medulla and cerebral white matter, stratified according to stage when cART was initiated and control participants



Abbreviations: CI = confidence intervals, cART = combination antiretroviral treatment

Legend: Bar chart representing [^{11}C] PBR28 total V_T from nine selected brain regions. Number of participants according to stage when ART was initiated: acute HIV infection ($n = 9$), chronic HIV infection ($n = 11$), control participants ($n = 15$).

Table 25: Regional brain [¹¹C] PBR V_T binding, stratified according to stage when cART was initiated and control participants

Brain regions	Participants who initiated cART during acute HIV infection n = 9	Participants who initiated cART during chronic HIV infection n = 11	Control participants n = 15	p-value
Whole brain	3.86 (1.27)	3.99 (1.25)	4.66 (1.22)	0.255
Occipital lobe	3.96 (1.24)	4.18 (1.27)	4.71 (1.26)	0.307
Insular cortex	4.35 (1.57)	4.36 (1.49)	5.22 (1.65)	0.280
Temporal lobe	4.10 (1.32)	4.04 (1.16)	4.96 (1.39)	0.152
Amygdala	4.85 (1.71)	4.75 (1.33)	5.94 (2.06)	0.263
Hippocampus	4.49 (1.46)	4.51 (1.32)	5.53 (1.68)	0.190
Frontal lobe	3.80 (1.29)	4.02 (1.46)	4.62 (1.19)	0.257
Cingulate cortex	4.12 (1.46)	4.31 (1.53)	4.98 (1.47)	0.244
Posterior cingulate gyrus	4.08 (1.44)	4.27 (1.46)	4.88 (1.40)	0.366
Anterior cingulate	4.15 (1.48)	4.33 (1.56)	5.04 (1.52)	0.251
Parietal lobe	3.80 (1.20)	3.98 (1.30)	4.58 (1.12)	0.246
Subcortical matter	4.04 (1.50)	4.12 (1.51)	4.91 (1.41)	0.199
Basal ganglia	3.75 (1.29)	3.88 (1.37)	4.45 (1.30)	0.421
Globus pallidus	4.46 (1.83)	4.91 (1.88)	5.23 (1.85)	0.583
Striatum	3.66 (1.27)	3.70 (1.31)	4.29 (1.20)	0.385
Left striatum	3.66 (1.25)	3.71 (1.32)	4.30 (1.20)	0.393
Right striatum	3.69 (1.30)	3.73 (1.30)	4.31 (1.23)	0.372
Caudate	3.05 (1.14)	3.50 (2.39)	3.78 (1.01)	0.216
Left caudate	3.20 (1.15)	3.71 (2.82)	3.61 (1.04)	0.694
Right caudate	3.00 (1.05)	3.60 (2.33)	3.94 (1.06)	0.137
Putamen	3.95 (1.31)	4.22 (1.52)	4.70 (1.50)	0.497
Left putamen	3.93 (1.25)	4.30 (1.65)	4.70 (1.54)	0.471
Right putamen	4.04 (1.45)	4.22 (1.44)	4.72 (1.47)	0.510
Thalamus	4.34 (1.73)	4.39 (1.64)	5.39 (1.54)	0.099
Cerebellum	4.06 (1.45)	4.21 (1.21)	4.87 (1.37)	0.260
Brainstem	4.90 (1.76)	4.89 (1.42)	6.08 (1.93)	0.158
Midbrain	5.48 (2.21)	5.27 (1.57)	6.61 (1.93)	0.153
Pons	4.83 (1.81)	4.96 (1.46)	6.06 (2.02)	0.151
Medulla	5.01 (1.97)	4.39 (1.23)	5.86 (2.48)	0.490
Cerebral white matter	3.55 (1.11)	3.73 (1.18)	4.33 (1.29)	0.321

Abbreviations: cART = combination antiretroviral treatment

Values are mean (standard deviation).

Legend: P-values calculated using the Kruskal-Wallis test.

Key findings: No statistically significant differences seen at any of the regions of interest between the three groups of participants

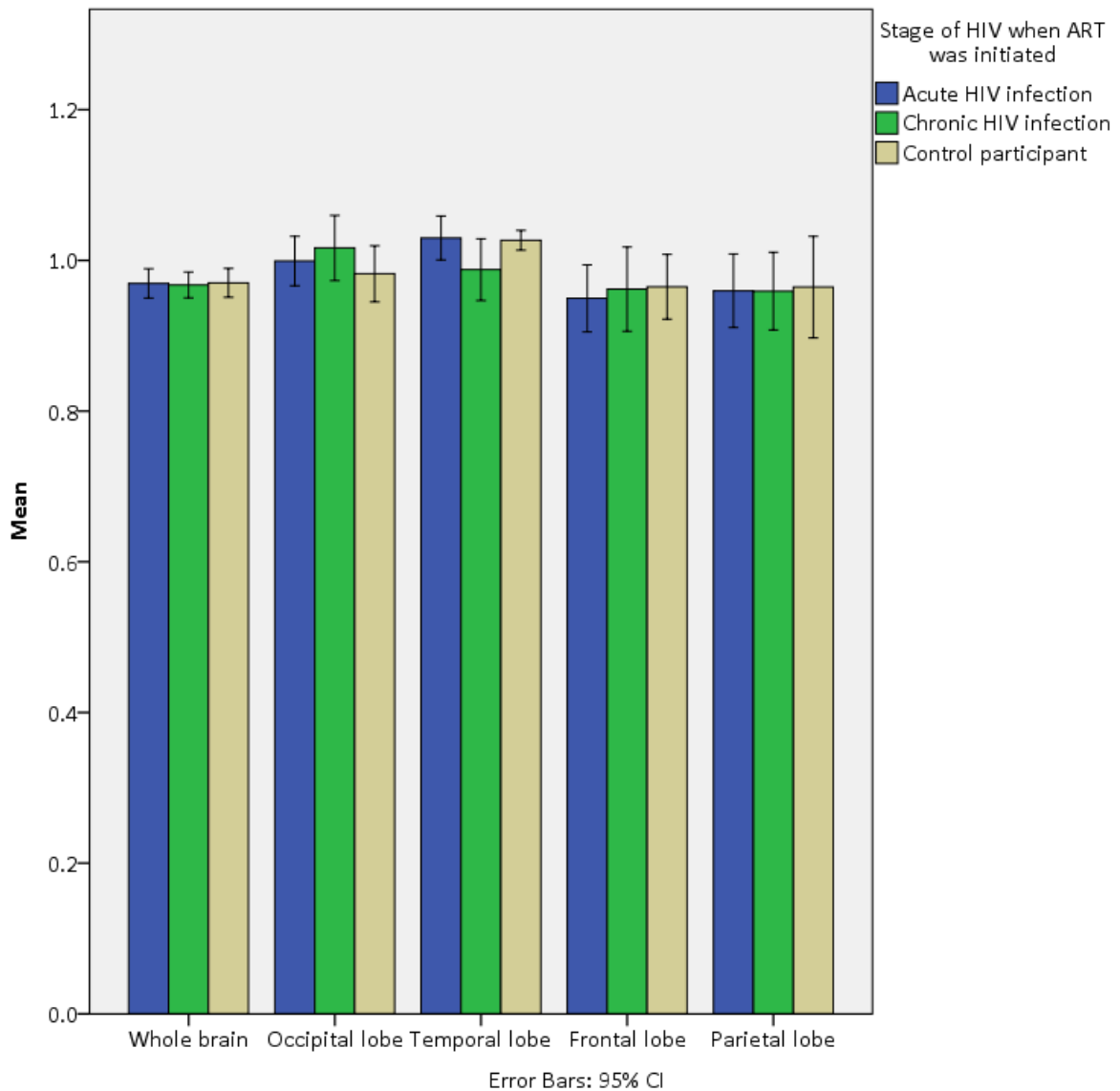
5.3.4.2 *[¹¹C] PBR28 normalised $V_{T(DVR)}$ results*

[¹¹C] PBR28 V_T binding was normalised to cortical grey matter (used as the reference region) and reported as [¹¹C] PBR28 normalised $V_{T(DVR)}$. Mean [¹¹C] PBR28 normalised $V_{T(DVR)}$ was broadly very similar across the three groups of participants at all the regions of interest, but especially across the whole brain and at the frontal lobe, parietal lobe, cingulate cortex, posterior cingulate, anterior cingulate, basal ganglia and cerebral white matter (Figures 66 – 68).

Mean [¹¹C] PBR28 normalised $V_{T(DVR)}$ binding was highest in the participants who initiated cART during chronic HIV infection compared to the other two groups of participants in the occipital lobe, globus pallidus and caudate (Figures 66 – 67) and mean [¹¹C] PBR28 normalised $V_{T(DVR)}$ binding was highest at the medulla in the participants who initiated cART during acute HIV infection compared to the other two groups of participants (Figure 68).

Mean [¹¹C] PBR V_T normalised $V_{T(DVR)}$ binding results at the selected brain regions, stratified according to participants who initiated cART during acute HIV infection, participants who initiated cART during chronic HIV infection and control participants are reported in Table 26. No statistically significant difference was seen in [¹¹C] PBR28 normalised $V_{T(DVR)}$ binding between the three groups of participants at any of the selected regions of interest ($p > 0.05$ by the Kruskal-Wallis test for differences between the three groups at all 30 regions of interest) (Table 26).

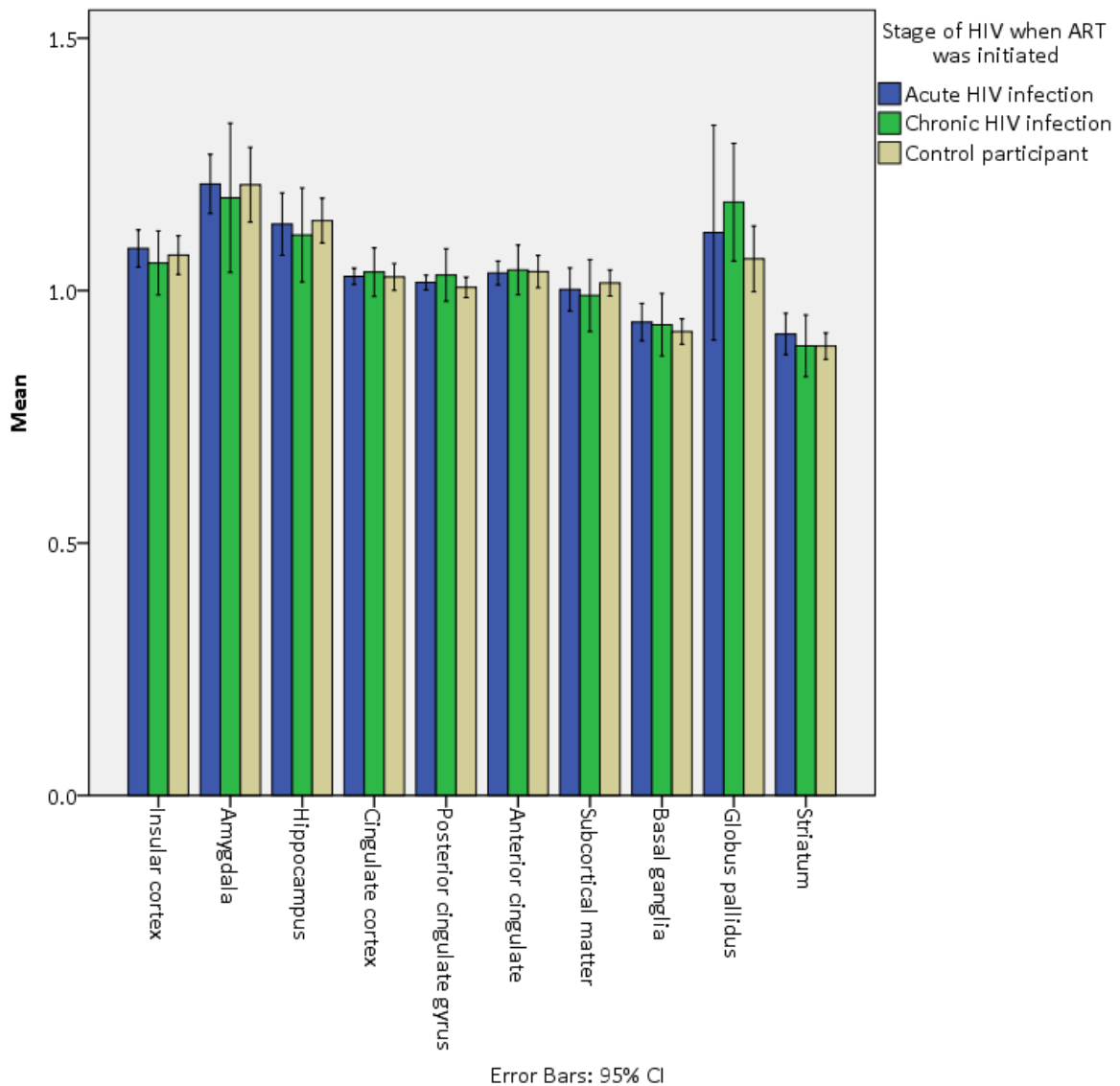
Figure 66: [^{11}C] PBR28 $V_{T(\text{DVR})}$ binding in selected brain regions: whole brain, occipital lobe, temporal lobe, frontal lobe and parietal lobe, stratified according to stage when cART was initiated and control participants



Abbreviations: CI = confidence intervals, cART = combination antiretroviral treatment

Legend: Bar chart representing [^{11}C] PBR28 $V_{T(\text{DVR})}$ from five selected brain regions. Number of participants according to stage when ART was initiated: acute HIV infection ($n = 9$), chronic HIV infection ($n = 11$), control participants ($n = 15$)

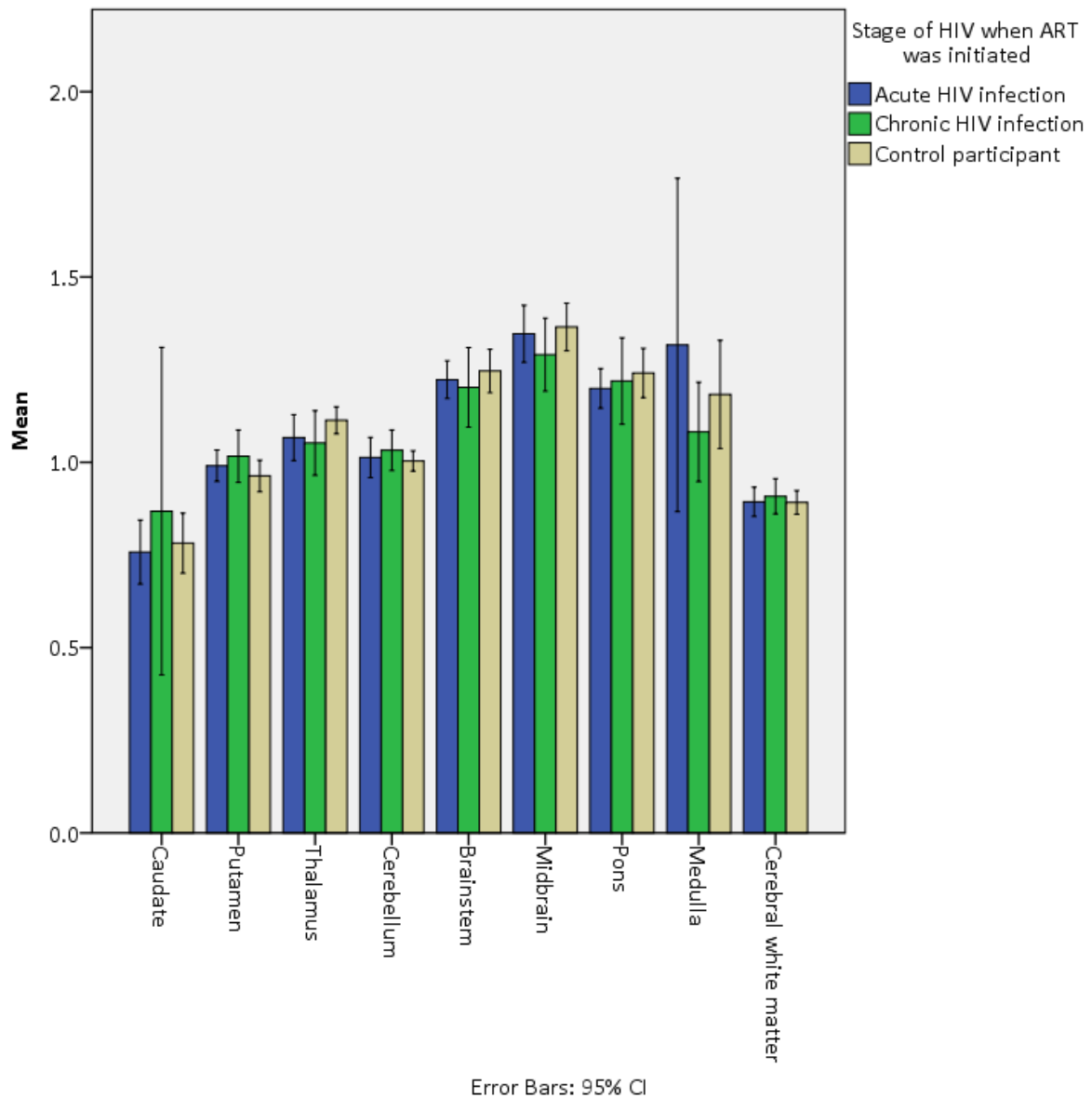
Figure 67: [^{11}C] PBR28 $V_{T(\text{DVR})}$ binding in selected brain regions: insular cortex, amygdala, hippocampus, cingulate cortex, posterior cingulate, anterior cingulate, subcortical matter, basal ganglia, globus pallidus and striatum, stratified according to stage when cART was initiated and control participants



Abbreviations: CI = confidence intervals, cART = combination antiretroviral treatment

Legend: Bar chart representing [^{11}C] PBR28 $V_{T(\text{DVR})}$ from ten selected brain regions. Number of participants according to stage when ART was initiated: acute HIV infection ($n = 9$), chronic HIV infection ($n = 11$), control participants ($n = 15$)

Figure 68: [^{11}C] PBR28 $V_{T(\text{DVR})}$ binding in selected brain regions: caudate, putamen, thalamus, cerebellum, brainstem, midbrain, pons, medulla and cerebral white matter, stratified according to stage when cART was initiated and control participants



Abbreviations: CI = confidence intervals, cART = combination antiretroviral treatment

Legend: Bar chart representing [^{11}C] PBR28 $V_{T(\text{DVR})}$ from nine selected brain regions. Number of participants according to stage when ART was initiated: acute HIV infection ($n = 9$), chronic HIV infection ($n = 11$), control participants ($n = 15$)

Table 26: Regional brain [^{11}C] PBR $V_{T(\text{DVR})}$ binding, stratified according to stage when cART was initiated and control participants

Brain region	Participants who initiated cART during acute HIV infection n = 9	Participants who initiated cART during chronic HIV infection n = 11	Control participants n = 15	p-value
Whole brain	0.97 (0.03)	0.97 (0.03)	0.97 (0.03)	0.879
Occipital lobe	1.00 (0.04)	1.02 (0.06)	0.98 (0.07)	0.550
Insular cortex	1.08 (0.05)	1.05 (0.09)	1.07 (0.07)	0.673
Temporal lobe	1.03 (0.04)	0.99 (0.06)	1.03 (0.02)	0.193
Amygdala	1.21 (0.08)	1.18 (0.22)	1.21 (0.13)	0.514
Hippocampus	1.13 (0.08)	1.11 (0.14)	1.14 (0.08)	0.833
Frontal lobe	0.95 (0.06)	0.96 (0.08)	0.97 (0.08)	0.948
Cingulate cortex	1.03 (0.02)	1.03 (0.07)	1.03 (0.05)	0.731
Posterior cingulate gyrus	1.02 (0.02)	1.03 (0.08)	1.00 (0.04)	0.556
Anterior cingulate	1.03 (0.03)	1.04 (0.07)	1.04 (0.06)	0.915
Parietal lobe	0.96 (0.06)	0.96 (0.08)	0.96 (0.12)	0.885
Subcortical matter	1.00 (0.06)	0.99 (0.11)	1.02 (0.05)	0.944
Basal ganglia	0.94 (0.05)	0.93 (0.09)	0.92 (0.05)	0.541
Globus pallidus	1.11 (0.28)	1.18 (0.17)	1.06 (0.12)	0.095
Striatum	0.91 (0.05)	0.89 (0.09)	0.89 (0.05)	0.499
Left striatum	0.91 (0.07)	0.89 (0.10)	0.89 (0.73)	0.620
Right striatum	0.92 (0.05)	0.90 (0.09)	0.89 (0.05)	0.427
Caudate	0.76 (0.11)	0.87 (0.66)	0.78 (0.14)	0.530
Left caudate	0.80 (0.12)	0.93 (0.80)	0.77 (0.20)	0.365
Right caudate	0.75 (0.11)	0.88 (0.61)	0.85 (0.23)	0.369
Putamen	0.99 (0.05)	1.01 (0.11)	0.96 (0.07)	0.144
Left putamen	0.99 (0.05)	1.03 (0.15)	0.96 (0.08)	0.113
Right putamen	1.01 (0.12)	1.02 (0.08)	0.97 (0.08)	0.136
Thalamus	1.07 (0.08)	1.05 (0.13)	1.12 (0.06)	0.278
Cerebellum	1.01 (0.07)	1.03 (0.08)	1.01 (0.05)	0.482
Brainstem	1.22 (0.07)	1.20 (0.16)	1.25 (0.10)	0.556
Midbrain	1.35 (0.10)	1.29 (0.15)	1.37 (1.08)	0.207
Pons	1.20 (0.07)	1.22 (0.17)	1.24 (0.11)	0.697
Medulla	1.32 (0.59)	1.08 (0.20)	1.20 (0.25)	0.409
Cerebral white matter	0.89 (0.05)	0.91 (0.07)	0.89 (0.05)	0.695

Abbreviations: cART = combination antiretroviral treatment

Values are mean (standard deviation)

Legend: P-values calculated using the Kruskal-Wallis test.

Key findings: No statistically significant differences seen at any of the regions of interest between the three groups of participants

5.3.5 Cerebrospinal fluid and plasma results

Among nine participants who initiated cART during acute HIV infection, 7/9 participants underwent cerebrospinal fluid collection, while of the 11 participants who initiated cART during chronic HIV infection, 10/11 participants underwent cerebrospinal fluid collection. Cerebrospinal fluid HIV RNA load, protein and white cell count were measured in all participants who underwent cerebrospinal fluid collection (Table 27).

Amongst the nine participants who initiated cART during acute HIV infection, stored samples were available for cerebrospinal fluid NfL analysis in all seven participants in this group who underwent lumbar puncture and plasma NfL analysis in all nine participants in this group (Table 27). Of the participants who initiated cART during chronic HIV infection, stored samples were available for cerebrospinal fluid NfL analysis in 3/11 participants and plasma NfL analysis in 6/11 participants in this group (Table 27).

I observed a trend towards significantly lower cerebrospinal fluid NfL concentrations among participants who initiated cART during acute versus chronic HIV infection (median (IQR) 397 (380, 431) vs 1039 (988, 1055) pg/mL, $p = 0.09$) (Table 27 and Figure 69) and significantly lower plasma NfL in participants who initiated cART during acute HIV infection compared to those who initiated cART during chronic HIV infection (median (IQR) 4.0 (4.0, 6.0) vs 8.1 (5.8, 10.4) pg/mL, $p = 0.045$) (Table 27 and Figure 70). Cerebrospinal fluid: plasma NfL ratios were broadly similar among participants who initiated cART during acute versus chronic HIV infection (median (IQR) 77.8 (74.6, 119.4) vs 97.7 (81.5, 130.8), $p = 0.91$) (Table 27).

All participants with cerebrospinal fluid collected had cerebrospinal fluid HIV RNA <40 copies/mL and cerebrospinal fluid protein and white cell count were broadly similar between the two groups of participants and within normal range (Table 27).

Table 27: Cerebrospinal fluid and plasma laboratory parameters

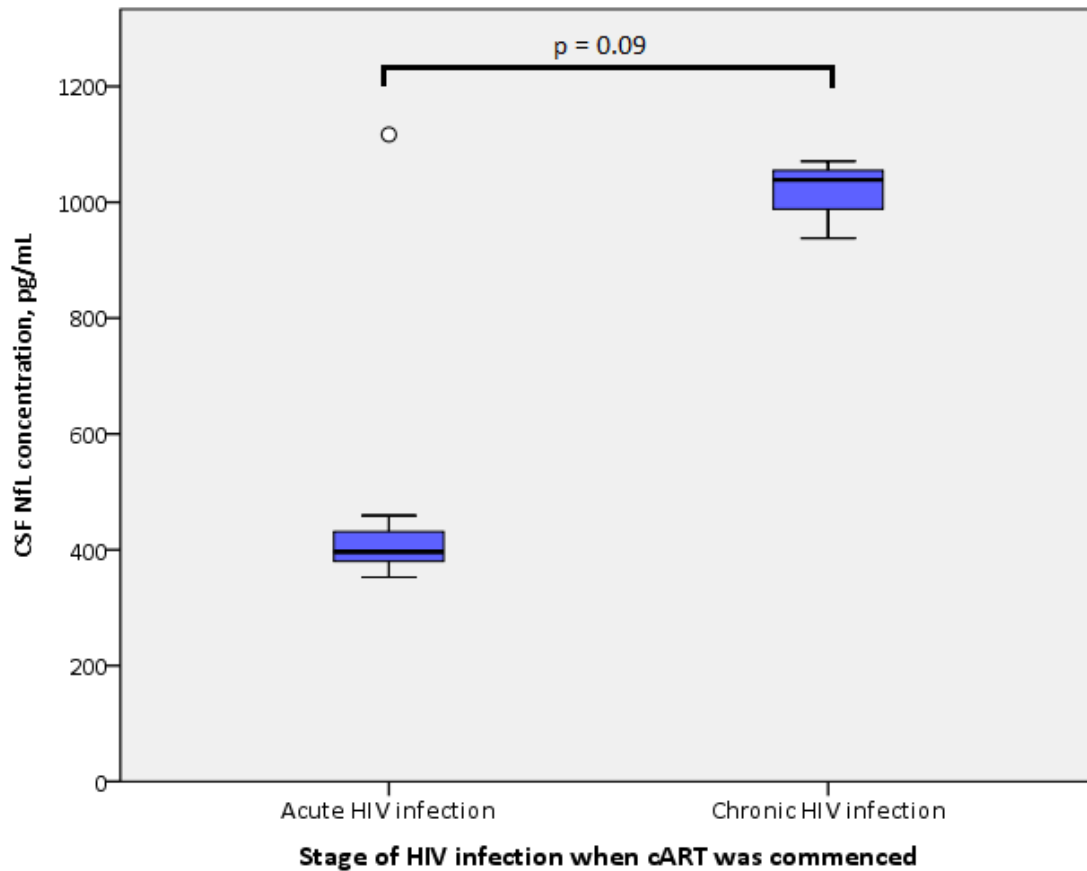
Variables, median (interquartile range) or n (%)	Participants who initiated cART during acute HIV infection n = 9	Participants who initiated cART during chronic HIV infection n = 11	p-value
CSF NfL, pg/mL	397 (380, 431) n = 7	1039 (988, 1055) n = 3	0.09
Plasma NfL, pg/mL	4.9 (4.0, 6.0) n = 9	8.1 (5.8, 10.4) n = 6	0.045*
CSF: plasma NfL	77.8 (74.6, 119.4) n = 7	97.7 (81.5, 130.8) n = 3	0.91
CSF HIV RNA viral load <40 copies/mL	7 (100) n = 7	10 (100) n = 10	
CSF protein,	0.28 (0.25, 0.50) n = 7	0.40 (0.38, 0.46) n = 10	0.70
CSF white cell count	2.0 (1.5, 2.0) n = 7	1.0 (1.0, 2.0) n = 10	0.48

Abbreviations: cART = combination antiretroviral treatment; CSF = cerebrospinal fluid; NfL = neurofilament light chain protein

Legend: P-values calculated using the Mann-Whitney U test.

** denotes results of statistical significance*

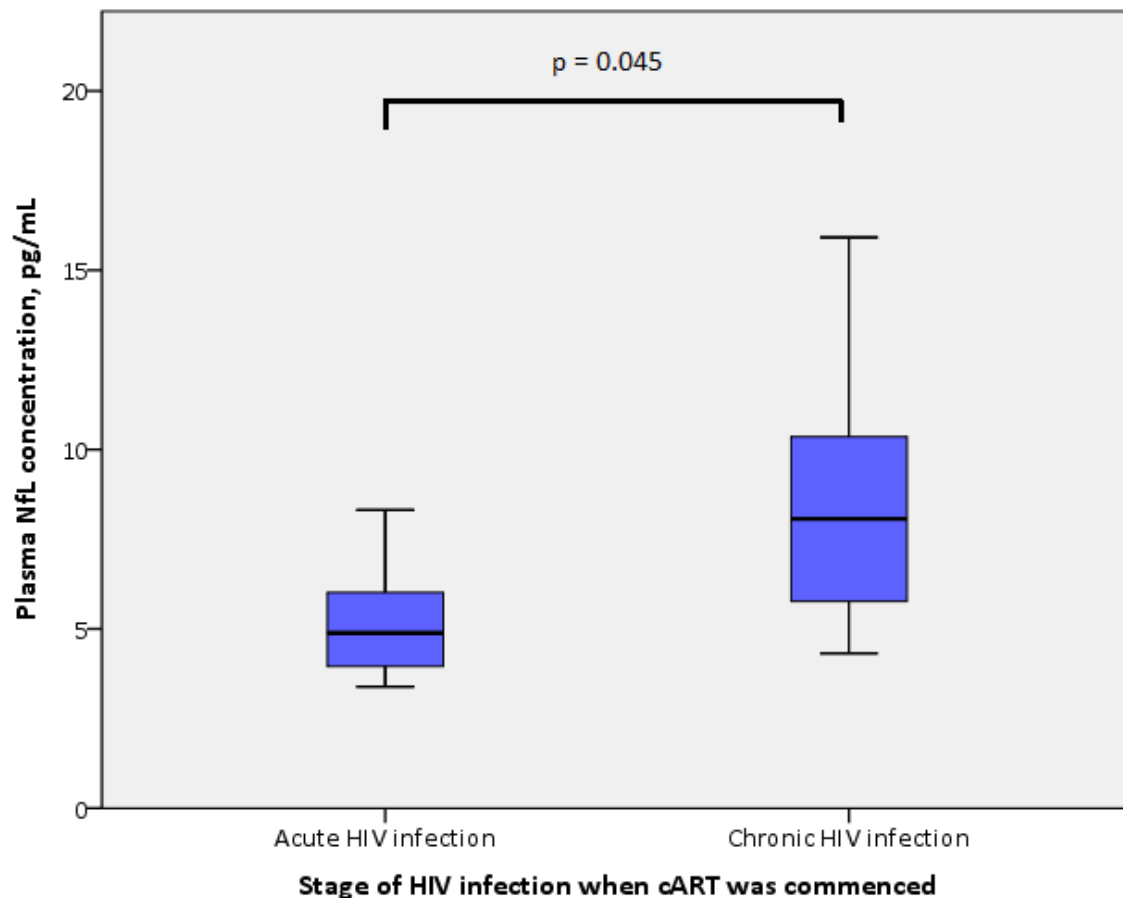
Figure 69: CSF NfL concentration in participants who commenced cART during acute HIV infections and participants who commenced cART during chronic HIV infection



Abbreviations: cART = combination antiretroviral treatment; CSF = cerebrospinal fluid; NfL = neurofilament light chain protein

Legend: P-value calculated using the Mann-Whitney U test. Number of participants according to stage when ART was initiated: acute HIV infection (n = 7), chronic HIV infection (n = 3). Error bars represent 95% confidence intervals

Figure 70: Plasma NfL concentration in participants who commenced cART during acute HIV infections and participants who commenced cART during chronic HIV infection



Abbreviations: cART = combination antiretroviral treatment; NfL = neurofilament light chain protein

Legend: P-value calculated using the Mann-Whitney U test. Number of participants according to stage when ART was initiated: acute HIV infection (n = 9), chronic HIV infection (n = 6). Error bars represent 95% confidence intervals

5.3.6 Cognitive testing results

Cognitive function test Z-score results were calculated using the complete cohort (n = 20) of participants enrolled into both the PET in AHI study and PET in CHI study, as the reference population. Results from the cognitive function test Z-scores for the seven cognitive domains, the three cognitive domain combinations (as specified by the manufacturer) and the global composite scores are presented in Table 28 and Figure 71, stratified according to participants who initiated cART during acute HIV infection and participants who initiated cART during chronic HIV infection.

Mean cognitive function z-scores in the seven cognitive domains and the three cognitive domain combinations, and median global composite T-score were consistently higher in

participants who initiated cART during acute HIV infection compared to participants who initiated cART during chronic HIV infection (Table 28). Statistically significant differences in z-scores between the study groups were seen in the visual motor processing domain (GMCT) (mean (standard deviation (SD)) 0.77 (0.75) vs -0.63 (0.70), $p = 0.001$), the executive function domain (GMLT) (mean (SD) 0.42 (0.49) vs -0.34 (1.19), $p = 0.02$), the combined 'working memory (ONB) and executive function (GMLT)' domains (mean (SD) 0.22 (0.27) vs -0.18 (0.57)) and global composite cognitive function T-score (median (interquartile range) 55.9 (47.3, 56.7) vs 46.7 (45.8, 49.5), $p = 0.03$) (Table 28).

Table 28: Cognitive function test results for participants who initiated cART during acute HIV infection versus chronic HIV infection

CogState™ neuropsychological test	Cognitive function test Z-score results normalized to the total cohort of participants enrolled into the PET in HIV studies		p-value
	Participants who initiated cART during acute HIV infection n = 9	Participants who initiated cART during chronic HIV infection n = 11	
Visual memory (CPAL)	0.24 (0.53)	-0.20 (1.26)	0.65
Psychomotor speed (DET)	0.06 (1.25)	-0.05 (0.80)	0.45
Visual motor processing (GMCT)	0.77 (0.75)	-0.63 (0.70)	0.001*
Executive function (GMLT)	0.42 (0.49)	-0.34 (1.19)	0.02*
Attention (IDN)	0.36 (1.18)	-0.29 (0.76)	0.09
Visual learning (OCL)	0.06 (0.75)	-0.05 (1.20)	0.85
Working memory (ONB)	0.01 (0.11)	-0.01 (0.09)	0.99
Psychomotor, attention and visual motor processing combined (DET + IDN + GMCT)	0.40 (0.98)	-0.32 (0.49)	0.07
Visual learning and visual memory combined (OCL + CPAL)	0.15 (0.34)	-0.13 (0.77)	0.21
Working memory and executive function combined (ONB + GMLT)	0.22 (0.27)	-0.18 (0.57)	0.02*
Global composite T-score ¹	55.9 (47.3, 56.7)	46.7 (45.8, 49.5)	0.03*

Abbreviations: cART = combination antiretroviral treatment, CPAL = Continuous Paired Associate Learning Test, DET = Detection, GMCT = Groton Maze Chase Test, GMLT = Groton Maze Learning Test, IDN = Identification, OCL = One Card Learning Test, ONB = One Back Test

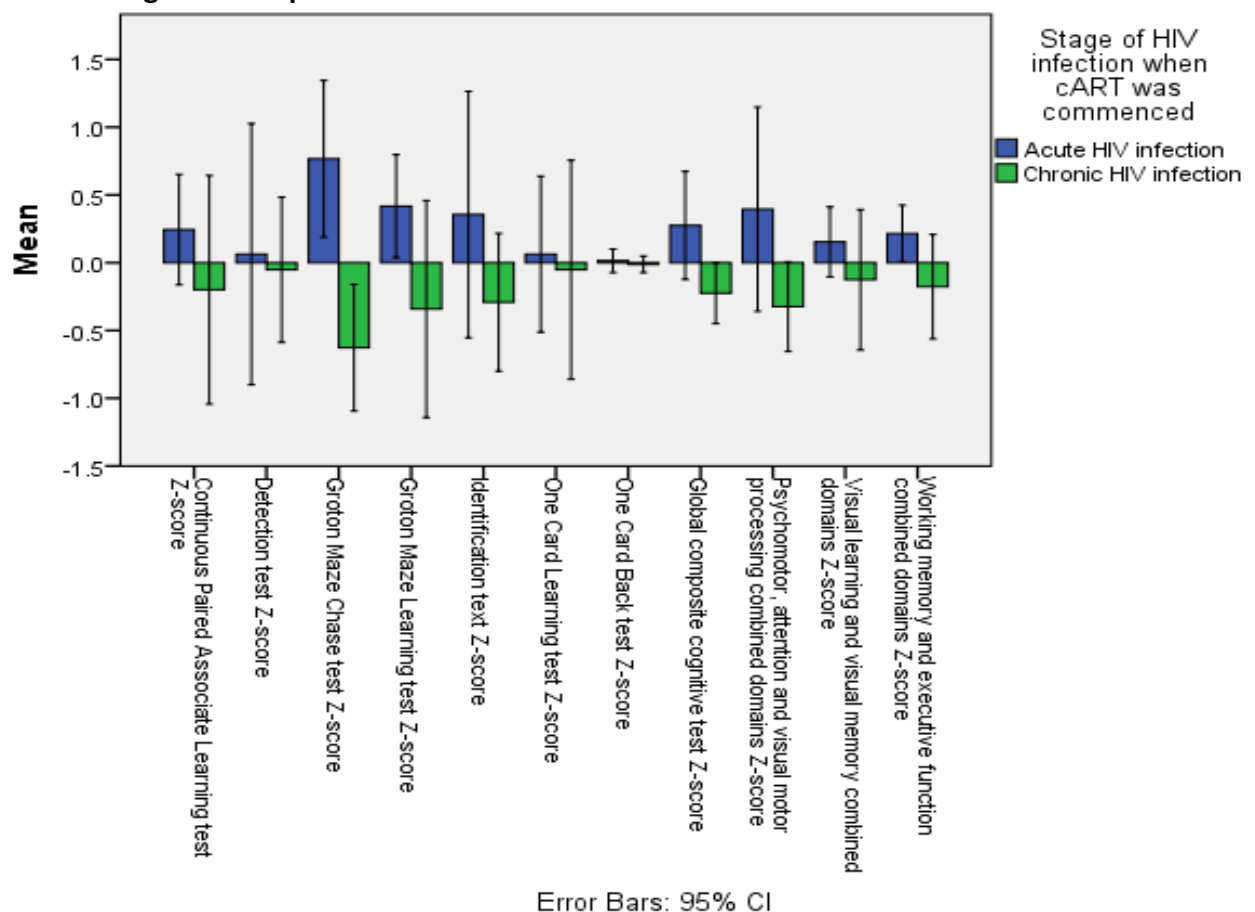
Results are mean (standard deviation) unless stated otherwise.¹ Results are median (interquartile range).

Legend: P-values calculated using the Mann-Whitney U test. Number of participants according to stage when ART was initiated: acute HIV infection (n = 9), chronic HIV infection (n = 11)

* denotes results of statistical significance.

The bar chart in Figure 71 illustrates the mean and 95% confidence interval results for cognitive function Z-scores in the seven separate cognitive domains, the global composite Z-score and the three combinations of domain, stratified according to participants who initiated cART during acute versus chronic HIV infection. Overall, mean cognitive Z-scores was consistently higher in participants who initiated cART during acute HIV infection in all the domains investigated. The 95% confidence intervals in the two groups overlapped in all the testing domains apart from the visual motor processing (GMCT) test, where participants who initiated cART during acute HIV infection had distinctly higher mean Z-score compared to those who initiated cART during chronic HIV infection (Figure 71).

Figure 71: Cognitive function test Z-score results for the individual domains, combined domains and global composite score



Abbreviations: cART = combination antiretroviral treatment, CPAL = Continuous Paired Associate Learning Test (CPAL), DET = Detection, GMCT = Groton Maze Chase Test, IDN = Identification, OCL = One Card Learning Test, ONB = One Back Test

Legend: Bar chart representing the mean cognitive function test Z-score results for seven separate domains, the global composite Z-score and the average score from three combinations of domains. Number of participants according to stage when ART was initiated: acute HIV infection (n = 9), chronic HIV infection (n = 11)

5.4 Discussion

In this chapter, I aimed to address my first hypothesis that smaller HIV reservoir size is associated with lower measures of neural activation. There is convincing evidence to support the notion that commencing cART earlier has beneficial effects on limiting HIV reservoir size[99]. Thus, **I compared a neural activation marker** (brain [^{11}C] PBR28 radioligand binding, an *in vivo* biomarker of microglial activation) **in people who initiated cART during acute HIV infection (who have smaller HIV reservoir size) versus people who initiated cART during chronic HIV infection (who have larger HIV reservoir size) versus control participants (who by definition, have no HIV reservoir).**

Using the [^{11}C] PBR28 total volume of distribution (V_T) and the [^{11}C] PBR28 V_T binding normalised to cortical grey matter ($V_{T(DVR)}$) methods of reporting brain [^{11}C] PBR28 binding, I observed **no statistically significant differences in regional brain [^{11}C] PBR28 binding between control participants, people who initiated cART during chronic HIV infection and people who initiated cART during acute HIV infection.** Thus, I had insufficient evidence to confirm my first hypothesis and **I was unable to corroborate my first hypothesis** that lower HIV reservoir size is associated with lower biomarkers of neural activation. An important limitation that needs to be taken into account when interpreting my findings is the small sample size of participants in the three groups included in this analysis which may have prevented the detection of significant regional differences in brain [^{11}C] PBR28 binding between the three groups of participants.

My findings are in contrast with the published results from the PET in CHI study, which observed higher [^{11}C] PBR28 ($V_{T(DVR)}$) binding in specific brain regions of HIV-positive participants, compared to control participants[210]. Several reasons may explain this discrepancy; firstly, the same control participants' data used in the PET in CHI study analysis were not available for me to use in my analysis. Secondly, Vera *et al* only included brain [^{11}C] PBR28 signals from grey matter[210], as at the time, it was felt that this would be the optimal strategy of identifying microglial activation. However, given that HIV disease affects both the grey and white matter throughout the brain, I have included brain [^{11}C] PBR28 signals from both grey and white matter in my analysis. The PET in HIV study performed by Vera *et al* included participants who were both high- ($n = 8$) and medium-affinity binders (n

= 4), and affinity binding status was adjusted for as a fixed factor in the statistical analysis, whereas all the participants included in my analysis for my PhD thesis were high affinity binders. Finally, while Vera *et al* estimated the continuous [^{11}C] PBR28 plasma-to-blood ratio using a constant model in the plasma input function[210], I observed that an exponential-approaching-constant model provided a much better fit for the data in the majority of the participants in my analysis.

The question of whether to use [^{11}C] PBR28 V_T binding or [^{11}C] PBR28 V_T binding normalised to a reference region to report regional brain [^{11}C] PBR28 binding remains hotly debated. Some studies have suggested a poor test-retest reproducibility with TSPO V_T binding due to intra-individual factors, such as normal physiology, stress responses, hormone-mediated changes in TSPO expression, blood cholesterol changes due to food intake and other genotypic factors that may affect TSPO radioligand uptake in the brain[317,417–419]. Several independent test-retest studies have indicated a trend towards higher TSPO V_T binding in the afternoons compared to the mornings[317,420]. This preliminary finding may be related to the diurnal changes in immune cells and cytokines[421] which can affect TSPO expression, hence indicating that diurnal changes in TSPO binding may be a potential confounder in clinical studies that warrants further investigation.

A study using [^{11}C] DPA-713, a second-generation TSPO radioligand, demonstrated poor test-retest reproducibility of [^{11}C] DPA-713 V_T binding, and significantly improved test-retest reproducibility when [^{11}C] DPA-713 V_T binding was normalised to a reference region, with the best test-retest reproducibility seen when normalised to total grey matter[317]. Nevertheless, a major concern with normalising TSPO binding to a reference region is losing the ability to detect changes in the region used as the reference region, by effectively cancelling out the signal in the region of interest by normalising to another region also displaying a signal. Ideally, the reference region chosen should be unaffected by the disease under investigation. However, HIV disease generally affects the whole central nervous system, and a reliable disease-free brain TSPO reference region in people with HIV has not been identified. Brain [^{11}C] PBR28 V_T binding once normalised to cortical grey matter ($V_{T(DVR)}$) caused blunting of signals when comparing the three groups of participants, suggesting that [^{11}C] PBR28 V_T signals are suppressed when normalised to cortical grey matter in this setting.

I observed that plasma NfL (a marker of neuro-axonal injury was lower and cognitive performance (global composite score, visual motor processing, executive function and combined working memory and executive function domains) were higher in people who initiated cART during acute HIV infection (who presumably have smaller HIV reservoirs) versus chronic HIV infection, thus **corroborating my first hypothesis that lower markers of neural activation are associated with lower HIV reservoir size**. However, median plasma NfL results in both groups were within normal range[293], despite statistically significant differences in plasma NfL between the study groups. While cerebrospinal fluid NfL was numerically but not statistically significantly higher in participants who initiated cART during chronic HIV infection, the participants' individual cerebrospinal fluid NfL results were considered normal upon adjusting for age[285]. My results demonstrated that average cognitive function test results were higher in all the domains tested in people who initiated cART during acute HIV infection, with statistically significant differences noted in the visual motor processing and executive function domains, and in the global composite T-score. While all the participants included in the PET in AHI and PET in CHI studies were deemed neuro-asymptomatic, the clear distinction seen in cognitive function scores across the domains between the two groups of participants is interesting and warrants further longitudinal investigation and adjustment for confounders. The cognitive function z-scores were calculated using the two HIV-positive groups of participants as the reference population, thus age, which was not adjusted for, may be a confounding factor.

Several other limitations may be relevant in the interpretation of my findings and need to be addressed. Overall, participants who initiated cART during chronic HIV infection were older and had higher body weight upon enrolment into the study, which may reflect the normal ageing processes. While control participants were on average younger than both groups of HIV-positive participants, 5/15 of the control participants were much older than all the other participants in the control group and in the two HIV-positive groups and were aged between 57 – 63 years. To date, published findings on the effect of age on TSPO expression have been inconclusive with some studies demonstrating higher TSPO binding with increasing age [422–426] and no effect in others[427–429]. Thus, it is possible that the numerically higher mean [^{11}C] PBR28 V_T binding in the control participants may be driven by the five older participants in the control arm. However, the other 10/15 control participants

were aged between 20 – 38, and the median age in the control group was lower than both the HIV-positive groups. Body mass index has been shown to negatively correlate with brain [^{11}C] PBR28 V_T binding[426], and while complete body mass index data was not available for my analysis, body weight data was available. My results demonstrating numerically lower mean [^{11}C] PBR28 V_T binding in the group of participants who initiated cART during chronic HIV infection (who overall, had higher body weight) compared to the control group are in keeping with published findings demonstrating a negative correlation between body mass index and [^{11}C] PBR28 V_T binding[426].

All the HIV-positive participants were male, whereas 4/15 control participants were female. Female participants were excluded from the 'PET in AHI' and 'PET in CHI' studies due to the unknown teratogenicity risks of [^{11}C] PBR28, as per ethics committee recommendations at the time. Once again, the published data on the effects of gender on TSPO binding to date have been contradictory with one study showing no effect of sex on [^{11}C] PBR28 V_T binding[424] and one study observing higher TSPO binding in females compared to males[426]. Thus, in my analysis, the female participants in the control group may have influenced the numerically higher mean [^{11}C] PBR28 V_T binding in the control group. Age, gender and body mass index were not adjusted for in my analysis. The majority of participants enrolled into the two PET in AHI and PET in CHI studies were men of white ethnicity who acquired HIV via sex between men and living in London, UK. Thus, the applicability of the results from this chapter to other populations such as women, or people in low and middle-income settings, is unclear.

Other potential confounders which may affect the results of my analysis were not considered, such as alcohol intake, recreational drug use and smoking history. While overt drug and alcohol dependency were contraindications to taking part in the study, occasional alcohol intake and recreational drug use was permitted, and the impact this may have on the markers of neural activation in this setting is unknown. Details on co-morbidities were collected at screening but undiagnosed co-infections such as hepatitis C or syphilis would not have been accounted for.

Astrocytes and microglia play very different roles in the central nervous system but emerging data suggests that reactive astrocytes may also have a contributory role to TSPO

expression signals[430] and it is becoming increasingly recognised that [^{11}C] PBR28 binding can represent pro-, anti- and mixed inflammatory phenotypes[497]. Thus, TSPO expression signals in this setting should be interpreted with caution and further studies are warranted to determine the phenotype (pro-, anti- and mixed inflammatory) of the signals identified and the cell type contributing to the [^{11}C] PBR28 binding signals (microglia and astrocytes), by correlating brain TSPO binding results with neuropathological specimens with histochemistry and fluid biomarkers.

PET-CT neuroimaging is expensive, and while consistent with other TSPO brain imaging studies, the sample size of participants in the PET in AHI and PET in CHI studies included in this analysis was small, and the sample size of participants with laboratory parameter measurements (cerebrospinal and plasma NFL) available was even smaller. Parametric brain [^{11}C] PBR28 V_T binding maps were not normalised to a standard scale, thus visual differences in degree of brain [^{11}C] PBR28 V_T binding between participants could not be directly compared. HIV testing was not a standard requirement for inclusion as a control participant, thus it is possible that participants with undiagnosed/ untreated HIV were included in the control participant cohort. Complete normative data for all seven cognitive domains tested was not available, thus cognitive function z-scores were generated using normative data from the combined participants in the PET in AHI and PET in CHI studies. When normative data for all seven cognitive domains becomes available, it would be interesting to re-evaluate the cognitive test results of the HIV-positive participants in the ‘PET in AHI’ and ‘PET in CHI’ studies using the general population age-matched normative data scores to generate z-scores, given these are neuro-asymptomatic cohorts. The HIV-positive participants were not randomised to the two groups, thus it is possible that the two groups of participants have other unmeasured confounders apart from the stage at which they initiated cART, which may impact our data and analysis. At study entry, I observed that while current CD4^+ T-cell counts were similar between the two groups of HIV-positive participants, participants who initiated cART during chronic HIV infection had lower nadir CD4^+ T-cell count, and lower $\text{CD4}^+/\text{CD8}^+$ ratio and higher CD8^+ T-cell counts despite much longer durations on cART. The persistently expanded CD8^+ T-cell compartment infers an aberration of CD8^+ T-cell function[431] and may have important implications for the development and maintenance of immune activation and inflammation in PLWH[432].

Ideally, to reduce confounding effects, the control participants and the two groups of HIV-positive participants (individuals who initiated cART during acute versus chronic HIV infection) should be matched for age, gender, weight, co-morbidities and lifestyle behaviours. In the case of the HIV-positive participants, antiretroviral drug regimen (historical and current) should also be similar between the two groups.

To date, the full extent of what increased TSPO expression indicates is not fully understood, and [^{11}C] PBR28 does not differentiate between anti- and pro-inflammatory states of microglial activation. Thus, there is an urgent need for immune cell markers with improved functional specificity that can differentiate between anti- and pro-inflammatory cell phenotypes.

5.5 Conclusion

In this chapter, I observed no significant differences in [^{11}C] PBR28 brain binding between control participants, people who initiated cART during acute HIV infection (when HIV reservoir size is generally lower) and people who initiated cART during chronic HIV infection (when HIV reservoir size is generally higher). Thus, I was unable to confirm my first hypothesis. The small sample size of participants included in this analysis may have precluded the detection of significant [^{11}C] PBR28 brain binding differences between the three groups of participants.

Chapter 6:

Cross-sectional analysis of the relationship between HIV reservoir measures and markers of neural activation

Chapter 6: Cross-sectional analysis of the relationship between HIV reservoir measures and markers of neural activation

6.1 Introduction

Studies have shown that markers of systemic immune activation remain persistently elevated in people living with chronic HIV on suppressive cART [433,434], and this has been linked with increased markers of inflammation[435] and increased risk of non-AIDS co-morbidities[146]. In people living with HIV on suppressive cART, chronic immune activation and neuroinflammation in the central nervous system has been observed[150,168,210,405] and is associated with cognitive dysfunction[205], with a reported prevalence rate of 20-50%[157,436,437]. Additional factors associated with the development of cognitive dysfunction in PLWH are extensively described in Chapter 1: Introduction, of my PhD thesis.

The mechanisms preventing full immune recovery following the initiation of cART are not fully understood, but are associated with longer duration of untreated HIV infection and lower nadir CD⁺ T-cell count[416,438,439]. It is thought that the complex interaction between HIV viral factors and immune and inflammatory responses in people living with treated HIV disease may be a contributing factor to the pathogenesis of HIV-associated cognitive dysfunction[158,270]. Early data in support of this hypothesis demonstrates associations between increased peripheral HIV DNA and neurocognitive impairment in people living with treated HIV disease[440–443].

My work in this chapter is a unique addition to the existing data exploring the interaction between viral, immune and inflammatory factors with cognitive function because I include a novel neuroimaging technique that identifies brain microglial activation. In this chapter, I aimed to address both my PhD hypotheses; firstly that **lower biomarker concentrations of HIV reservoir size** (using **total HIV DNA per circulating CD4⁺ T-cell** as a surrogate measure of HIV reservoir size) are associated with **lower biomarker measures of neural activation** (as

measured by **markers of microglial activation (radiolabelled [¹¹C] PBR28 TSPO binding on PET brain imaging), neuro-axonal injury and cognitive function)**, and that **increased biomarker concentrations of HIV viral transcription (as measured by ultra-sensitive plasma HIV RNA)** are associated with **higher biomarker concentrations of neural activation (as measured by markers of microglial activation (radiolabelled [¹¹C] PBR28 TSPO binding on PET brain imaging), neuro-axonal injury and cognitive function)**.

Increased biomarker evidence of microglial activation is seen in neuro-asymptomatic PLWH on virologically suppressive cART[48,210,211], however less data is available investigating the precise factors associated with increased microglial activation in this setting. Thus, I sought to further **characterise the associations between microglial activation (as measured by radiolabelled [¹¹C] PBR28 TSPO binding on PET brain imaging)** with **age, HIV-specific clinical and immunological factors, biomarkers of central nervous system injury, cognitive function and antiretroviral agents**.

6.2 Methods

6.2.1 Clinical studies

The ‘PET in AHI’ and ‘PET in CHI’ studies are described in detail in Chapter 2 and Chapter 5 of my PhD thesis. Data on HIV-specific clinical and immunological factors from participants enrolled in the ‘PET in AHI’ and ‘PET in CHI’ studies were obtained from the participants’ electronic hospital medical records, with participant consent. This data includes including current cART regimen, most recent CD4⁺ and CD8⁺ T-cell counts, nadir CD4⁺ T-cell count, pre-treatment plasma HIV RNA load, most recent plasma HIV RNA load, date of HIV diagnosis and date when participant commenced cART.

Data on brain [¹¹C] PBR28 binding on PET imaging in control participants were obtained from previous studies conducted at the Imanova Centre for Imaging Sciences, as described in Chapter 2: Materials and Methods.

6.2.2 Previously described biomarker analysis

The methodologies used for analysing brain [¹¹C] PBR28 ligand binding on PET imaging, cerebrospinal fluid and plasma NfL and cognitive function test scores from participants

enrolled in the 'PET in AHI' and 'PET in CHI' studies are described in Chapter 2 and Chapter 5 of my PhD thesis. Cerebrospinal fluid protein and white cell count were measured at the Clinical Biochemistry and Microbiology laboratories at Imperial College Healthcare NHS Trust, London. Given the global blunting of signals seen once [^{11}C] PBR28 V_T binding was normalised to cortical grey matter (DVR), I elected to analyse associations with regional brain [^{11}C] PBR28 V_T binding.

6.2.3 Statistical analysis

Due to the data being not Normally distributed, Spearman's rank correlation was utilised to analyse associations between cerebral [^{11}C] PBR V_T binding and age in the:

- two HIV-positive cohorts combined ('PET in AHI' and 'PET in CHI')
- control participants cohort
- three cohorts combined ('PET in AHI', 'PET in CHI' and control participants)

In the two cohorts combined ('PET in AHI' and 'PET in CHI'), Spearman's rank correlation was used to analyse the associations between cerebral [^{11}C] PBR V_T binding on PET-CT and:

- HIV-specific clinical parameters: duration since HIV diagnosis, duration since commencing cART, duration between HIV diagnosis and cART commencement (i.e., duration of known untreated HIV disease) and pre-treatment HIV RNA load
- HIV-specific immunological parameters: most recent CD4⁺ count, most recent CD8⁺ count, most recent CD4/CD8 ratio and nadir CD4⁺ count)
- biomarkers of central nervous system injury (cerebrospinal fluid NfL, plasma NfL, cerebrospinal fluid white cell count and cerebrospinal fluid protein)
- cognitive function test scores

The analysis in this chapter is predominantly exploratory and adjustments for multiple hypothesis testing were not performed at this stage. Based on these initial exploratory analyses, further statistical analyses will be performed to identify associations with regional

[¹¹C] PBR28 binding, prior to manuscript submission. Differences in [¹¹C] PBR V_T binding on PET-CT at the 30 pre-selected regions of interest by antiretroviral agent class (NNRTI, PI and INSTI) were analysed using the Kruskal-Wallis test. Where significant associations ($p < 0.05$) were observed, I planned to use the Mann-Whitney U test to identify significant differences in [¹¹C] PBR V_T binding on PET-CT, between the three antiretroviral classes (NNRTI vs PI, NNRTI vs INSTI, PI vs INSTI). Non-parametric tests were utilised throughout this analysis as these tests do not make assumptions with regards to the distribution of the data.

6.3 Results

6.3.1 Associations between total HIV DNA concentrations in circulating CD4⁺ T-cells and regional brain [¹¹C] PBR28 V_T binding on PET imaging

I intended to investigate the associations between total HIV DNA concentrations in circulating CD4⁺ T-cells isolated from peripheral blood mononuclear cells with regional [¹¹C] PBR28 V_T binding. However, due to restrictions to laboratory access during the COVID-19 pandemic, I was unable to perform the measurement of total HIV DNA concentrations and hence unable to investigate the associations between regional brain [¹¹C] PBR28 binding on PET imaging with total HIV DNA concentrations.

6.3.2 Associations between plasma and cerebrospinal fluid HIV RNA loads and regional brain [¹¹C] PBR28 V_T binding on PET imaging

Where measured, all the participants included in this study had plasma and cerebrospinal fluid HIV RNA loads below the limit of detection using conventional assays for clinical purposes (<20 copies HIV RNA/mL). Due to the COVID-19 pandemic, I have been unable to access the Molecular Diagnostics Unit laboratory at Imperial College London to perform ultra-sensitive HIV RNA measurements on the participants' plasma and cerebrospinal fluid samples. Thus, I was unable to investigate the associations between regional brain [¹¹C]

PBR28 binding on PET imaging with ultra-sensitive plasma and cerebrospinal fluid HIV RNA loads.

6.3.3 Associations between [^{11}C] PBR28 V_T binding with age

In the cohort of HIV-positive participants, higher brain [^{11}C] PBR28 V_T binding was associated with younger age at all the pre-specified regions of interest (Table 29). Negative correlations between age and [^{11}C] PBR28 V_T binding were statistically significant at the caudate ($r = -0.535$, $p = 0.015$), left caudate ($r = -0.527$, $p = 0.017$) and right caudate ($r = -0.492$, $p = 0.028$) only (Table 29).

In the cohort of control participants, higher brain [^{11}C] PBR28 V_T binding was associated with older age at most of the regions of interest, with significant correlations noted at the amygdala ($r = 0.526$, $p = 0.044$), cingulate cortex ($r = 0.529$, $p = 0.042$), anterior cingulate ($r = 0.528$, $p = 0.040$), putamen ($r = 0.533$, $p = 0.041$) and right putamen ($r = 0.530$, $p = 0.044$) (Table 29).

In the combined cohort of HIV-positive and control participants, higher regional brain [^{11}C] PBR28 V_T binding was generally associated with older age in most of the regions of interest, apart from the parietal lobe, caudate, left caudate and right caudate, where higher regional brain [^{11}C] PBR28 V_T binding was associated with younger age (Table 29). However, no statistically significant correlations were observed in this combined cohort with $p > 0.05$ at all the regions of interest.

Table 29: Associations between [¹¹C] PBR28 VT binding with age

Region of interest	Correlation coefficient, rho		
	Age (HIV-positive participants only) n = 20	Age (Control participants only) n = 15	Age (HIV-positive participants and control participants, n = 35)
Whole brain	-0.160	0.271	0.056
Occipital lobe	-0.178	0.201	0.05
Insular cortex	-0.085	0.513	0.198
Temporal lobe	-0.121	0.493	0.163
Amygdala	-0.054	0.526*	0.195
Hippocampus	-0.033	0.437	0.152
Frontal lobe	-0.227	0.177	-0.006
Cingulate cortex	-0.085	0.529*	0.196
Posterior cingulate gyrus	-0.067	0.468	0.162
Anterior cingulate	-0.078	0.528*	0.196
Parietal lobe	-0.242	-0.047	-0.082
Subcortical matter	-0.197	0.405	0.079
Basal ganglia	-0.245	0.446	0.127
Globus pallidus	-0.194	0.446	0.135
Striatum	-0.273	0.446	0.094
Left striatum	-0.224	0.432	0.086
Right striatum	-0.257	0.405	0.091
Caudate	-0.535*	0.062	-0.238
Left caudate	-0.527*	-0.019	-0.291
Right caudate	-0.492*	0.294	-0.2
Putamen	-0.025	0.533*	0.251
Left putamen	0.009	0.506	0.26
Right putamen	-0.041	0.530*	0.249
Thalamus	-0.201	0.307	0.012
Cerebellum	-0.139	0.45	0.138
Brainstem	-0.056	0.426	0.178
Midbrain	-0.063	0.379	0.111
Pons	-0.066	0.407	0.175
Medulla	-0.198	0.49	0.144
Cerebral white matter	-0.160	0.419	0.151

Legend: Correlations were analysed using Spearman's rank correlation. Blue signifies a positive correlation, red signifies a negative correlation. Darker shades represent stronger correlations. Key findings: Higher regional brain [¹¹C] PBR28 VT binding was generally associated with younger age in the HIV-positive participants, older age in the control participants and older age in the combined cohort of HIV-positive and control participants.

* denotes statistically significant correlations (p<0.05).

6.3.4 Associations between [¹¹C] PBR28 V_T binding with i) duration since HIV diagnosis and ii) duration since commencing cART

No statistically significant correlations were observed between [¹¹C] PBR28 V_T binding with duration since HIV diagnosis and duration since commencing cART at any of the regions of interest (Table 30).

Table 30: Association between [¹¹C] PBR28 V_T binding with duration since HIV diagnosis (n = 19) and duration since commencing cART (n=19)

Region of interest	Correlation coefficient, rho	
	Duration since HIV diagnosis, n = 19	Duration since commencing cART, n = 19
Whole brain	0.066	-0.091
Occipital lobe	0.122	-0.015
Insular cortex	0.105	-0.021
Temporal lobe	0.081	-0.046
Amygdala	0.050	-0.075
Hippocampus	0.115	-0.039
Frontal lobe	-0.005	-0.155
Cingulate cortex	0.083	-0.060
Posterior cingulate gyrus	0.068	-0.075
Anterior cingulate	0.079	-0.066
Parietal lobe	-0.007	-0.169
Subcortical matter	-0.051	-0.174
Basal ganglia	-0.100	-0.228
Globus pallidus	-0.028	-0.134
Striatum	-0.097	-0.220
Left striatum	-0.074	-0.205
Right striatum	-0.081	-0.199
Caudate	-0.337	-0.440
Left caudate	-0.230	-0.288
Right caudate	-0.331	-0.454
Putamen	0.076	-0.054
Left putamen	0.140	0.012
Right putamen	0.119	-0.006
Thalamus	-0.040	-0.154
Cerebellum	0.076	-0.047
Brainstem	0.075	-0.039
Midbrain	0.083	-0.011
Pons	0.074	-0.032
Medulla	-0.097	-0.252
Cerebral white matter	-0.047	-0.195

Legend: Correlations were analysed using Spearman's rank correlation. Blue signifies a positive correlation, red signifies a negative correlation. Darker shades represent stronger correlations. Key finding: No statistically significant associations were seen, $p > 0.05$ at all regions of interest.

6.3.5 Associations between [^{11}C] PBR28 V_T binding with i) duration of known untreated HIV disease and ii) pre-treatment HIV RNA load

Higher [^{11}C] PBR28 V_T binding was associated with longer duration of known untreated HIV disease at most of the regions of interest (Table 31). Specifically, the correlations between higher [^{11}C] PBR28 V_T binding and longer duration of known untreated HIV disease were statistically significant across the whole brain ($r = 0.496$, $p = 0.031$) and at the temporal lobe ($r = 0.460$, $p = 0.048$), frontal lobe ($r = 0.477$, $p = 0.039$), cingulate cortex ($r = 0.474$, $p = 0.041$), posterior cingulate gyrus ($r = 0.465$, $p = 0.045$), anterior cingulate ($r = 0.511$, $p = 0.026$), parietal lobe ($r = 0.458$, $p = 0.049$) and cerebral white matter ($r = 0.472$, $p = 0.041$). No statistically significant associations were seen between [^{11}C] PBR28 V_T binding and pre-treatment HIV RNA load at any of the 30 regions of interest (Table 31).

Table 31: Association between [^{11}C] PBR28 VT binding with duration of known untreated HIV disease and pre-treatment HIV RNA load

Region of interest	Correlation coefficient, rho	
	Duration of known untreated HIV disease n = 19	Pre-treatment HIV RNA load n = 17
Whole brain	0.496*	0.127
Occipital lobe	0.428	0.159
Insular cortex	0.371	0.208
Temporal lobe	0.460*	0.135
Amygdala	0.261	0.176
Hippocampus	0.440	0.184
Frontal lobe	0.477*	0.127
Cingulate cortex	0.474*	0.081
Posterior cingulate gyrus	0.465*	0.061
Anterior cingulate	0.511*	0.125
Parietal lobe	0.458*	0.142
Subcortical matter	0.306	0.115
Basal ganglia	0.356	0.145
Globus pallidus	0.364	-0.037
Striatum	0.356	0.186
Left striatum	0.306	0.216
Right striatum	0.377	0.169
Caudate	0.254	0.245
Left caudate	0.173	0.221
Right caudate	0.363	0.211
Putamen	0.357	0.123
Left putamen	0.373	0.103
Right putamen	0.426	0.100
Thalamus	0.343	0.135
Cerebellum	0.423	0.164
Brainstem	0.343	0.123
Midbrain	0.289	0.015
Pons	0.389	0.127
Medulla	0.188	0.240
Cerebral white matter	0.472*	0.081

Legend: Correlations were analysed using Spearman's rank correlation. Blue signifies a positive correlation, red signifies a negative correlation. Darker shades represent stronger correlations.

*Key findings: Higher regional brain [^{11}C] PBR28 V_T binding was generally associated with longer duration of known untreated HIV disease (time between HIV diagnosis and cART commencement) and higher pre-treatment HIV RNA load. * denotes statistically significant correlations ($p < 0.05$).*

6.3.6 Associations between [^{11}C] PBR28 V_T binding with markers of immune system function: i) most recent CD4^+ count, ii) most recent CD8^+ count, iii) most recent $\text{CD4}/\text{CD8}$ ratio and iv) nadir CD4^+ count

Overall, higher [^{11}C] PBR28 V_T binding at most of the regions of interest were associated with lower most recent CD4^+ count, lower most recent current CD8^+ count and lower nadir CD4^+ count (Table 32). [^{11}C] PBR28 V_T binding poorly correlated with most recent $\text{CD4}/\text{CD8}$ ratio (Table 32). Of the four markers of immune system function, the degree of negative correlation was most apparent between [^{11}C] PBR28 V_T binding and current CD8^+ count (Table 32). However, none of the correlations between [^{11}C] PBR28 V_T binding and any of the four markers of immune system function were statistically significant; $p > 0.05$ at all the regions of interest (Table 32).

6.3.7 Associations between [^{11}C] PBR28 V_T binding with biomarkers of central nervous system injury: i) cerebrospinal fluid NfL, ii) plasma NfL, iii) cerebrospinal fluid white cell count and iv) cerebrospinal fluid protein

No statistically significant correlations were observed between [^{11}C] PBR28 V_T binding with cerebrospinal fluid NfL and plasma NfL at any of the regions of interest, $p > 0.05$ at all regions of interest (Table 33).

Higher cerebrospinal fluid white cell count was associated with higher [^{11}C] PBR28 V_T binding at all the brain regions of interest with statistically significant correlations seen in the subcortical matter ($r = 0.503$, $p = 0.040$), basal ganglia ($r = 0.490$, $p = 0.046$), globus pallidus ($r = 0.551$, $p = 0.022$), striatum ($r = 0.513$, $p = 0.035$), right striatum ($r = 0.525$, $p = 0.030$), left caudate ($r = 0.524$, $p = 0.031$) and thalamus ($r = 0.500$, $p = 0.041$) (Table 33).

Lower cerebrospinal fluid protein was associated with higher [^{11}C] PBR28 V_T binding at all the regions of interest, but no statistically significant correlations were seen, $p > 0.05$ at all regions of interest (Table 33).

Table 32: Association between [^{11}C] PBR28 V_T binding with current CD4 count, current CD8 count, current CD4:CD8 and nadir CD4 count

Region of interest	Correlation coefficient, rho			
	Current CD4 count n = 20	Current CD8 count n = 20	Current CD4:CD8 n = 20	Nadir CD4 count n = 20
Whole brain	-0.132	-0.262	-0.015	-0.194
Occipital lobe	-0.065	-0.175	-0.047	-0.183
Insular cortex	-0.12	-0.210	-0.078	-0.104
Temporal lobe	-0.117	-0.269	-0.006	-0.095
Amygdala	-0.171	-0.260	-0.062	-0.122
Hippocampus	-0.168	-0.145	-0.167	-0.245
Frontal lobe	-0.12	-0.250	0.009	-0.186
Cingulate cortex	-0.119	-0.247	-0.059	-0.150
Posterior cingulate gyrus	-0.149	-0.254	-0.075	-0.185
Anterior cingulate	-0.123	-0.215	-0.087	-0.185
Parietal lobe	-0.129	-0.288	0.042	-0.164
Subcortical matter	-0.152	-0.253	-0.047	-0.101
Basal ganglia	-0.149	-0.269	-0.009	-0.065
Globus pallidus	-0.259	-0.187	-0.140	-0.140
Striatum	-0.17	-0.266	-0.015	-0.083
Left striatum	-0.195	-0.366	0.047	-0.071
Right striatum	-0.144	-0.231	-0.029	-0.084
Caudate	-0.269	-0.186	-0.057	-0.075
Left caudate	-0.138	-0.087	-0.062	-0.021
Right caudate	-0.361	-0.214	-0.122	-0.176
Putamen	-0.126	-0.237	-0.075	-0.096
Left putamen	-0.137	-0.261	-0.068	-0.111
Right putamen	-0.081	-0.155	-0.120	-0.119
Thalamus	-0.171	-0.213	-0.099	-0.123
Cerebellum	-0.141	-0.211	-0.080	-0.102
Brainstem	-0.194	-0.188	-0.152	-0.170
Midbrain	-0.129	-0.202	-0.098	-0.111
Pons	-0.227	-0.169	-0.183	-0.238
Medulla	-0.284	-0.339	-0.008	-0.053
Cerebral white matter	-0.233	-0.281	-0.077	-0.179

Legend: Correlations were analysed using Spearman's rank correlation. Blue signifies a positive correlation, red signifies a negative correlation. Darker shades represent stronger correlations.

Key findings: Higher regional brain [^{11}C] PBR28 V_T binding was generally associated with lower most recent CD4⁺ T-cell count, lower most recent CD8⁺ T-cell count, lower most recent CD4/CD8 ratio and lower nadir CD4⁺ T-cell count. No statistically significant associations were seen, $p > 0.05$ at all regions of interest.

Table 33: Associations between [¹¹C] PBR28 VT binding with CSF NfL, plasma NfL, CSF white cell count and CSF protein

Region of interest	Correlation coefficient, rho			
	CSF NfL	Plasma NfL	CSF white cell count	CSF protein
	n = 10	n = 15	n = 17	n = 17
Whole brain	0.115	-0.129	0.393	-0.411
Occipital lobe	0.115	-0.143	0.466	-0.400
Insular cortex	0.224	0.014	0.431	-0.325
Temporal lobe	0.200	-0.057	0.422	-0.400
Amygdala	-0.006	-0.011	0.403	-0.332
Hippocampus	0.152	-0.039	0.332	-0.252
Frontal lobe	0.018	-0.164	0.445	-0.454
Cingulate cortex	0.200	-0.057	0.378	-0.437
Posterior cingulate gyrus	0.200	-0.079	0.378	-0.437
Anterior cingulate	0.176	-0.011	0.369	-0.414
Parietal lobe	-0.091	-0.143	0.396	-0.434
Subcortical matter	0.030	-0.089	0.503*	-0.442
Basal ganglia	0.030	-0.075	0.490*	-0.433
Globus pallidus	-0.115	-0.104	0.551*	-0.479
Striatum	0.030	-0.100	0.513*	-0.444
Left striatum	-0.030	-0.107	0.459	-0.434
Right striatum	0.030	-0.086	0.525*	-0.434
Caudate	-0.285	-0.286	0.472	-0.444
Left caudate	-0.127	-0.286	0.524*	-0.480
Right caudate	-0.224	-0.196	0.393	-0.421
Putamen	-0.176	-0.007	0.408	-0.340
Left putamen	0.248	0.050	0.356	-0.308
Right putamen	0.261	-0.014	0.457	-0.341
Thalamus	0.115	-0.104	0.500*	-0.447
Cerebellum	0.200	-0.064	0.342	-0.362
Brainstem	0.115	-0.075	0.414	-0.369
Midbrain	0.091	-0.132	0.471	-0.391
Pons	0.091	-0.068	0.462	-0.426
Medulla	-0.091	-0.214	0.150	-0.132
Cerebral white matter	-0.018	-0.082	0.364	-0.468

Legend: Correlations were analysed using Spearman's rank correlation. Blue signifies a positive correlation, red signifies a negative correlation. Darker shades represent stronger correlations.

Abbreviations: CSF = cerebrospinal fluid, NfL = neurofilament light chain protein.

Key findings: Higher regional brain [¹¹C] PBR28 V_T binding was generally associated with lower plasma NfL concentration, higher cerebrospinal fluid white cell count and lower cerebrospinal fluid protein. Regional brain [¹¹C] PBR28 V_T binding correlated poorly with cerebrospinal fluid NfL concentration. * denotes statistically significant correlations (p<0.05).

6.3.8 Associations between [^{11}C] PBR28 V_T binding with cognitive function

Overall, higher brain [^{11}C] PBR28 V_T binding was associated with higher cognitive function z-scores in the following domains: psychomotor speed (DET), attention (IDN), visual motor processing (GMCT) and executive function (GMLT), whereas higher brain [^{11}C] PBR28 V_T binding was associated with lower cognitive function z-scores in the following domains: visual memory (CPAL), visual learning (OCL) and working memory (ONB) (Table 34).

However, no statistically significant associations were noted between the seven cognitive domains and any of the 30 brain regions of interest, $p > 0.05$ at all regions of interest (Table 34).

In terms of the domain combinations, higher brain [^{11}C] PBR28 V_T binding was weakly associated with higher average z-scores in the combined psychomotor speed (DET), attention (IDN) and visual motor processing (GMCT) domains and the combined executive function (GMLT) and working memory (ONB) domains (Table 35), but no statistically significant associations were observed, $p > 0.05$ at all regions of interest (Table 35). On the other hand, higher brain [^{11}C] PBR28 V_T binding was associated with lower average z-scores in the combined visual memory (CPAL) and visual learning (OCL) domains, with the correlation coefficients meeting statistical significance in many of the regions of interest (Table 35).

Generally, cognitive function global composite T-scores correlated poorly with regional brain [^{11}C] PBR28 V_T binding on PET imaging (Table 35).

Table 34: Associations between [¹¹C] PBR28 V_T binding and cognitive function domains

Region of interest	Psychomotor speed n=20	Attention n=20	Visual motor processing n=20	Visual memory n=20	Visual learning n=20	Executive function n=20	Working memory n=20
Whole brain	0.153	0.110	0.068	-0.086	-0.168	0.101	-0.175
Occipital lobe	0.150	0.111	-0.021	-0.149	-0.121	0.080	-0.198
Insular cortex	0.165	0.119	-0.023	-0.167	-0.134	0.095	-0.243
Temporal lobe	0.083	0.082	0.073	-0.093	-0.149	0.117	-0.271
Amygdala	0.306	0.225	0.000	-0.041	-0.324	0.115	-0.107
Hippocampus	0.186	0.033	-0.072	-0.005	-0.285	-0.013	-0.147
Frontal lobe	0.145	0.097	0.098	-0.071	-0.132	0.107	-0.208
Cingulate cortex	0.096	0.059	-0.011	-0.149	-0.206	0.080	-0.226
Posterior cingulate gyrus	0.150	0.081	-0.021	-0.135	-0.243	0.059	-0.182
Anterior cingulate	0.111	0.067	0.013	-0.121	-0.191	0.073	-0.194
Parietal lobe	0.175	0.144	0.138	-0.049	-0.142	0.148	-0.154
Subcortical matter	0.241	0.129	-0.017	-0.142	-0.148	0.071	-0.220
Basal ganglia	0.181	0.120	0.093	-0.084	-0.103	0.097	-0.272
Globus pallidus	0.143	0.114	0.019	-0.056	-0.070	-0.007	-0.287
Striatum	0.191	0.120	0.095	-0.078	-0.063	0.098	-0.280
Left striatum	0.297	0.175	0.091	-0.107	-0.130	0.149	-0.147
Right striatum	0.154	0.108	0.099	-0.035	-0.082	0.090	-0.296
Caudate	0.140	0.023	0.192	0.232	0.080	0.012	-0.300
Left caudate	0.034	0.002	0.102	0.211	0.108	-0.017	-0.318
Right caudate	0.153	0.001	0.194	0.223	0.066	-0.023	-0.252
Putamen	0.196	0.129	-0.035	-0.152	-0.232	0.085	-0.176
Left putamen	0.206	0.164	-0.035	-0.163	-0.273	0.104	-0.155
Right putamen	0.039	-0.014	-0.096	-0.141	-0.190	0.008	-0.296
Thalamus	0.223	0.109	-0.026	-0.121	-0.113	0.040	-0.263
Cerebellum	0.169	0.147	0.082	-0.119	-0.105	0.091	-0.261
Brainstem	0.259	0.176	-0.007	-0.122	-0.208	0.034	-0.185
Midbrain	0.197	0.201	-0.011	-0.114	-0.268	0.067	-0.223
Pons	0.267	0.186	0.004	-0.093	-0.185	0.026	-0.159
Medulla	0.423	0.266	0.208	-0.001	-0.145	0.083	-0.015
Cerebral white matter	0.187	0.105	0.098	-0.047	-0.157	0.081	-0.189

Legend: Data relates to the correlation coefficient, rho, results. Correlations were analysed using Spearman's rank correlation. Key findings: No statistically significant associations were observed, p>0.05 at all regions of interest.

Table 35: Associations between [¹¹C] PBR28 VT binding and cognitive function combined domains

Region of interest	Correlation coefficient, rho			
	Combined psychomotor speed, attention and visual motor processing domains n=20	Combined visual memory and visual learning domains n=20	Combined executive function and working memory domains n=20	Global composite T-score n=20
Whole brain	0.153	-0.441	0.042	0.017
Occipital lobe	0.114	-0.454*	0.012	-0.009
Insular cortex	0.147	-0.514*	0.041	-0.024
Temporal lobe	0.128	-0.460*	0.053	0.003
Amygdala	0.238	-0.522*	0.054	-0.023
Hippocampus	0.078	-0.511*	-0.077	-0.162
Frontal lobe	0.135	-0.408	0.030	0.024
Cingulate cortex	0.093	-0.532*	0.008	-0.062
Posterior cingulate gyrus	0.126	-0.547*	-0.011	-0.063
Anterior cingulate	0.110	-0.499*	0.012	-0.051
Parietal lobe	0.179	-0.376	0.093	0.069
Subcortical matter	0.153	-0.507*	-0.006	-0.018
Basal ganglia	0.158	-0.433	0.029	0.030
Globus pallidus	0.137	-0.394	-0.078	-0.006
Striatum	0.158	-0.405	0.029	0.045
Left striatum	0.227	-0.454*	0.087	0.066
Right striatum	0.140	-0.385	0.015	0.027
Caudate	0.048	-0.054	-0.054	0.045
Left caudate	-0.036	-0.006	-0.086	0.017
Right caudate	0.063	-0.086	-0.071	0.042
Putamen	0.167	-0.564*	0.030	-0.057
Left putamen	0.197	-0.591*	0.051	-0.033
Right putamen	0.012	-0.562*	-0.066	-0.155
Thalamus	0.138	-0.471*	-0.042	-0.024
Cerebellum	0.195	-0.426	0.042	0.041
Brainstem	0.220	-0.519*	-0.023	-0.027
Midbrain	0.211	-0.537*	0.000	-0.012
Pons	0.227	-0.481*	-0.032	-0.012
Medulla	0.371	-0.313	0.101	0.132
Cerebral white matter	0.173	-0.421	0.023	0.009

Legend: Correlations were analysed using Spearman's rank correlation Blue signifies a positive correlation, red signifies a negative correlation. Darker shades represent stronger correlations.

Key findings: Higher regional brain [¹¹C] PBR28 VT binding was statistically significantly associated with lower average z-scores in the combined visual memory and visual learning domains

** denotes statistically significant correlations (p<0.05).*

6.3.9 Associations between [¹¹C] PBR28 V_T binding with antiretroviral agent class

No significant difference in brain [¹¹C] PBR28 V_T binding was observed at any of the regions of interest, when the participants were stratified according to antiretroviral third agent class (p>0.05 at all the regions of interest, as shown in Table 36).

Table 36: [¹¹C] PBR28 V_T binding at regions of interest, with participants stratified according to antiretroviral third agent class

Region of interest	NNRTI N = 5	PI N = 7	INSTI N = 8	p-value ¹
Whole brain	4.36 (1.21)	4.11 (1.19)	3.52 (1.30)	0.302
Occipital lobe	4.50 (1.22)	4.33 (1.18)	3.60 (1.25)	0.236
Insular cortex	4.91 (1.39)	4.42 (1.55)	3.96 (1.57)	0.195
Temporal lobe	4.41 (1.06)	4.16 (1.18)	3.76 (1.37)	0.293
Amygdala	4.95 (1.01)	4.93 (1.61)	4.58 (1.74)	0.522
Hippocampus	4.78 (0.83)	4.65 (1.54)	4.19 (1.53)	0.201
Frontal lobe	4.46 (1.58)	4.12 (1.20)	3.41 (1.33)	0.331
Cingulate cortex	4.75 (1.49)	4.35 (1.47)	3.79 (1.49)	0.259
Posterior cingulate gyrus	4.71 (1.33)	4.32 (1.46)	3.73 (1.46)	0.251
Anterior cingulate	4.78 (1.57)	4.37 (1.48)	3.82 (1.51)	0.224
Parietal lobe	4.30 (1.30)	4.17 (1.16)	3.41 (1.23)	0.423
Subcortical matter	4.74 (1.29)	4.16 (1.47)	3.62 (1.56)	0.201
Basal ganglia	4.45 (1.16)	3.88 (1.28)	3.38 (1.38)	0.218
Globus pallidus	5.44 (1.86)	4.61 (1.66)	4.33 (2.04)	0.418
Striatum	4.32 (1.07)	3.74 (1.26)	3.24 (1.32)	0.201
Left striatum	4.29 (1.08)	3.76 (1.26)	3.24 (1.32)	0.346
Right striatum	4.37 (1.07)	3.75 (1.27)	3.26 (1.32)	0.183
Caudate	4.84 (2.88)	3.04 (1.23)	2.56 (1.17)	0.175
Left caudate	5.14 (3.56)	3.27 (1.26)	2.63 (1.25)	0.185
Right caudate	4.84 (2.70)	3.12 (1.40)	2.57 (1.05)	0.121
Putamen	4.78 (1.50)	4.19 (1.37)	3.60 (1.34)	0.258
Left putamen	4.75 (1.73)	4.26 (1.46)	3.64 (1.29)	0.416
Right putamen	4.97 (1.35)	4.19 (1.32)	3.59 (1.40)	0.085
Thalamus	5.08 (1.36)	4.44 (1.68)	3.86 (1.77)	0.169
Cerebellum	4.49 (1.09)	4.32 (1.27)	3.77 (1.48)	0.411
Brainstem	5.32 (1.03)	5.06 (1.58)	4.50 (1.83)	0.308
Midbrain	5.78 (1.19)	5.48 (1.79)	4.99 (2.30)	0.353
Pons	5.37 (1.14)	5.11 (1.69)	4.43 (1.77)	0.251
Medulla	4.78 (0.61)	4.61 (1.20)	4.66 (2.33)	0.812
Cerebral white matter	4.05 (0.92)	3.76 (1.28)	3.30 (1.13)	0.337

Values are mean (standard deviation). Abbreviations: NNRTI = non-nucleoside reverse transcriptase inhibitors, PI = protease inhibitors, INSTI = integrase strand transferase inhibitors.

¹ Kruskal-Wallis test used to assess for differences in [¹¹C] PBR28 binding when the participants were stratified according to antiretroviral third agent class

Key finding: No significant differences were noted between regional brain [¹¹C] PBR28 V_T binding and antiretroviral third agent class.

6.3.10 Summary of associations with regional brain [^{11}C] PBR28 V_T binding

Higher regional brain [^{11}C] PBR28 V_T binding significantly correlated with:

- younger age in the HIV-positive participants in the caudate, left caudate and right caudate
- older age in the control participants in the amygdala, cingulate cortex, anterior cingulate, putamen and right putamen
- longer duration of known untreated HIV disease (time between HIV diagnosis and cART commencement) across the whole brain, temporal lobe, frontal lobe, cingulate cortex, posterior cingulate cortex, anterior cingulate, parietal lobe and cerebral white matter
- higher cerebrospinal fluid white cell count in the subcortical matter, basal ganglia, globus pallidus, striatum, right striatum, left caudate and thalamus
- lower combined visual memory and visual learning average z-score in the occipital lobe, insular cortex, temporal lobe, amygdala, hippocampus, cingulate cortex, posterior cingulate gyrus, anterior cingulate, subcortical matter, left striatum, putamen, left putamen, right putamen, thalamus, brainstem, midbrain and pons

No significant correlations were observed between regional brain [^{11}C] PBR28 V_T binding with:

- age in the combined cohort of HIV-positive and control participants
- duration between HIV diagnosis and PET imaging
- duration between cART commencement and PET imaging
- pre-treatment HIV RNA load
- most recent CD4^+ T-cell count

- most recent CD8⁺ T-cell count
- most recent CD4/CD8 ratio
- nadir CD4⁺ T-cell count
- cerebrospinal fluid and plasma NfL concentration
- cerebrospinal fluid protein
- psychomotor speed z-score
- attention z-score
- visual motor processing z-score
- visual memory z-score
- visual learning z-score
- executive function z-score
- working memory z-score
- combined psychomotor speed, attention and visual motor processing average z-score
- combined executive function and working memory average z-score
- global composite cognitive function T-score

No significant differences were observed in regional brain [¹¹C] PBR28 V_T binding according to antiretroviral third agent class used by the participants.

6.3.11 Summary of significant associations with regional brain [^{11}C] PBR28 V_T binding

Table 37 summarises the statistically significant associations observed between increased [^{11}C] PBR28 V_T binding at specific brain regions and participant age, HIV-related factors, laboratory parameters and cognitive domain function. Current knowledge indicates that certain brain regions may be associated with function in particular cognitive domains. For example, the basal ganglia is thought to play a role in motor control and executive function[444]. In terms of the cognitive domain functions, none of the associations with increased [^{11}C] PBR28 V_T binding were consistent with typically matched anatomical sites.

Table 37: Summary of significant associations with regional brain [^{11}C] PBR28 V_T binding

Regions of interest	Associations with [^{11}C] PBR28 V_T binding
Whole brain	Longer duration of known untreated HIV disease
Frontal lobe	Longer duration of known untreated HIV disease
Parietal lobe	
Cerebral white matter	
Occipital lobe	Lower average combined visual memory and visual learning z-score
Temporal lobe	Longer duration of known untreated HIV disease
Posterior cingulate gyrus	Lower average combined visual memory and visual learning z-score
Caudate	Lower age in HIV-positive participants
Right caudate	
Left caudate	Lower age in HIV-positive participants Higher cerebrospinal fluid white cell count
Striatum	Higher cerebrospinal fluid white cell count
Globus pallidus	
Right striatum	
Basal ganglia	
Thalamus	Higher cerebrospinal fluid white cell count
Subcortical white matter	Lower average combined visual memory and visual learning z-score
Putamen	Older age in control participants
Right putamen	Lower combined visual memory and visual learning z-score
Left putamen	Lower combined visual memory and visual learning z-score
Left striatum	
Brainstem	
Midbrain	
Pons	
Hippocampus	
Insular cortex	
Cingulate cortex	Older age in control participants
Anterior cingulate	Longer duration of known untreated HIV disease Lower average combined visual memory and visual learning z-score
Amygdala	Older age in control participants Lower average combined visual memory and visual learning z-score

6.4 Discussion

Due to the COVID-19 pandemic, I was unable to gain access to the laboratories where I planned to perform the total HIV DNA and ultra-sensitive HIV RNA measurement, as the laboratories had paused all non-essential research work to focus on COVID-19 related laboratory research, with no estimated date for non-essential research work resumption. Thus, I was unable to address my first and second PhD hypotheses in this chapter, due to the unavailability of total HIV DNA and ultra-sensitive HIV RNA measurement results. When I am granted laboratory access, I plan to measure total HIV DNA in circulating CD4⁺ T-cells, and plasma and cerebrospinal fluid HIV RNA using single-copy assay (SCA) in all participants with available samples, which will then enable me to adequately test both my first and second PhD hypotheses in this chapter.

Several important limitations of the correlation analyses reported in this chapter need to be highlighted and taken into account when interpreting my results. At this stage, the analysis in this chapter is exploratory and the aim is to have a broad overview of the data, hence adjustments for multiple hypothesis testing was not performed. Given the large number of regions of interest under investigation in my exploratory analysis, multiple comparisons may have led to erroneous inferences. Some significant associations may have occurred due to chance in view of the large number of correlational analyses performed and small number of participants included. More in-depth statistical analyses to detect subtle associations with regional [¹¹C] PBR28 signals with adjustments for multiple hypothesis testing will be performed, prior to manuscript submission. The small sample size of participants included in the PET in AHI and PET in CHI studies (limited due to the costly nature of neuroimaging) may have restricted our ability to detect additional significant associations. By increasing the sample size of participants included, statistically significant correlations could be further refined. While significant associations have been observed, unmeasured confounders may have affected the associations seen, and the direction of causality within the correlational analyses cannot be determined. Further longitudinal studies are warranted to investigate the causality between immune activation, neuroinflammation, HIV reservoir size, neural activation and cART regimens with neurological clinical sequelae such as cognitive function. Finally, cART regimen (historical and current) differed between the two groups of HIV-

positive participants and may have conferred a confounding effect. Participants who initiated cART during chronic HIV infection were more treatment-experienced and tended to be on NNRTI- or PI-based cART regimens at the time of PET scanning, whereas participants who initiated cART during acute HIV infection were more likely to have only ever been on one or two combinations of antiretroviral drug regimens and were more likely to be on INSTI-based cART regimens at the time of PET scanning.

To date, published findings on the effect of age on TSPO expression have been inconclusive with some studies demonstrating higher TSPO binding with increasing age [422–425] and no effect in others [427–429]. My findings demonstrate higher regional brain [^{11}C] PBR28 V_T binding with increasing age in control participants, but not in HIV-positive participants, suggesting that HIV-related confounders may impact this association. Possible confounders include durations since HIV diagnosis, duration of untreated HIV disease and antiretroviral toxicities. The precise underlying mechanisms of microglial activation in the normal ageing process remain to be elucidated, but is thought to be related to neurodegeneration, immune senescence and inflammation [445].

I observed higher regional brain [^{11}C] PBR28 V_T binding with longer duration of known untreated HIV disease (which may reflect a legacy effect of a longer period with uncontrolled HIV viral replication). For the purposes of my analysis, the duration of untreated HIV disease for the participants in the CHI study was calculated as the duration between HIV diagnosis date and cART initiation date. The date of HIV seroconversion was not known for the participants in the PET in CHI study, thus the actual duration of untreated HIV disease in these participants is likely even longer. The impact of the individual antiretroviral ART agents and antiretroviral history were not explored given the small sample size and poor reliability of historical ART data.

Higher cerebrospinal fluid white cell count [446,447] may reflect higher HIV reservoir size and viral transcription, due to the production of viral antigens causing central nervous system inflammation. My observations suggest an association between higher regional brain [^{11}C] PBR28 V_T binding with higher cerebrospinal fluid white cell count. However, the overall cerebrospinal fluid white cell counts in the participants included in this analysis were largely

similar between the two groups ('PET in AHI' and 'PET in CHI' studies) and mostly within normal range.

Overall, higher regional brain [^{11}C] PBR28 V_T binding was observed to be significantly associated with increased average z-scores of the combined visual memory and visual learning domains only. Of note, all the participants included in this analysis were neuro-asymptomatic, and the pattern of associations between regional brain [^{11}C] PBR28 V_T binding and cognitive domains may differ in people living with treated HIV reporting cognitive impairment, where the commonly affected cognitive domains include attention, executive function, fine movement and learning.

Central nervous system side effects are well-described in people with HIV taking certain antiretroviral agents, but when I stratified regional [^{11}C] PBR28 V_T binding according to antiretroviral agent class, no significant associations were observed. This may be due to the small and unequal number of participants in the three antiretroviral third agent classes, and further studies with larger sample size are warranted to investigate this in more detail given the central nervous system clinical signals seen in people taking certain antiretroviral agents[448].

6.5 Conclusion

Due to the unavailability of total HIV DNA and ultra-sensitive HIV RNA measurement results, I was unable to address my first and second PhD hypotheses in this chapter. When permitted, I plan to measure total HIV DNA and ultra-sensitive HIV RNA in stored samples from the participants, which will enable me to test both my first and second PhD hypotheses in this chapter. Further studies are needed to determine whether HIV persistence in the central nervous system is causally related to neuroinflammation, leading to neurological sequelae such as cognitive impairment.

Chapter 7

Investigating neuro-axonal injury following a HIV “shock and kill” strategy and immediate cART compared to immediate cART alone, initiated during primary HIV infection: the RIVER study

Chapter 7: Investigating neuro-axonal injury following a HIV “shock and kill” strategy and immediate cART compared to immediate cART alone, initiated during primary HIV infection: the RIVER study

7.1 Introduction

While cART has significantly improved prognosis and extended life expectancy for PLWH, cART alone cannot cure HIV and upon stopping cART, plasma viral load rebounds in the majority of PLWH within four to eight weeks[60]. The source of rebounding virus is considered to be cells latently infected with HIV, termed the reservoir[58,64–66]. Following HIV acquisition, viral genetic material inserts into the host cell genome, and can remain integrated for the lifetime of the host cell[65]. The latently infected cells are established soon after HIV acquisition[68], remain stable despite cART[65,69,70], do not express viral antigens and thus, evade immune system detection[71]. However, activation of latently infected cells can lead to viral transcription and expression of viral antigens[65,75], which can in turn lead to immune recognition and eradication or to virally-mediated cell death[255]. The main HIV latent reservoir is in resting CD4⁺ T-cells in circulation and lymph nodes[65,75], but emerging evidence suggests that additional important anatomic reservoir compartments include the lungs, genital tract and central nervous system [64,75,266,403,449,450], comprising of latently infected macrophages, monocytes and microglial cells.

To date, two individuals are considered to be in long-term HIV remission[451,452] through chemotherapy, radiotherapy and bone marrow transplantation; but this is not generalisable or safe, hence alternative strategies are being explored. One HIV cure approach termed “shock and kill” [252] involves forcing viral transcription from latently infected cells using a LRA[453], followed by the targeted removal of HIV-expressing cells by immune-mediated

direct cytopathic action (via pre-vaccination to direct and enhance HIV-specific cytolytic T-cell activity)[252].

Reported significant central nervous system adverse effects following “shock and kill” are rare[263,264], but data remain limited. “Shock and kill” is still a novel HIV-remission approach and to date, trials involving this method have not extended beyond phase I and II studies. In general, “shock and kill” HIV remission strategies aim to reduce HIV reservoir size. HIV reservoirs in the central nervous system have been implicated in viral antigen production, causing persistent immune activation[147] and neuroinflammation[205], which can lead to poorer neurocognitive performance [85,205], possibly due to neuronal injury. Thus, a “shock and kill” strategy which aims to reduce HIV reservoir size may be associated with lower neuronal injury. On the other hand, a major concern with “shock and kill” within the central nervous system is that the LRAs used could be directly neurotoxic[265] or the HIV viral transcription triggered by the LRAs could lead to neuro-inflammation and immune-activation[454], which may then cause neuronal injury[267,455].

In a trial of SIV-infected macaques receiving cART and two LRAs, one macaque experienced significant viral rebound (higher in the cerebrospinal fluid compared to plasma), increased central nervous system immuno-activation and neuronal injury biomarkers and neurological symptoms that necessitated euthanasia[268].

NfL is a major structural component of myelinated axons and maintains the functional integrity of axons[279,281]. NfL measured in the cerebrospinal fluid has been shown to be a sensitive and dynamic biomarker of central nervous system neuro-axonal injury[279,281] with elevated concentrations reported in range of neurological conditions, including untreated HIV disease[269,279,281,285,456]. However, given the invasive nature of cerebrospinal fluid collection by lumbar puncture, frequent cerebrospinal fluid NfL measurement is challenging. A new assay measuring blood NfL concentration has been developed[293], and data suggests that blood NfL correlates moderately to strongly with cerebrospinal fluid NfL[293,368,369,372] and as per my results in Chapter 3, including in PLWH on suppressive cART as per my results in Chapter 4. This removes the barriers faced by repeated cerebrospinal fluid collection and allows more frequent measurements given that blood is easier to obtain.

RIVER[338] is the first open-label randomised “shock and kill” trial assessing cART-alone versus cART plus a LRA (vorinostat, a histone deacetylase inhibitor) plus a prime/boost T-cell vaccination (ChAdV63.HIVconsv and MVA.HIVconsv[337], (ART+V+V)) in individuals who initiated cART during primary HIV infection. The following measurements were performed within the context of the RIVER trial: total and integrated HIV DNA in CD4⁺ T-cells isolated from peripheral blood (as surrogate measures of HIV reservoir size), ultra-sensitive plasma HIV RNA and cell-associated unspliced HIV RNA in CD4⁺ T-cells isolated from peripheral blood (as surrogate measures of HIV viral transcription) and vaccine-induced HIV-specific polyfunctional CD4⁺ and CD8⁺ T-cell responses (as a surrogate measure of HIV viral control).

In this chapter, I test both my first hypothesis that lower biomarker concentrations of HIV reservoir size are associated with lower biomarker concentrations of neural activation and **my second hypothesis** that higher biomarker concentrations of HIV viral transcription are associated with higher biomarker concentrations of neural activation, within the context of a HIV “shock and kill” intervention.

To test my first hypothesis, I explored the association between a biomarker of neuro-axonal injury (plasma NfL) with total HIV DNA per 10⁶ CD4⁺ T-cells (as a measure of HIV reservoir size) following a HIV “shock and kill” intervention. To test my second hypothesis, I explored the association between plasma NfL and ultra-sensitive plasma HIV RNA (as a measure of HIV viral transcription) following a HIV “shock and kill” intervention. This work was performed participants enrolled into the randomised controlled RIVER trial[338] which compared cART plus a “shock and kill” strategy versus cART-alone. Furthermore, I explored demographic, clinical and additional biomarker factors associated with plasma NFL in the context of a HIV “shock and kill” remission strategy.

7.2 Methods

The RIVER trial design is described in detail in Chapter 2: Materials and Methods.

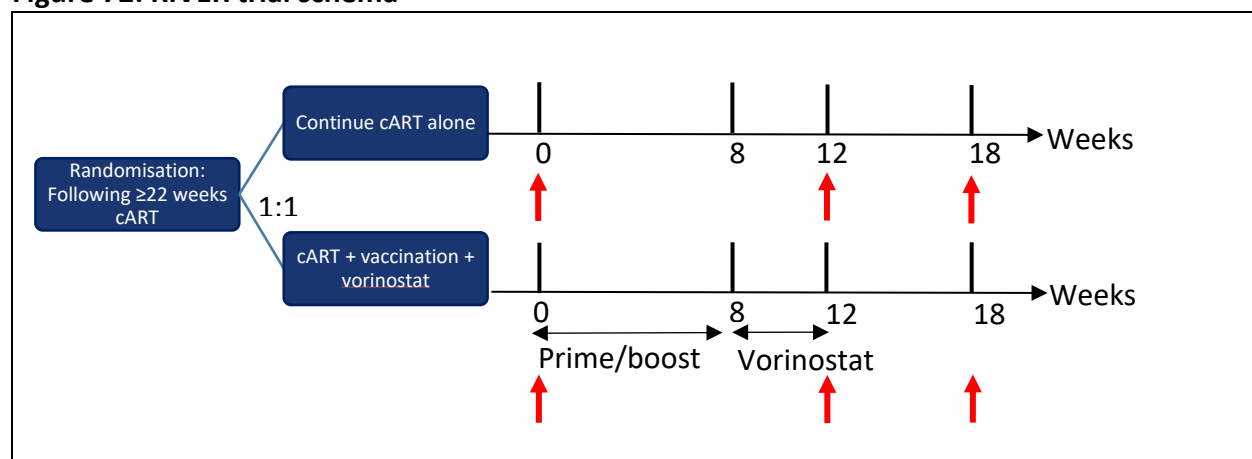
I submitted a sub-study proposal to the RIVER Trial Steering Committee, who approved my request to conduct this work. I was successful in my application to the British HIV Association (BHIVA) for a competitive research grant to pay for the work in this sub-study.

7.2.1 Participants and sample timepoints

All participants enrolled into the RIVER trial who consented for their data and stored blood samples to be used for future research purposes were included in this sub-study. Plasma NfL concentration measurements were performed on stored plasma samples from the following three timepoints (Figure 72 and Table 38):

- i. On the day of randomisation: After the participants had received at least 22 weeks of continuous cART, the participants were randomised 1:1 to either continue cART alone or to continue cART plus vaccination with ChAdV63.HIVconsV prime (given within one week of randomisation) and MVA.HIVconsV boost (given eight weeks apart) followed by vorinostat taken orally in 10 doses of 400mg every 3 days for 28 days (ART+V+V)
- ii. At week 12 post-randomisation: before receiving the tenth and final vorinostat and four weeks after completing the vaccination course in the intervention arm
- iii. At week 18 post-randomisation

Figure 72: RIVER trial schema



RIVER trial schema demonstrating the 1:1 randomisation into the two study arms. The red arrows signify the time points when blood samples were analysed for NfL. Abbreviations: NfL = neurofilament light chain protein; cART = combination antiretroviral treatment

7.2.2 Laboratory measurements

I measured plasma NfL using the commercially available NF-light assay on a HD-X Simoa instrument (Quanterix™, USA) at the UK Dementia Research Institute, University College London, UK[342], as described in Chapter 2: Materials and Methods.

The following research parameters were measured previously in the main RIVER study[338] (Table 38):

1. **Total HIV DNA in CD4⁺ T-cells isolated from peripheral blood** (by qPCR)[338] at randomisation, week 12 and week 18
2. **Integrated HIV DNA in CD4⁺ T-cells isolated from peripheral blood** (by qPCR)[338] at randomisation and week 18
3. **Cell-associated unspliced HIV RNA in CD4⁺ T-cells isolated from peripheral blood** (by qPCR)[338] at randomisation and week 18
4. **Ultra-sensitive plasma HIV RNA** (by single-copy assay)[338] at randomisation, week 12 and week 18
5. **HIV-specific CD8⁺ and CD4⁺ T-cell frequencies isolated from peripheral blood** (by intracellular cytokine staining using multiparameter flow cytometry) to detect IFN- γ ⁺, IL-2, TNF α ⁺, CD107a⁺ (a marker of T-cell degranulation) and CD154⁺ (a marker of T-cell activation) expression at randomisation and week 12 only.

The following routine clinical parameters were measured previously in the main RIVER study.

1. **Serum creatinine and estimated glomerular filtration rate** (using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) calculator)
2. **CD4⁺ and CD8⁺ T-cell counts**

Table 38: Research biomarkers analysed in the RIVER trial sub-study, method of measurement and time points when the biomarkers were measured

Biomarker	Method	Week 0 (following ≥ 22wks of cART)	Week 12 (on final day of vorinostat dosing and 4 weeks after second vaccination dose)	Week 18
Plasma NfL, pg/mL	Simoa	✓	✓	✓
Total HIV DNA in CD4 ⁺ T-cells isolated from peripheral blood	qPCR	✓	✓	✓
Integrated HIV DNA in CD4 ⁺ T-cells isolated from peripheral blood	qPCR	✓		✓
Cell-associated unspliced HIV RNA in CD4 ⁺ T-cells isolated from peripheral blood	qPCR	✓		✓
Ultra-sensitive plasma HIV RNA, copies/mL	Single-copy assay	✓	✓	✓
HIV-specific CD8 ⁺ and CD4 ⁺ T-cell frequencies	Intracellular cytokine staining	✓	✓	

Abbreviations: cART = combination antiretroviral treatment, NfL = neurofilament light chain protein, Simoa = single molecule array digital immunoassay, qPCR = quantitative polymerase chain reaction

7.2.3 Statistical analysis

Where appropriate, plasma NfL, total HIV DNA, integrated HIV DNA, cell-associated unspliced HIV RNA and ultra-sensitive HIV RNA were log₁₀-transformed to reduce data skewness. Plasma NfL <13 pg/mL was considered normal[293]. Given the normal distributions of the data, differences in laboratory parameters (plasma NfL, total HIV DNA, integrated HIV DNA, cell-associated unspliced HIV RNA, ultra-sensitive plasma HIV RNA, % CD154⁺ IFN-γ⁺ CD4⁺ cells and % CD107a⁺ IFN-γ⁺ CD8⁺ cells) by study arms at each time point were analysed using Student's t-test and longitudinal changes in plasma NfL were analysed using mixed models. Correlations between plasma NfL and total HIV DNA,

integrated HIV DNA, cell-associated unspliced HIV RNA, ultra-sensitive plasma HIV RNA and HIV-specific CD4⁺ and CD8⁺ polyfunctional T-cell responses at the time points were analysed using Spearman's rank statistics, which enables a more accurate measure of association between the two variables if their relationship was non-linear.

A priori, I identified a selection of factors (age, body mass index, ethnicity, duration since primary HIV infection diagnosis, estimated glomerular filtration rate (calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula)[344], CD4⁺ T-cell count, CD8⁺ T-cell count, total HIV DNA in peripheral CD4⁺ T-cells and ultra-sensitive plasma HIV RNA) that may be associated with plasma NfL at baseline. All these factors were included in the multivariable linear regression model. There is supporting evidence that certain factors may be associated with plasma NfL, thus the *a priori* method of including all factors potentially associated with plasma NfL in the multivariable regression model was considered more appropriate in this context, compared with a stepwise automatic selection process which runs the risk of excluding factors known to be biologically significant, on the basis of lack of significance in the stepwise regression models.

Formal sample size calculation was not performed. RIVER was the largest randomised controlled trial of "shock and kill" in humans at the time and the precise impact of "shock and kill" on NfL was unknown. My proposal to measure serial plasma NfL concentrations in participants enrolled into the RIVER study was reviewed and the statistical plans included in this chapter were approved by the RIVER Trial Steering Committee, which included experienced biostatisticians, who felt that this work would be a valuable contribution to the field, and our analysis using the sample size available was likely to produce an informative result. Statistical analysis for this chapter was performed by myself, under the supervision of Professor Abdel Babiker and Dr Wolfgang Stohr, the RIVER trial biostatisticians, based at the MRC Clinical Trials Unit at University College London.

7.3 Results

In total, 58 out of the 60 participants enrolled into the RIVER study were included in this sub-study. 2 participants were excluded (one each from the ART-only arm and the ART+V+V arm), as they did not consent for their samples to be used for future research purposes.

7.3.1 Participant characteristics

All participants included in this analysis were male, 69% were of white ethnicity and 91% acquired HIV via sex between men (Table 39). At randomisation, overall median age of the participants was 32 years (interquartile range (IQR) 28, 40), median duration since primary HIV infection diagnosis was 28 weeks (IQR 27, 37), median CD4⁺ T-cell count was 696 cells/ μ L (IQR 566, 785), median CD4⁺/CD8⁺ ratio was 1.1 (IQR 0.8, 1.4), and 57/58 participants included in the analysis had plasma HIV RNA <50 copies/mL (Table 39). One participant had plasma viral load between 50-200 HIV RNA copies/mL at randomisation. However, on repeat testing one week later, plasma viral load had resuppressed to <50 HIV RNA copies/mL, and this participant proceeded with randomisation. Most participants harboured clade B virus (66%) and 78% of participants were commenced on a four-drug combination antiretroviral regimen (Table 39). Overall, median body mass index was 24.0 kg/m² (IQR 21.7, 26.6) and estimated glomerular filtration rate was 110 ml/min/1.73m² (IQR 99, 117). Participant characteristics at randomisation were well-balanced by study arm.

Table 39: Participant characteristics at randomisation

Median (IQR) or total (%)	Total (n = 58)	ART-only arm (n=29)	ART+V+V (n = 29)
Age, years	32 (28, 40)	31 (29, 38)	34 (28, 44)
Male	58 (100)	29 (100)	29 (100)
Ethnicity			
White	40 (69.0)	15 (51.8)	25 (86.3)
Black African	2 (3.5)	2 (6.9)	0 (0)
Black Caribbean or African American	2 (3.5)	2 (6.9)	0 (0)
Hispanic/ Latino	5 (8.6)	3 (10.3)	2 (6.9)
South Asian	1 (1.7)	0 (0)	1 (3.4)
South East Asian	1 (1.7)	1 (3.4)	0 (0)
Mixed ethnicity	6 (10.3)	5 (17.3)	1 (3.4)
Other	1 (1.7)	1 (3.4)	0 (0)
Mode of HIV acquisition			
MSM	53 (91.3)	25 (86.3)	28 (96.6)
MSW	2 (3.5)	1 (3.4)	1 (3.4)
MSM and IDU	2 (3.5)	2 (6.9)	0 (0)
Unknown	1 (1.7)	1 (3.4)	0 (0)
Duration between PHI diagnosis and randomisation, weeks	28.1 (27, 37.1)	28.0 (26.6, 42)	28.1 (27.1, 35.8)
CD4 ⁺ T-cell count at randomisation, cells/ μ L	696 (566, 785)	675 (561, 817)	704 (573, 750)
CD8 ⁺ T-cell count at randomisation, cells/ μ L	660 (452, 833)	670 (458, 963)	642 (438, 746)
CD4/CD8 ratio at randomisation	1.1 (0.8, 1.4)	1.1 (0.7, 1.3)	1.1 (0.9, 1.5)
Plasma HIV RNA at randomisation, copies/mL			
<50	57 (98.3)	28 (96.6)	29 (100)
50 - <200	1 (1.7)	1 (3.4)	0 (0)
cART			
3 drug regimens	13 (22.4)	6 (20.7)	7 (24.1)
4 drug regimens	45 (77.6)	23 (79.3)	22 (75.9)
HIV clade			
B	38 (65.5)	19 (65.6)	19 (65.5)
A	1 (1.7)	0 (0)	1 (3.4)
F	3 (5.2)	3 (10.3)	0 (0)
Recombinant	9 (15.5)	3 (10.3)	6 (20.7)
Not known	7 (12.1)	4 (13.8)	3 (10.3)
Serum creatinine at randomisation, μ mol/L	78 (71, 86)	83 (74, 90)	75 (71, 83)
eGFR at randomisation, ml/min/1.73m ²	110 (99, 117)	107 (94, 118)	111 (103, 119)
Weight, kg	75 (71, 84)	74 (69, 84)	75 (71, 86)
Height, cm	179 (173, 182)	178 (173, 183)	180 (172, 182)
Body mass index, kg/m ²	24.0 (21.7, 26.6)	24.0 (21.6, 26.3)	23.9 (22.3, 26.9)

Abbreviations: cART = combination antiretroviral treatment; ART-only = antiretroviral treatment only; ART+V+V = combination antiretroviral treatment plus HIV-specific vaccination plus vorinostat; MSM = men who have sex with men; MSW = men who have sex with women; IDU = injection drug use; PHI = primary HIV infection; eGFR = estimated glomerular filtration rate

7.3.2 Laboratory parameter results by study arm

In the overall cohort, estimated geometric mean of plasma NfL was 6.9 pg/mL at randomisation, 7.4 pg/mL at week 12, and 6.9 pg/mL at week 18 (Table 40). Plasma NfL concentrations in the ART-only and ART+V+V arms were similar at each timepoint, with no significant difference seen in plasma NfL concentrations by study arm at randomisation ($p = 0.16$), week 12 ($p = 0.22$) and week 18 ($p = 0.74$) (Table 40).

Furthermore, I observed no significant difference by study arm in total HIV DNA, integrated HIV DNA and cell-associated unspliced HIV RNA in peripheral CD4⁺ T-cells and ultra-sensitive plasma HIV RNA measurements, at the time points when these markers were measured (Table 40).

While no significant difference in HIV-specific CD4⁺ and CD8⁺ T-cell responses were observed at randomisation (pre-intervention in the ART+V+V arm), participants in the ART+V+V arm demonstrated significantly higher HIV-specific CD4⁺ T-cell responses (% CD154⁺ IFN- γ ⁺ CD4⁺ cells) and HIV-specific CD8⁺ T-cell responses (% CD107a⁺ IFN- γ ⁺ CD8⁺ cells) at week 12 (four weeks after the booster vaccination dose), compared to participants in the ART-only arm (Table 40).

Table 40: Research biomarker results at each time point

		Randomisation: Following ≥ 22 weeks continuous ART)	Week 12 (on final day of intervention in the ART+V+V arm)	Week 18
Plasma NfL, pg/mL ¹	All participants	6.9 (6.2, 7.6)	7.4 (6.5, 8.3)	6.9 (6.3, 7.7)
	ART only	7.4 (6.5, 8.4)	8.0 (6.6, 9.7)	7.1 (6.2, 8.0)
	ART+V+V	6.4 (5.4, 7.6)	6.9 (5.8, 8.1)	6.8 (5.7, 8.1)
	p-value	0.16	0.22	0.74
Total HIV DNA, copies/10 ⁶ peripheral CD4 ⁺ cells	All participants	1581 (569, 2939)	1177 (527, 2399)	1501 (554, 2741)
	ART only	1597 (571, 2915)	1121 (413, 2114)	1501 (476, 2814)
	ART+V+V	1564 (583, 2893)	1375 (621, 2371)	1347 (558, 1933)
	p-value	0.49	0.28	0.45
Integrated HIV DNA, copies/10 ⁶ peripheral CD4 ⁺ cells	All participants	827 (333, 1617)	n/a	789 (271, 1428)
	ART only	808 (405, 1864)		773 (232, 1493)
	ART+V+V	945 (232, 1471)		789 (356, 1356)
	p-value	0.83		0.65
Cell-associated unspliced HIV RNA, copies/ng	All participants	0.134 (0.007, 0.678)	n/a	0.189 (0.006, 1.081)
	ART only	0.179 (0.008, 0.745)		0.228 (0.006, 0.880)
	ART+V+V	0.061 (0.003, 0.537)		0.157 (0.000, 1.487)
	p-value	0.56		0.43
Ultra-sensitive plasma HIV RNA, copies/mL	All participants	14 (4, 25)	5 (1, 12)	6 (1, 16)
	ART only	17 (3, 30)	9 (2, 14)	6 (1, 20)
	ART+V+V	13 (5, 23)	5 (1, 9)	6 (1, 14)
	p-value	0.26	0.47	0.30
% CD154 ⁺ IFN- g ⁺ CD4 ⁺ cells	All participants	0.009 (0.000, 0.023)	0.031 (0.005, 0.112)	n/a
	ART only	0.010 (0.000, 0.024)	0.006 (0.000, 0.014)	
	ART+V+V	0.009 (0.000, 0.020)	0.112 (0.050, 0.214)	
	p-value	0.65	<0.001	
% CD107a ⁺ IFN- g ⁺ CD8 ⁺ cells	All participants	0.074 (0.008, 0.289)	0.125 (0.033, 0.291)	n/a
	ART only	0.076 (0.000, 0.262)	0.063 (0.008, 0.110)	
	ART+V+V	0.069 (0.009, 0.362)	0.274 (0.143, 0.620)	
	p-value	0.90	<0.001	

Abbreviations: NfL = neurofilament light chain protein; ART = combination antiretroviral treatment; ART+V+V = antiretroviral treatment and vaccination and vorinostat; n/a: not assessed at this time point

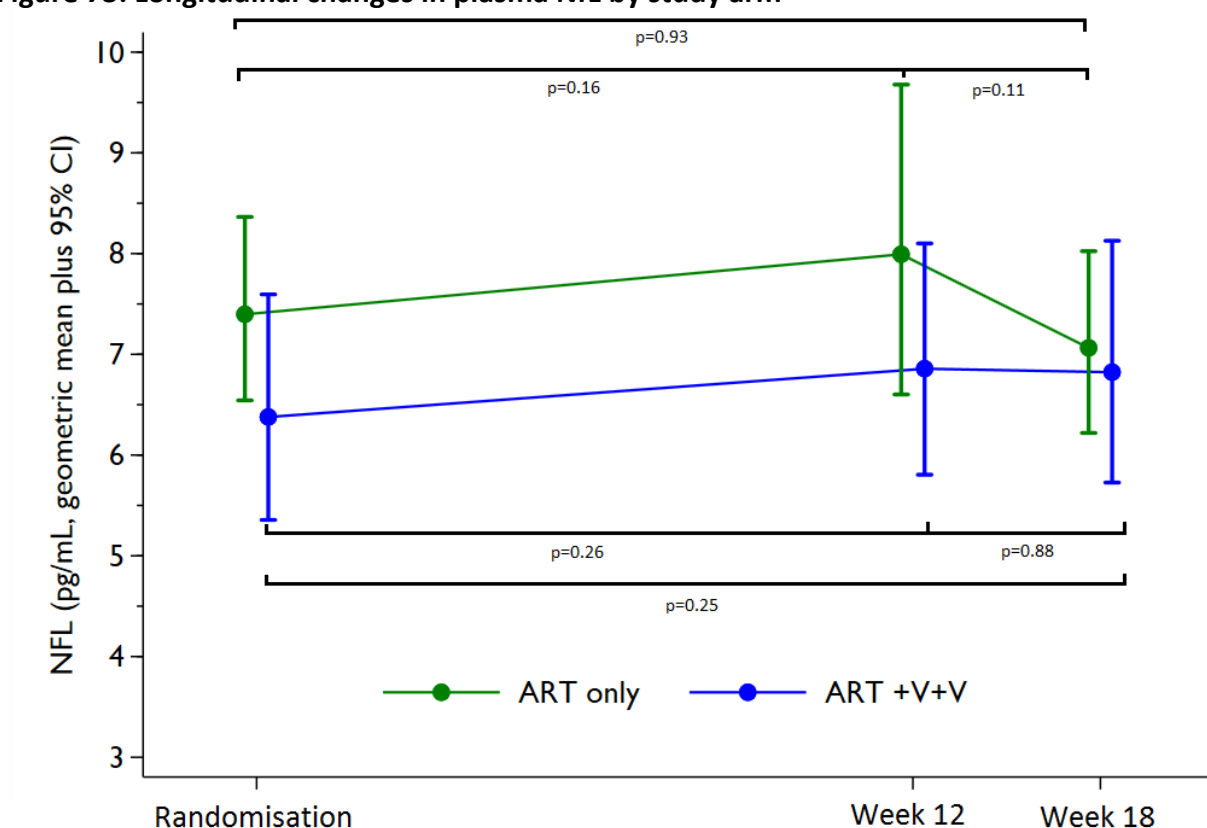
Values are median (IQR) unless stated otherwise. ¹Geometric mean (95% confidence interval)

Legend: P-values relate to differences in the measured biomarker by study arm at each time point and were analysed using Student's t-test. Number of participants in the ART only arm (n = 29), number of participants in the ART+V+V arm (n = 29)

7.3.3 Plasma NfL longitudinal trends

There was no significant difference in terms of change in plasma NfL concentration across the three time points. Specifically, no significant change in plasma NfL concentration was seen in the ART-only and ART+V+V arms between randomisation and week 12 ($p=0.16$ and $p=0.26$, respectively), between randomisation and week 18 ($p=0.93$ and $p=0.25$, respectively) or between week 12 and week 18 ($p = 0.11$ and $p = 0.88$, respectively) (Figure 73).

Figure 73: Longitudinal changes in plasma NfL by study arm



Abbreviations: NfL = neurofilament light chain protein; ART = antiretroviral treatment, ART+V+V = antiretroviral treatment and vaccination and vorinostat.

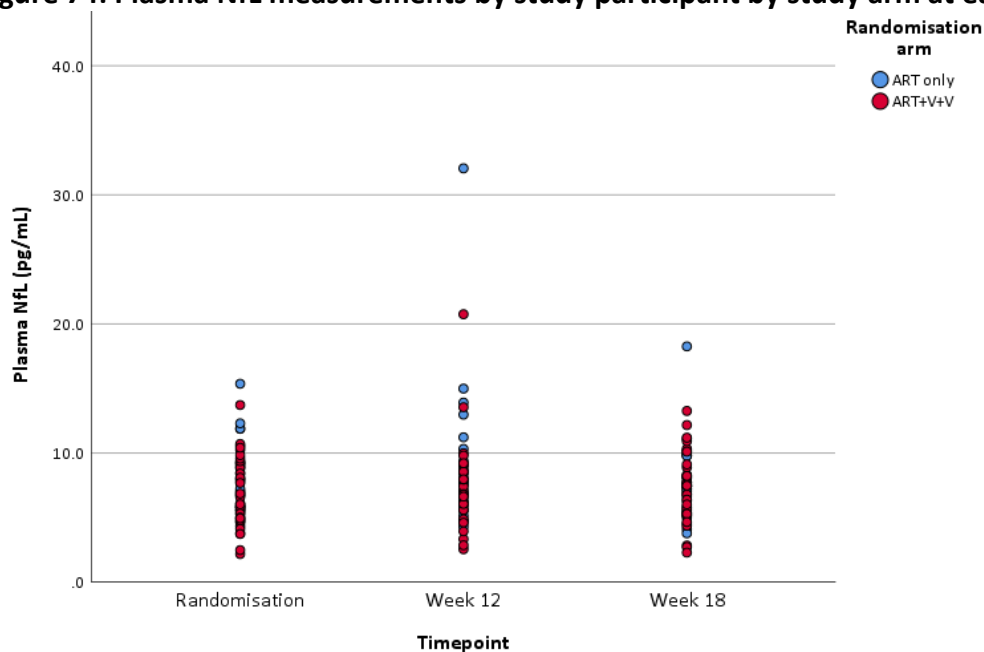
Legend: The p-values above the line graphs relate to the ART-only arm and the p-values below the line graphs relate to the ART+V+V arm. P-values were analysed using mixed models. Error bars denote 95% confidence intervals. Number of participants in the ART only arm ($n = 29$), number of participants in the ART+V+V arm ($n = 29$)

7.3.4 Participant(s) with outlying plasma NfL measurement(s)

At randomisation, all but two participants had plasma NfL <13pg/mL (Figure 74). One participant with plasma NfL 13.7 pg/mL at randomisation was allocated to the ART+V+V arm, and plasma NfL in this participant remained stable throughout the trial (plasma NfL 13.6 pg/mL and 13.3 pg/mL at weeks 12 and 18, respectively). The other participant had plasma NfL measuring 15.4 pg/mL at randomisation and was allocated to the ART-only arm; while plasma NfL remained stable for this participant at week 12 (15.0 pg/mL), plasma NfL rose to 18.3pg/mL at week 18.

At week 12, plasma NfL was elevated in two other participants, one in a participant allocated to the ART-only arm (plasma NfL 32.1 pg/mL, having been 10.2 pg/mL at randomisation; no sample was available for plasma NfL measurement at week 18) and one in a participant allocated to the ART+V+V arm (plasma NfL 20.8 pg/mL, having been 9.8 pg/mL at randomisation; plasma NfL declined back to normal by week 18 at 9.1 p/mL).

Figure 74: Plasma NfL measurements by study participant by study arm at each time point



Abbreviations: NfL = neurofilament light chain protein

Legend: Scatter plot demonstrating plasma NfL distribution by study arm, at each time point.

7.3.5 Participants reporting neurological symptoms during the trial

During the trial, five participants from the ART+V+V arm and one participant from the ART-only reported the onset of new neurological symptoms (Table 41). None of these participants had plasma NfL concentrations above 13 pg/mL measured at any of the time points.

Table 41: Description of the neurological symptoms reported by participants during the trial and serial plasma NfL measurement

	Study arm	Symptom	Grade	Time point	Duration	Plasma NfL concentration		
						Randomisation, pg/mL	Week 12-PR, pg/mL	Week 18-PR, pg/mL
Subject 1	ART+V+V	Headache	1	Week 16-PR	1 month	5.3	4.6	2.8
Subject 2	ART+V+V	Headache	1	Week 9-PR	1 month	6.9	5.6	6.3
Subject 3	ART+V+V	Headache	1	Week 11-PR	1 day	3.8	3.3	6.4
Subject 4	ART+V+V	Dizziness	1	Week 6-PR	Months	5.8	4.8	5.0
		Somnolence	1	Week 9-PR	1 day			
Subject 5	ART+V+V	Dizziness	1	Week 8-PR	1 month	9.2	8.7	7.4
Subject 6	ART-only	Memory impairment	1	Week 2-PR	3 months	9.2	8.7	7.4

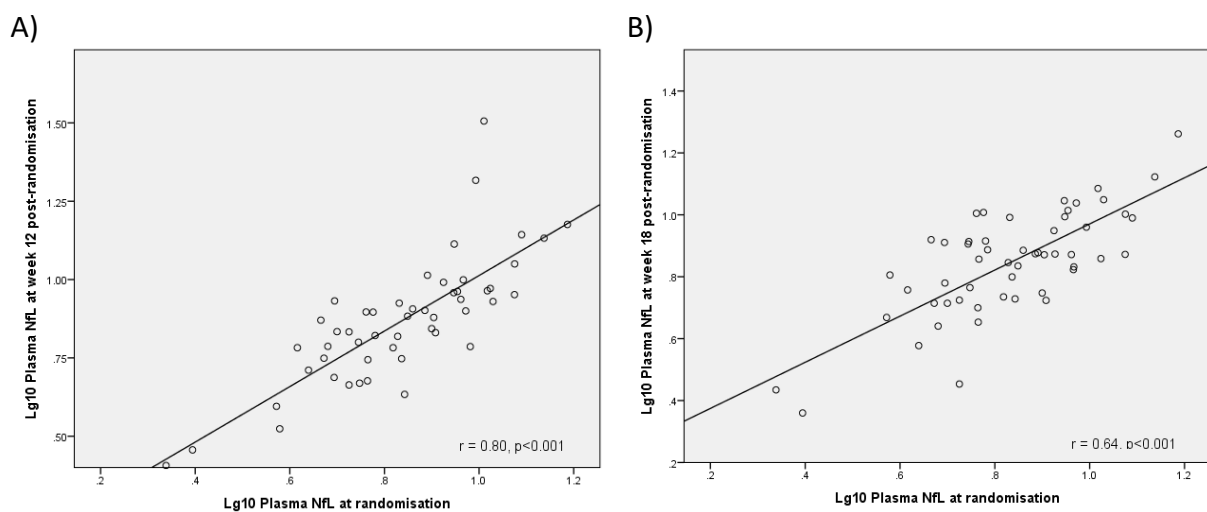
Abbreviations: NfL = neurofilament light chain protein; PR = post-randomisation; ART = antiretroviral treatment; V+V = vaccination and vorinostat; n/a = data not available

7.3.6 Correlations with plasma NfL

7.3.6.1 *Correlations between plasma NfL at different time points*

In the overall cohort, plasma NfL at randomisation positively correlated with plasma NfL at week 12, $r = 0.80$, $p < 0.001$ (Figure 75A), with no significant difference by study arm, $p = 0.78$. Similarly, plasma NfL at randomisation positively correlated with plasma NfL at week 18, $r = 0.64$, $p < 0.001$ (Figure 75B), with no significant difference seen by study arm, $p = 0.45$.

Figure 75: Correlation between plasma NfL at the different time points



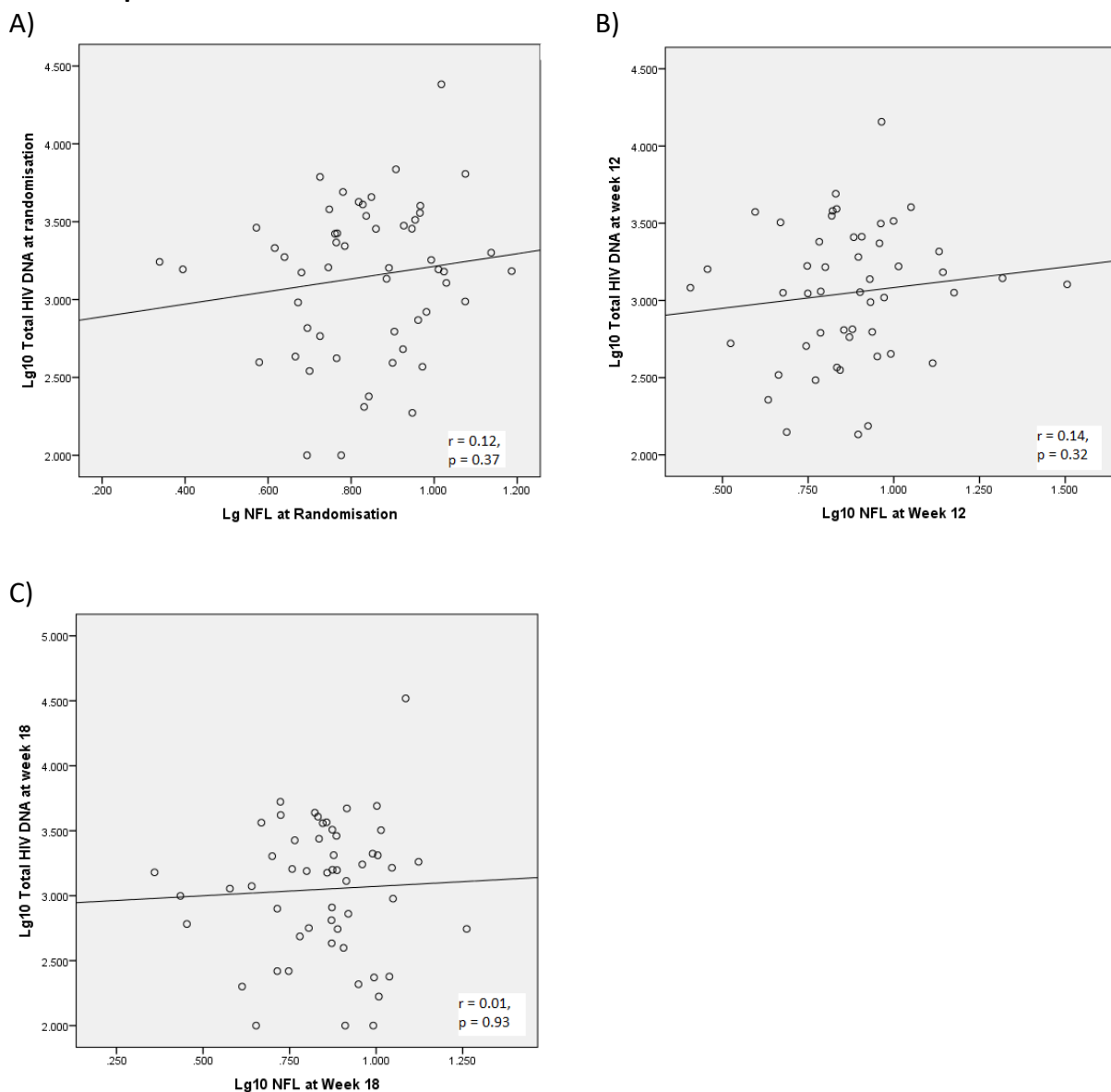
Abbreviation: NfL = neurofilament light chain protein

Legend: Correlations between plasma NfL at A) randomisation and week 12 ($n = 51$) and B) randomisation and week 18 ($n = 54$) were analysed using Spearman's rank statistics.

Correlations with total HIV DNA

No significant associations were seen between plasma NfL and total HIV DNA in the overall cohort at randomisation ($r = 0.12$, $p = 0.37$) (Figure 76A), week 12 ($r = 0.14$, $p = 0.32$) (Figure 76B) and week 18 ($r = 0.01$, $p = 0.93$) (Figure 76C).

Figure 76: Correlation between plasma NfL and total HIV DNA in the overall cohort, at each time point

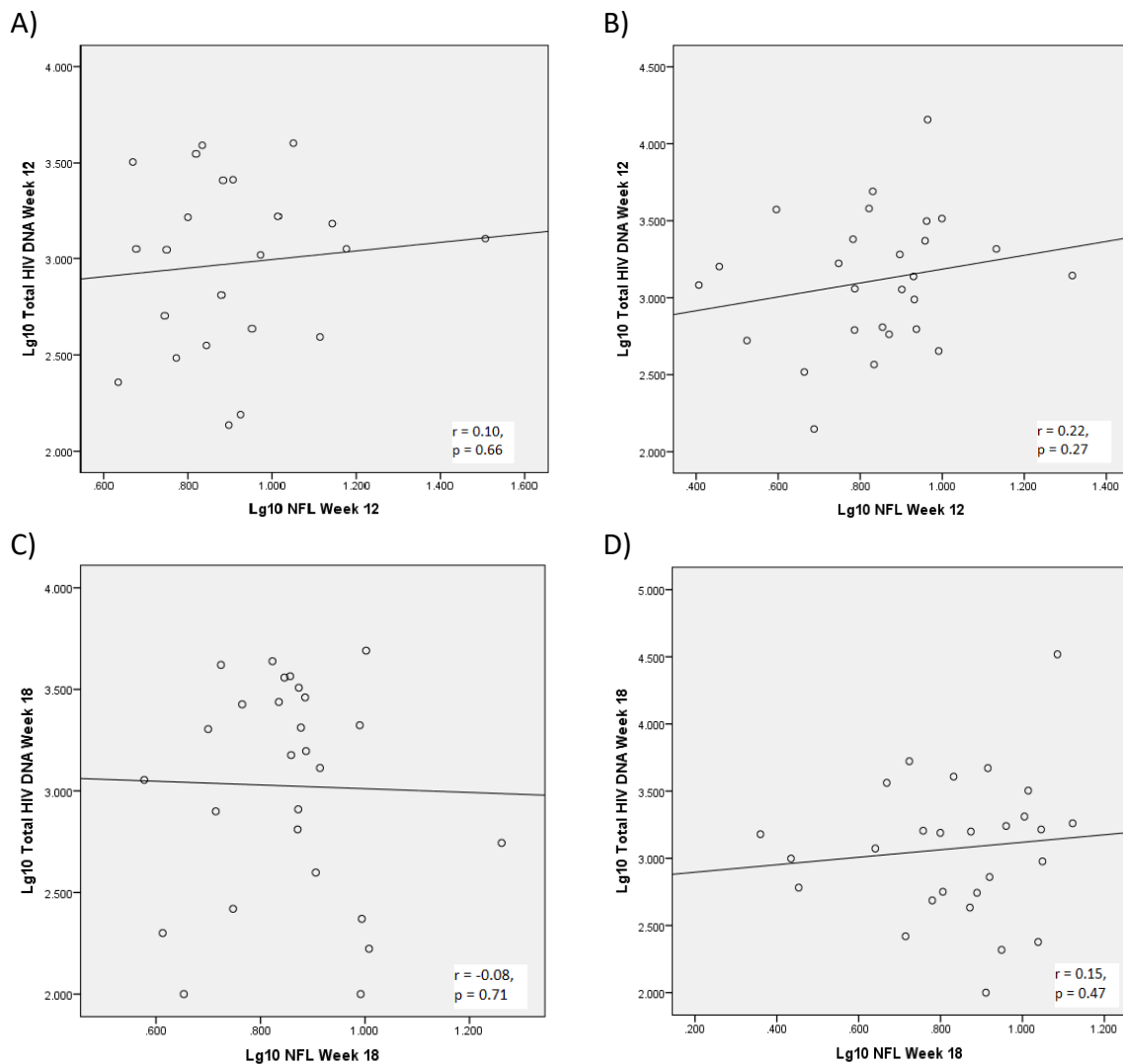


Abbreviation: NfL = neurofilament light chain protein

Legend: Correlations between plasma neurofilament light chain protein and total HIV DNA in the overall cohort at A) randomisation ($n = 54$), B) week 12 ($n = 50$) and C) week 18 ($n = 55$) were analysed using Spearman's rank statistics.

Additionally, no significant associations were seen between plasma NfL and total HIV DNA in the separate study arms at week 12 (ART-only: $r = 0.10$, $p = 0.66$ (Figure 77A) versus ART+V+V: $r = 0.22$, $p = 0.27$ (Figure 77B)) and week 18 (ART-only: $r = -0.08$, $p = 0.71$ (Figure 77C) versus ART+V+V: $r = 0.15$, $p = 0.47$) (Figure 77D).

Figure 77: Correlation between plasma NfL and total HIV DNA at weeks 12 and 18, by study arm



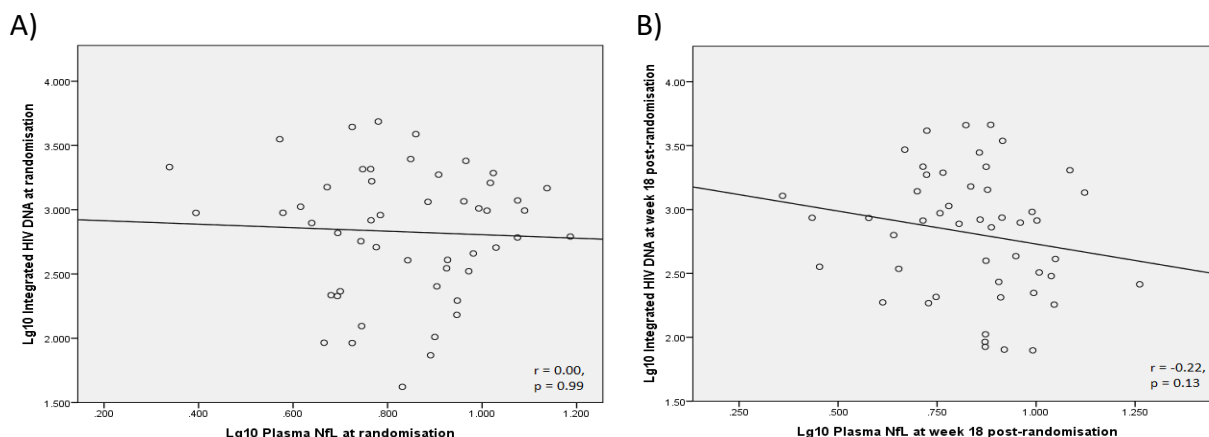
Abbreviation: NfL = neurofilament light chain protein

Legend: Correlations between plasma neurofilament light chain protein and total HIV DNA at A) week 12 in the ART-only arm ($n = 23$), B) week 12 in the ART+V+V arm ($n = 27$), C) week 18 in the ART-only arm ($n = 27$) and D) week 18 in the ART+V+V arm ($n = 28$) were analysed using Spearman's rank statistics.

7.3.6.2 Correlations with integrated HIV DNA

In the overall cohort, no significant correlations were seen between plasma NfL and integrated HIV DNA at randomisation ($r = 0.00$, $p = 0.99$) (Figure 78A) and week 18 ($r = -0.22$, $p = 0.13$) (Figure 78B).

Figure 78: Correlation between plasma NfL and integrated HIV DNA in the overall cohort at randomisation and week 18

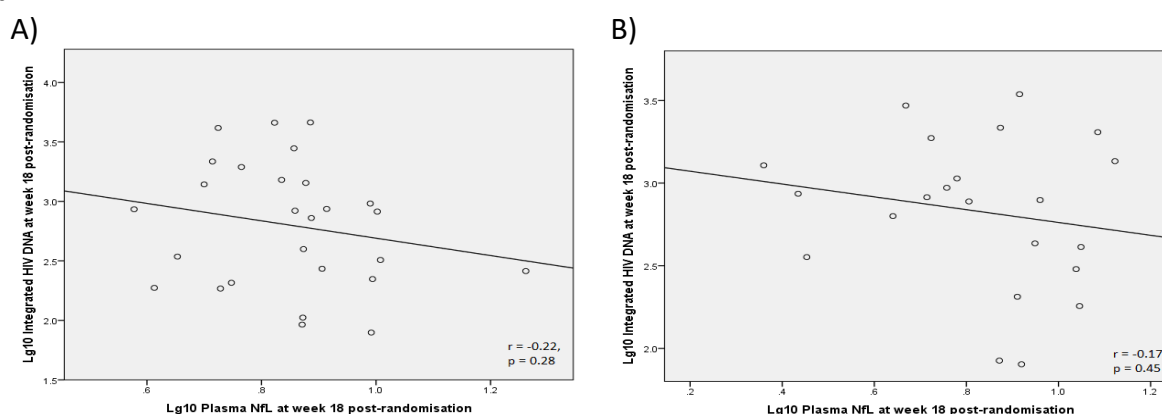


Abbreviation: NfL = neurofilament light chain protein

Legend: Correlations between plasma neurofilament light chain protein and integrated HIV DNA in the overall cohort at A) randomisation ($n = 50$) and B) week 18 ($n = 49$) were analysed using Spearman's rank statistics.

Additionally, no significant correlations were seen between plasma NfL and integrated HIV DNA in the separate study arms at week 18 (ART-only: $r = -0.22$, $p = 0.28$ (Figure 79A) versus ART+V+V: $r = -0.17$, $p = 0.45$) (Figure 79B).

Figure 79: Correlation between plasma NfL and integrated HIV DNA at week 18, by study arm



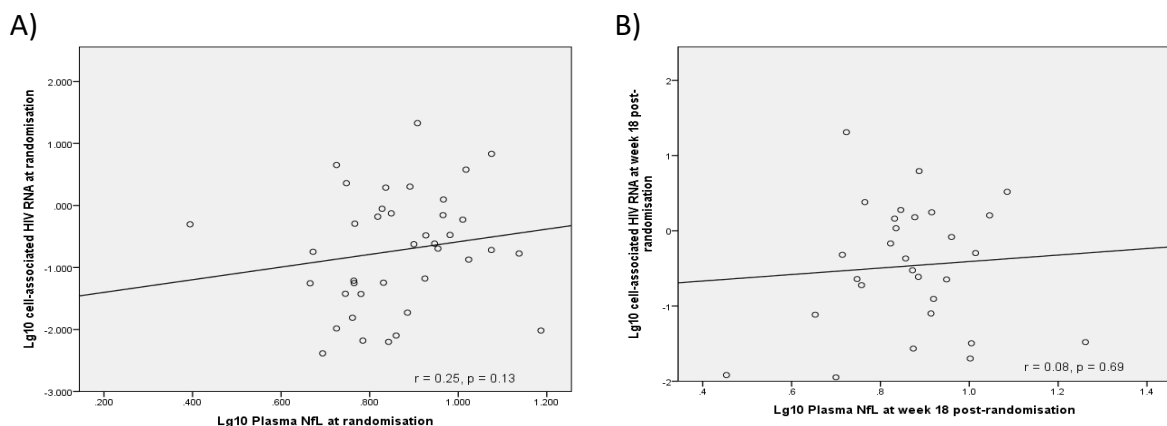
Abbreviation: NfL = neurofilament light chain protein

Legend: Correlations between plasma neurofilament light chain protein and integrated HIV DNA at week 18 in the A) ART-only arm ($n = 27$) and B) ART+V+V arm ($n = 22$) were analysed using Spearman's rank statistics.

Correlations with cell-associated unspliced HIV RNA

In the overall cohort, no significant correlations were observed between plasma NfL and cell-associated unspliced HIV RNA at randomisation ($r = 0.25$, $p = 0.13$) (Figure 80A) or at week 18 ($r = 0.08$, $p = 0.69$) (Figure 80B).

Figure 80: Correlation between plasma NfL and cell-associated unspliced HIV RNA in the overall cohort at randomisation and week 18

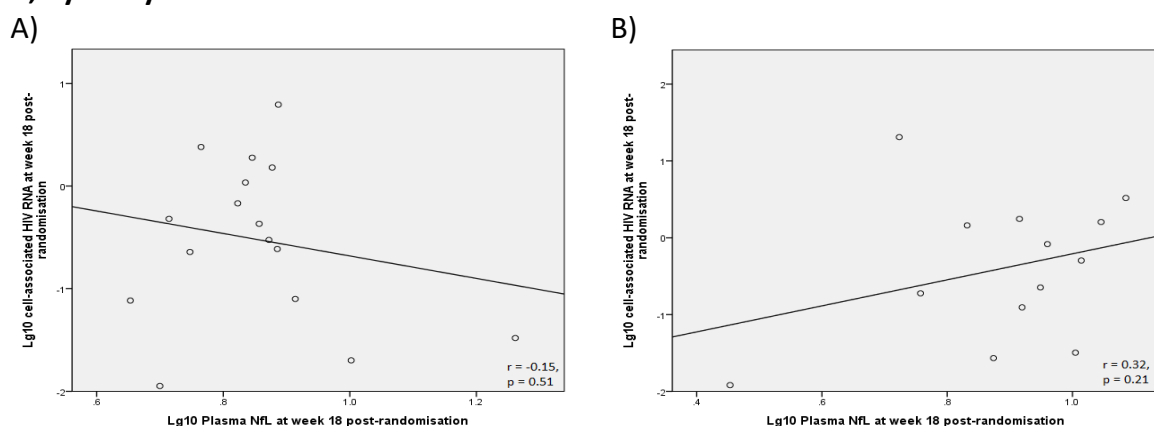


Abbreviation: NfL = neurofilament light chain protein

Legend: Correlations between plasma neurofilament light chain protein and cell-associated unspliced HIV RNA in the overall cohort at A) randomisation ($n = 48$) and B) week 18 ($n = 38$) were analysed using Spearman's rank statistics.

In the separate study arms at week 18, no significant correlations were seen between plasma NfL and cell-associated unspliced HIV RNA (ART-only: $r = -0.15$, $p = 0.51$) (Figure 81A) versus ART+V+V: $r = 0.32$, $p = 0.21$) (Figure 81B).

Figure 81: Correlation between plasma NfL and cell-associated unspliced HIV RNA at week 18, by study arm

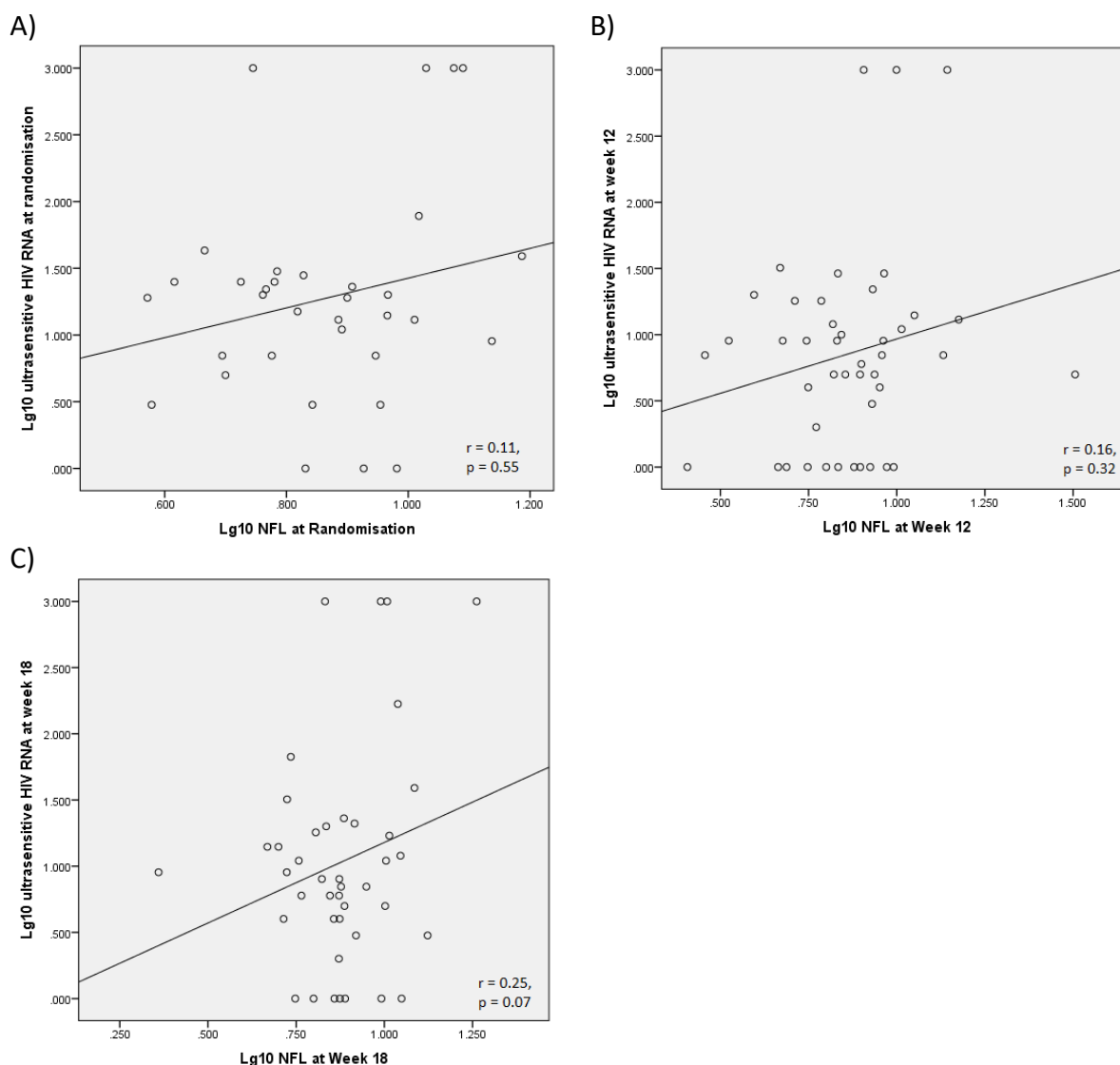


Abbreviation: NfL = neurofilament light chain protein. Legend: Correlations between plasma neurofilament light chain protein and cell-associated unspliced HIV RNA at week 18 in the A) ART-only arm ($n = 21$) and B) ART+V+V arm ($n = 17$) were analysed using Spearman's rank statistics.

7.3.6.3 Correlations with ultra-sensitive HIV RNA

With regards to ultra-sensitive plasma HIV RNA, no significant correlations were seen with plasma NfL in the overall cohort at randomisation ($r = 0.11$, $p = 0.55$) (Figure 82A), week 12 ($r = 0.16$, $p = 0.32$) (Figure 82B) and week 18 ($r = 0.25$, $p = 0.07$) (Figure 82C).

Figure 82: Correlation between plasma NfL and ultra-sensitive HIV RNA in the overall cohort, at each time point

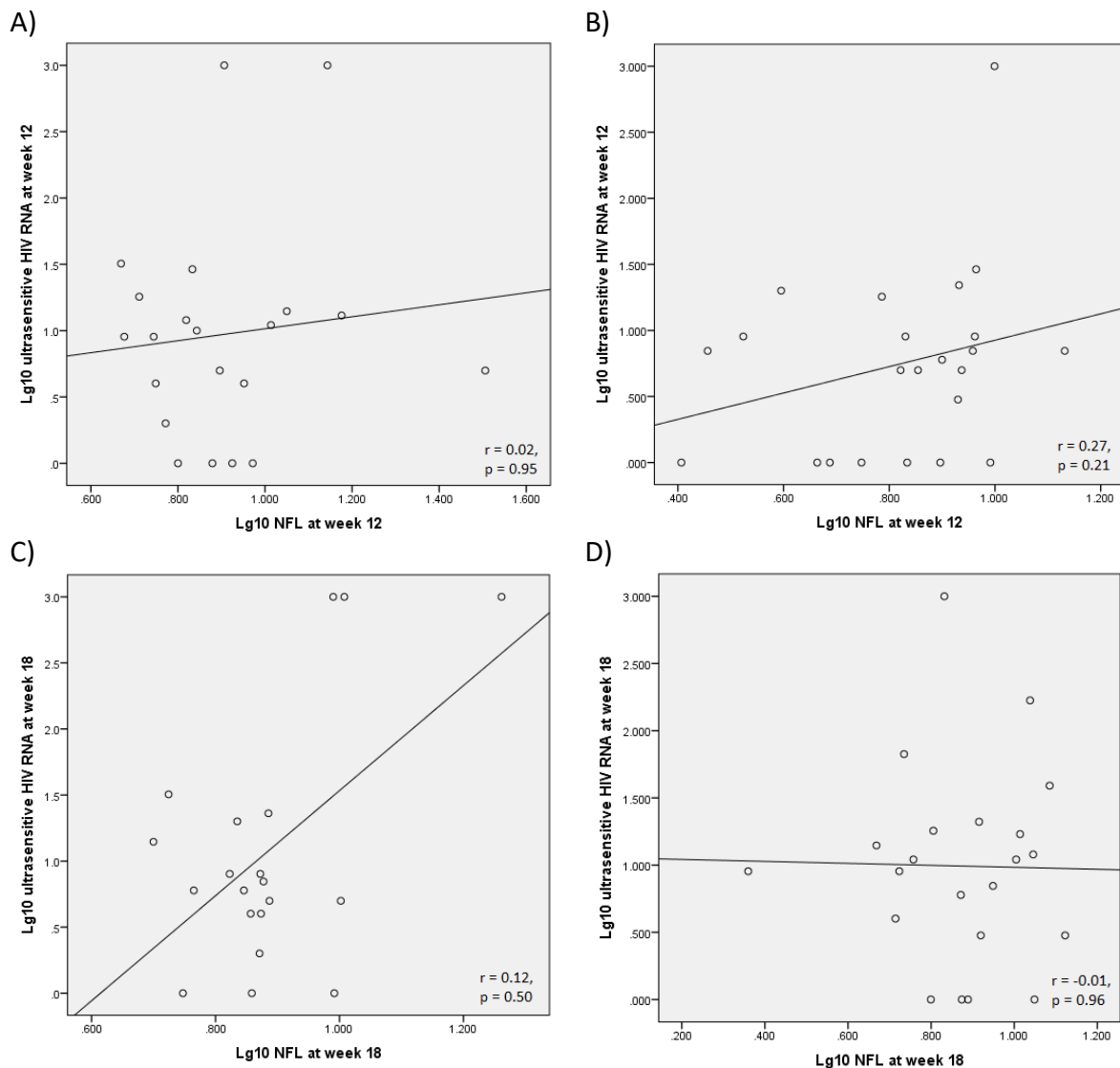


Abbreviation: NfL = neurofilament light chain protein

Legend: Correlations between plasma NfL and ultra-sensitive HIV RNA in the overall cohort at A) randomisation ($n = 32$), B) week 12 ($n = 44$) and C) week 18 ($n = 44$) were analysed using Spearman's rank statistics.

Furthermore, no significant correlations were observed between plasma NfL and ultra-sensitive plasma HIV RNA in the separate study arms at week 12 (ART-only: $r = 0.02$, $p = 0.95$ (Figure 83A) versus ART+V+V: $r = 0.27$, $p = 0.21$ (Figure 83B)) and week 18 (ART-only: $r = 0.12$, $p = 0.50$ (Figure 83C) versus ART+V+V: $r = -0.01$, $p = 0.96$ (Figure 83D)).

Figure 83: Correlation between plasma NfL and ultra-sensitive plasma HIV RNA at weeks 12 and 18, by study arm



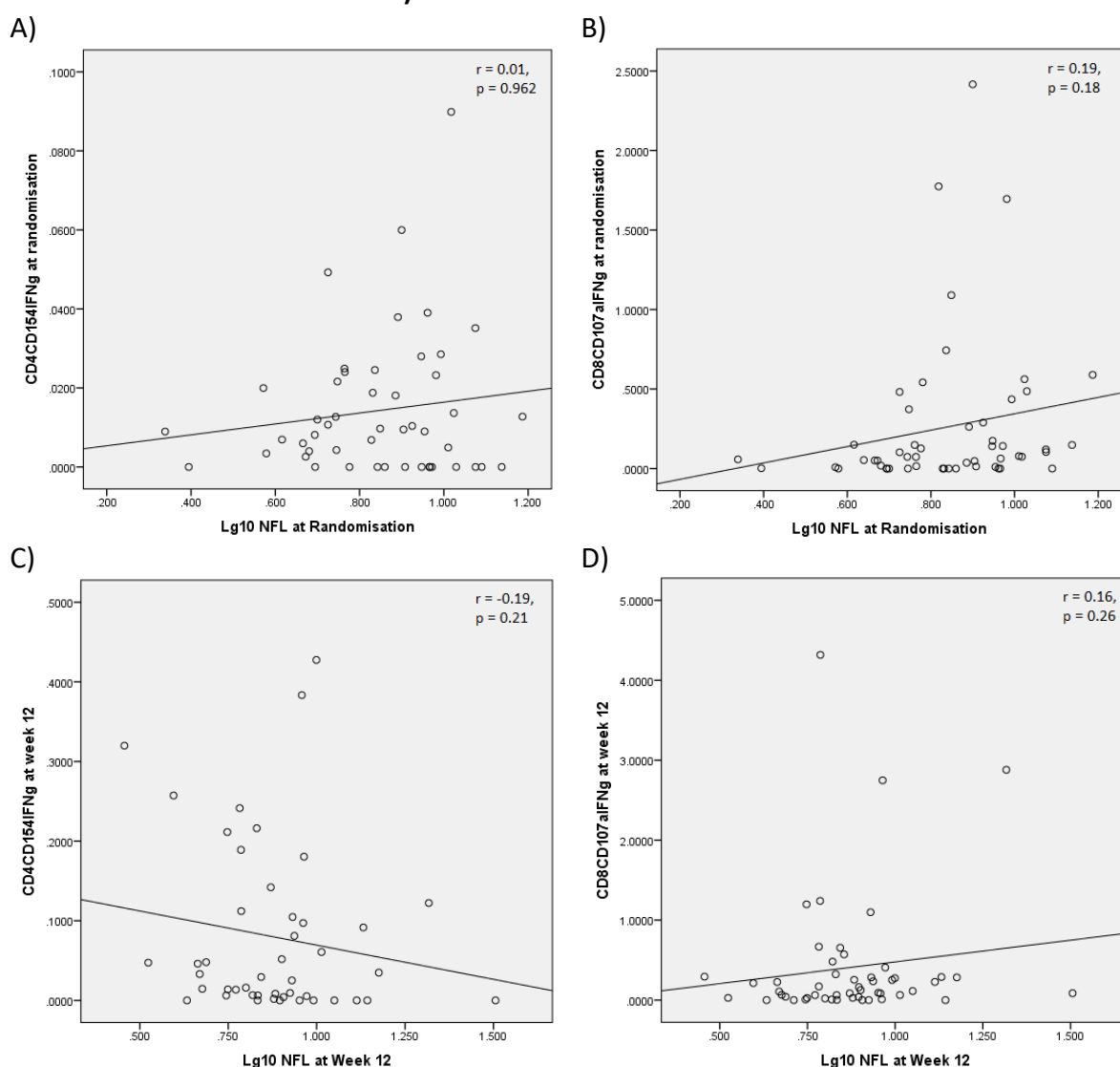
Abbreviation: NfL = neurofilament light chain protein

Legend: Correlation between plasma NfL and ultra-sensitive plasma HIV RNA at A) week 12 in the ART-only arm ($n = 21$), B) week 12 in the ART+V+V arm ($n = 23$), C) week 18 in the ART-only arm ($n = 21$) and D) week 18 in the ART+V+V arm ($n = 23$) were analysed using Spearman's rank statistics.

7.3.6.4 Correlations with polyfunctional CD4⁺ T-cell and CD8⁺ T-cell responses

No significant correlations were observed between plasma NfL in the overall cohort and polyfunctional CD4⁺ T-cell and CD8⁺ T-cell responses at randomisation ($r = 0.01$, $p = 0.96$ (Figure 84A) and $r = 0.19$, $p = 0.18$ (Figure 84B), respectively) and week 12 ($r = -0.19$, $p = 0.21$ (Figure 84C) and $r = 0.16$, $p = 0.26$ (Figure 84D), respectively).

Figure 84: Correlations between plasma NfL and polyfunctional CD4⁺ and CD8⁺ T-cell responses in the overall cohort at randomisation and week 12 (4 weeks after the final vaccination in the ART+V+V arm)

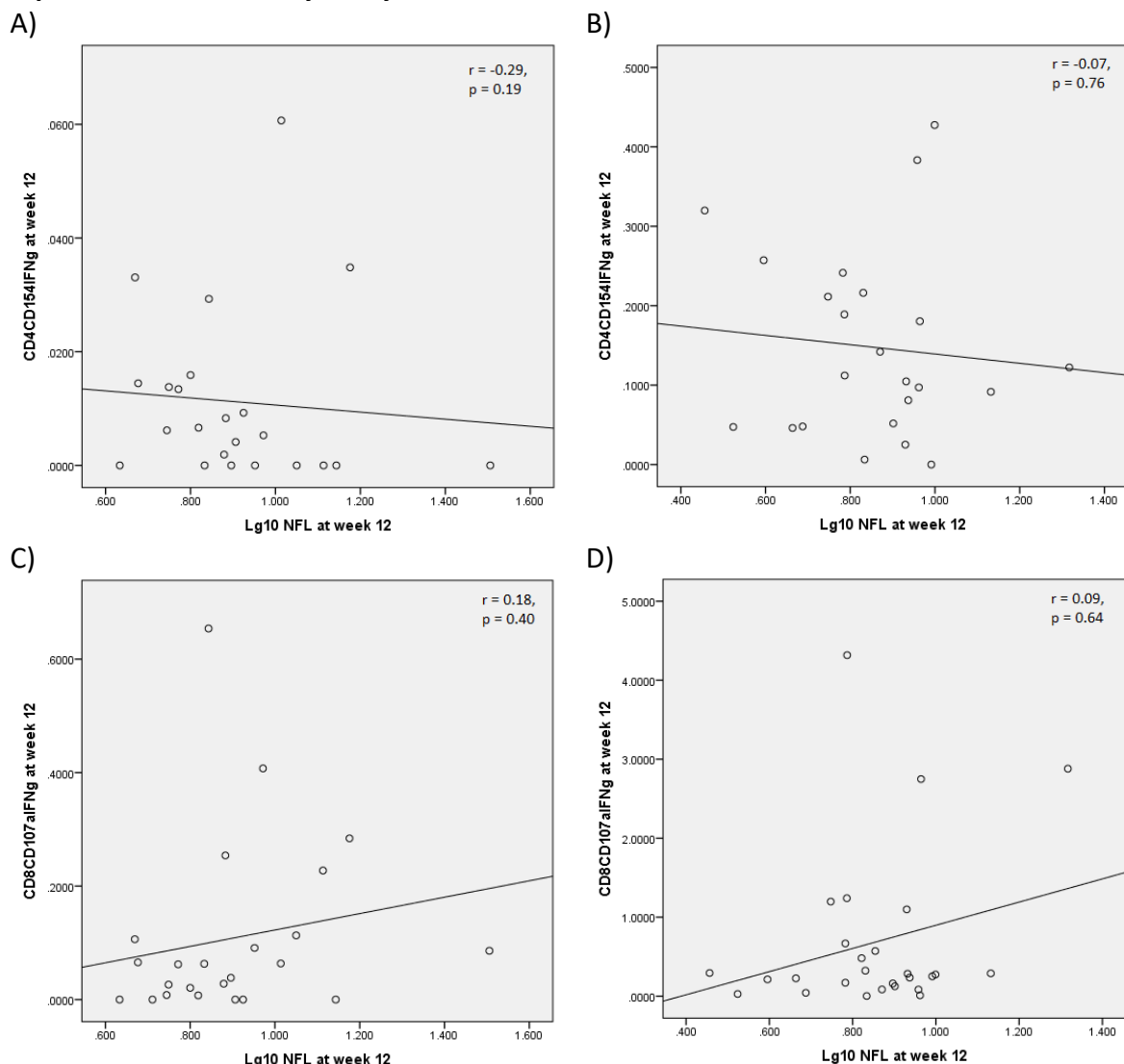


Abbreviation: NfL = neurofilament light chain protein

Legend: Correlations between plasma NfL and A) polyfunctional CD4⁺ T-cell responses at randomisation ($n = 50$), B) polyfunctional CD8⁺ T-cell responses at randomisation ($n = 54$), C) polyfunctional CD4⁺ T-cell responses at week 12 ($n = 46$) and D) polyfunctional CD8⁺ T-cell responses at week 12 ($n = 51$) were analysed using Spearman's rank statistics.

Similarly, no significant correlations were noted between plasma NfL and polyfunctional CD4⁺ T-cell and CD8⁺ T-cell responses by study arm at week 12. For polyfunctional CD4⁺ T-cell responses in the ART-only versus ART+V+V arms: $r = -0.29$, $p = 0.19$ (Figure 85A) versus $r = -0.07$, $p = 0.76$ (Figure 85B), respectively and for polyfunctional CD8⁺ T-cell responses in the ART-only versus ART+V+V arms: $r = 0.18$, $p = 0.40$ (Figure 85C) and $r = 0.09$, $p = 0.64$ (Figure 85D), respectively.

Figure 85: Correlation between plasma NfL and polyfunctional CD4⁺ and CD8⁺ T-cell responses at week 12, by study arm



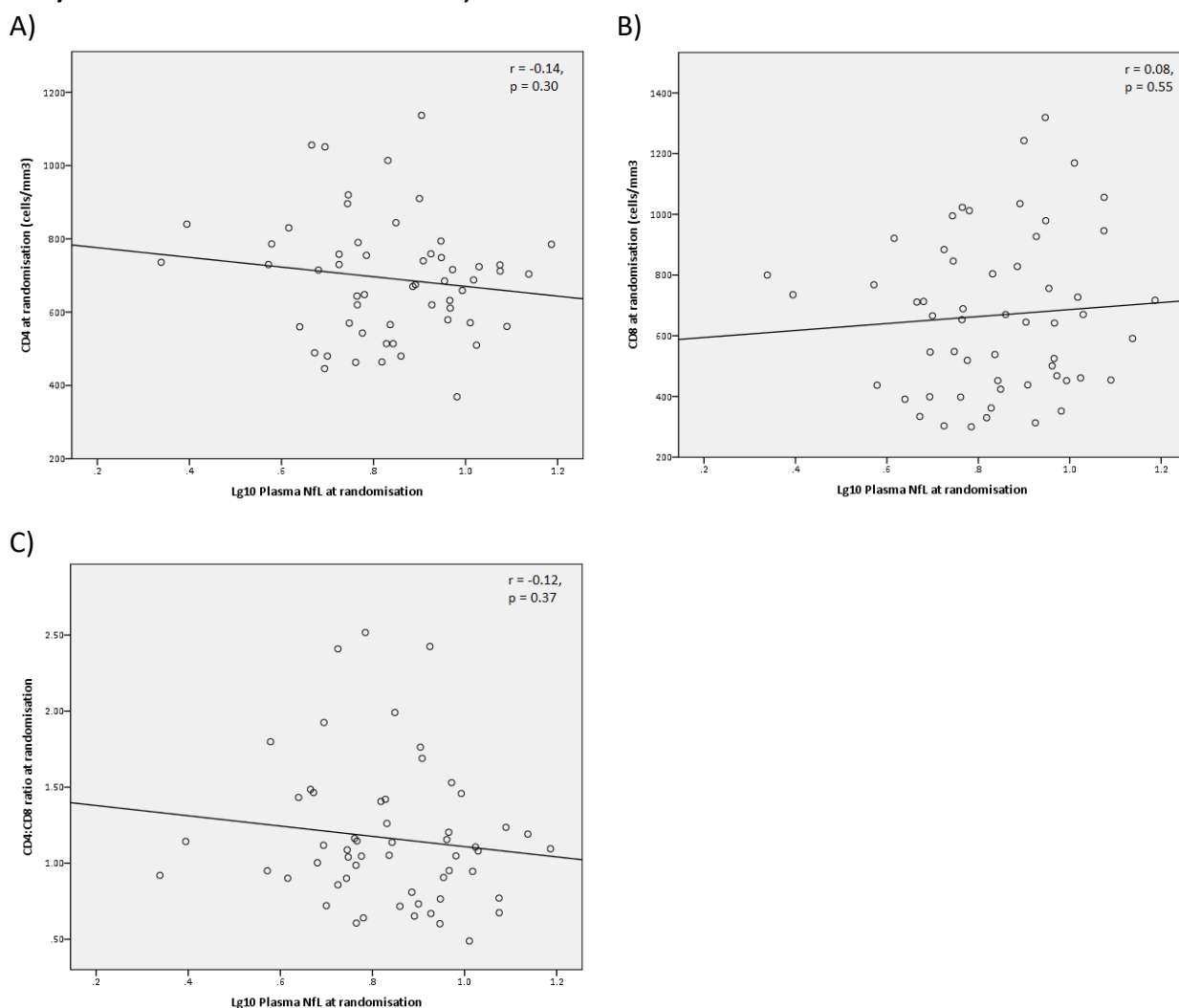
Abbreviation: NfL = neurofilament light chain protein

Legend: Correlation between plasma NfL at week 12 and polyfunctional CD4⁺ T-cell responses in the A) ART-only arm (n = 23), B) ART+V+V arm (n = 23), and polyfunctional CD8⁺ T-cell responses in the C) ART-only arm (n = 24) and D) ART+V+V arm (n = 27) were analysed using Spearman's rank statistics.

7.3.6.5 Correlations with CD4⁺ T-cell count, CD8⁺ T-cell count and CD4⁺/CD8⁺ ratio at randomisation

At randomisation, no significant associations were seen between plasma NfL and CD4⁺ T-cell count ($r = -0.14$, $p = 0.30$) (Figure 86A), CD8⁺ T-cell count ($r = 0.08$, $p = 0.55$) (Figure 86B) and CD4⁺/CD8⁺ ratio ($r = -0.12$, $p = 0.37$) (Figure 86C).

Figure 86: Correlation between plasma NfL and CD4⁺ T-cell count, CD8⁺ T-cell count and CD4⁺/CD8⁺ ratio in the overall cohort, at randomisation



Abbreviation: NfL = neurofilament light chain protein

Legend: Correlations between plasma neurofilament light chain protein and A) CD4⁺ T-cell count ($n = 58$), B) CD8⁺ T-cell count ($n = 58$) and C) CD4⁺/CD8⁺ ratio ($n = 58$) at randomisation were analysed using Spearman's rank statistics.

7.3.7 Factors associated with baseline plasma NfL

In multivariate linear regression analysis, higher plasma NfL at randomisation was independently associated with age (0.12 (95% confidence interval (CI) 0.05, 0.19) $\log_{10}$ NfL higher per 10 years older, $p = 0.001$), body mass index (-0.02 (95% CI -0.03, 0.00) $\log_{10}$ NfL per kg/m^2 , $p = 0.01$) and CD8^+ T-cell count (0.02 (95% CI 0.01, 0.04) $\log_{10}$ NfL higher per 100 cells/ μL increase, $p = 0.01$) (Table 42).

Table 42: Multivariable linear regression model to identify factors associated with plasma NFL at randomisation, $n = 58$

Variable	Parameter estimate ¹ (95% confidence interval)	p -value
Age (per 10 years older)	0.12 (0.05, 0.19)	0.001*
Body mass index, kg/m^2	-0.02 (-0.03, 0.00)	0.01*
White ethnicity	-0.02 (-0.13, 0.09)	0.66
Time since PHI diagnosis (per 5 weeks increase)	0.01 (-0.01, 0.03)	0.25
eGFR (per 10 $\mu\text{mol}/\text{L}/\text{min}$ increase)	0.00 (-0.04, 0.03)	0.78
CD4^+ T-cell count, cells/ μL (per 100 cells/ μL increase)	-0.02 (-0.03, 0.02)	0.20
CD8^+ T-cell count, cells/ μL (per 100 cells/ μL increase)	0.02 (0.01, 0.04)	0.01*
Total HIV DNA (per 1 $\log_{10}$ copy/ 10^6 CD4^+ T-cell increase)	-0.05 (-0.15, 0.05)	0.28
Ultra-sensitive plasma HIV RNA (per 1 $\log_{10}$ copies/mL increase)	0.03 (-0.04, 0.09)	0.37

¹Parameter estimates reflect the associated impact of each independent variable in the model on $\log_{10}$ plasma NFL.

7.4 Discussion

In this chapter, I set out to investigate the association between plasma NfL, a surrogate marker of neuro-axonal injury and thus, neural activation and measures of 1) HIV reservoir size and 2) HIV viral transcription.

Using two surrogate measures of HIV reservoir size, I observed no evidence of significant associations between total HIV DNA and integrated HIV DNA in CD4⁺ T-cells isolated from PBMCs with a marker of neuro-axonal injury at any of the time points when these markers were measured, as an overall group nor in the separate study arms. **With these findings, I am unable to confirm my first hypothesis that lower biomarker concentrations of HIV reservoir size are associated with lower biomarker concentrations of neural activation.**

As mentioned in Chapter 1: Introduction, to date, the optimal method(s) for quantifying the HIV reservoir is still being debated. Investigating the correlation between plasma NfL and HIV reservoir concentration measured using one of the more novel and accurate assays such as IPDA or Q4PCR in participants in the RIVER trial may enhance my ability to identify associations between these factors.

By investigating cell-associated unspliced HIV RNA in CD4⁺ T-cells isolated from PBMCs and ultra-sensitive plasma HIV RNA as direct measures of HIV viral transcription, I observed no significant association with plasma NfL (a measure of neuro-axonal injury) at any of the time points when these markers were measured and in either study arm. **Therefore, I was not able to confirm my second hypothesis that higher biomarker concentrations of HIV viral transcription are associated with higher biomarker concentrations of neural activation.**

Given that the basis of my PhD is to investigate the effect of “shock and kill” in the central nervous system, ideally, I would evaluate cerebrospinal fluid biomarkers of neuro-axonal injury, inflammation, HIV reservoir size and HIV viral transcription, and include neuroimaging studies as part of the assessment of participants taking part in HIV “shock and kill” studies, to provide further insight into the impact of “shock and kill” strategies on the central nervous system.

Despite evidence of robust vaccine-induced HIV-specific T-cell responses and vorinostat activity as measured by histone deacetylation[338], I observed no evidence of increased neuro-axonal injury using plasma NfL as a surrogate biomarker following this “shock and kill” strategy in participants who initiated ART during primary HIV infection. Specifically, I saw no evidence of increased neuro-axonal injury by study arm or longitudinally following this “shock and kill” strategy in this setting. My results are in keeping with published literature demonstrating no evidence of central nervous system adverse effects as assessed by cerebrospinal fluid biomarkers following panobinostat and vorinostat (histone deacetylase inhibitors) administration[264,457]. However, the observed lack of effect of this “shock and kill” intervention in the RIVER trial on the different measures of the HIV reservoir size in circulating CD4⁺ T-cells and plasma viral transcription suggests that the LRA used here may have had limited impact on latency reversal, which may then also explain the absence of evidence of neuro-axonal injury.

I postulated that the HIV-specific CD4⁺ and CD8⁺ T-cells induced by the prime/boost T-cell vaccination (ChAdV63.HIVconsv and MVA.HIVconsv) used in the RIVER trial would specifically target the viral antigens expressed on latently infected cells as a result of vorinostat-induced viral transcription and cause higher HIV-killing activity, thus leading to higher measures of neuronal injury. However, despite robust vaccine-induced HIV-specific peripheral CD4⁺ and CD8⁺ T-cell responses in participants post-vaccination, I observed no significant associations between HIV-specific peripheral CD4⁺ and CD8⁺ T-cell responses and measures of neuro-axonal injury before and after HIV-specific vaccination in the ART+V+V arm and in the ART+V+V arm at both time points. This suggests that the prime/boost T-cell vaccination used in the RIVER trial is safe from a neurological perspective.

CD8⁺ T-cells are associated with enhanced control of viral replication during acute HIV infection[458,459], leading to reductions in HIV viral transcription, increased viral reservoir decay[460] and total CD4⁺ T-cell count recovery (a validated surrogate marker of HIV disease progression[461]). Low CD4⁺/CD8⁺ T-cell ratio in treated HIV infection has been independently associated with persistently elevated T-cell activation markers[462] and measures of HIV viral reservoir[463]. However, my data demonstrates no significant association between measures of neuro-axonal injury and CD4⁺ T-cell count, CD8⁺ T-cell

count (as a measure associated with HIV reservoir size and HIV viral transcription) and CD4⁺/CD8⁺ T-cell ratio (as a measure associated with HIV reservoir size) at randomisation.

On the other hand, higher CD8⁺ T-cell count, older age and lower body mass index were independently associated with higher plasma NfL in multivariable regression analysis. The independent association between higher CD8⁺ T-cell count and higher plasma NfL is novel and unexpected, given the poor correlation in univariate analysis. This finding may indicate that higher CD8⁺ T-cell counts may be associated with increased neuronal injury, potentially via enhanced control of HIV viral replication, or may indicate a collinearity effect with another unmeasured factor. Older age and lower body mass index have been previously demonstrated to be associated with higher NfL[294,389,456,464]. However, while age-related reference values for cerebrospinal fluid NfL have been defined[285], similar reference values for plasma NfL are yet to be ascertained. In keeping with our findings from the COBRA cohort in Chapter 4, lower bodyweight appears to be associated with higher plasma NfL, perhaps indicative of a lower volume of distribution and hence higher plasma NfL concentration. Of note, neither total HIV DNA nor ultra-sensitive HIV RNA independently associated with plasma NfL at randomisation (following ≥ 22 weeks cART and prior to intervention in the ART+V+V arm).

The significance of the longitudinal intra-individual variations in plasma NfL is unclear; several participants had outlying plasma NfL measurements in this sub-study. However, none of these participants reported concomitant central nervous system adverse events when increased plasma NfL concentrations were measured. On the other hand, six participants reported central nervous system adverse events over the course of the RIVER trial, none of whom had plasma NfL concentrations above what is considered normal at any time point in this analysis.

Strengths of this analysis include the random allocation of participants in the RIVER trial to the control arm (ART only) and the “shock and kill” arm (ART+V+V), which enables the assessment of the impact of “shock and kill” without risk of bias through confounding by indication. However, several limitations need to be addressed; all participants enrolled into the RIVER trial initiated cART during primary HIV infection, when HIV reservoir size is presumed to be low but also erratic[112]. The impact of “shock and kill” on markers of

neuronal injury in participants who initiated cART during chronic HIV infection when HIV reservoir size is larger but stable, is unknown. Clinical trials investigating various HIV remission strategies in participants with chronic HIV infection are currently underway[465], which will provide more insight into this question. Whilst the results I present are reassuring, the majority of participants enrolled into the RIVER trial were young, white men who have sex with men, and it is unclear whether these results can be extrapolated to other populations, such as women and children. High proportions of activated CD4⁺ T-cells harbouring HIV DNA have been demonstrated in adipose tissue, suggesting this is an additional tissue reservoir to consider[466]. Men and women have well-described differences in body fat content and distribution, and adipocyte function is regulated by oestrogen. Therefore, the size and distribution of the HIV reservoir between men and women could be very different and the outcomes (systemic and central nervous system) following “shock and kill” approaches may vary by gender. A concern is that the central nervous system may be particularly vulnerable to latency reversal, reactivation of viral transcription, immune activation and neuroinflammation, given the limited space for expansion within the skull. This issue could be particularly detrimental in children whose brains are not yet fully developed and may be more susceptible to neuronal injury and long-lasting damages following HIV remission strategies such as “shock and kill”.

The extent to which this finding can be translated to more potent LRAs is unknown and it is possible that viral transcription with other LRAs may lead to more neuronal inflammation and injury. Clinical parameters, such as patient-reported outcome measures of central nervous system function or cognitive testing, were not performed. Such parameters may have provided further clinical insights, however, given the bluntness of such clinical tools, we may be unlikely to observe any changes in such parameters in this cohort of neuro-asymptomatic participants.

Published studies report a variation in the degree of correlation between cerebrospinal fluid and blood NfL, with stronger correlations seen in conditions with higher cerebrospinal fluid and blood NfL concentration. In both study arms of this sub-study, plasma NfL concentrations were generally low and within the range of plasma NfL concentrations seen in HIV-negative controls (median plasma NfL (IQR) 9.3 pg/mL (5.9, 13.1)[293], and the current plasma NfL assay may have been insufficiently sensitive to detect subtle changes at

these low plasma NfL concentrations. Given the low plasma NfL concentration ranges seen in this sub-study, the correlation between blood and cerebrospinal fluid in this setting is likely to be moderate and the lack of concurrent cerebrospinal fluid NfL restricts an accurate interpretation about the corresponding NfL trends in the central nervous system. I selected the time points to measure plasma NfL as a best estimate for when I was likely to see a signal in plasma NfL concentration following the intervention. It is possible that the time points chosen missed the changes in plasma NfL concentration due to the intervention, and more frequent serial plasma NfL measurements may have detected changes related to the intervention.

While increased peripheral histone acetylation was observed following vorinostat dosing, vorinostat concentration and histone acetylation in the brain was not measured, thus I was unable to conclude whether vorinostat crossed the blood-brain barrier and acted on the central nervous system HIV reservoir. Preliminary studies suggest that the current form of vorinostat does not cross the blood-brain barrier, but vorinostat encased in a lipid chelator and polyethylene glycol may improve central nervous system bioavailability[467]. However, the central nervous system will have to be monitored closely following the administration of vorinostat in this modified form, given that a higher central nervous system penetration and possibly larger effect on viral replication, immune activation and inflammation might be anticipated.

The RIVER trial was powered for its primary endpoint (total HIV DNA per 10^6 CD4+ T-cells isolated from PBMCs at post-randomisation week 18, by study arm), but this sub-study may have been insufficiently powered to detect subtle but possible clinically relevant changes in plasma NfL in this setting.

7.5 Conclusions

I observed no clear evidence of associations between markers of HIV reservoir size and HIV viral transcription with measures of neuro-axonal injury in the control or “shock and kill” arm in this setting. Furthermore, despite evidence of vaccine-induced HIV-specific T-cell responses and histone deacetylation[338], I observed no evidence of increased neuro-axonal injury using plasma NfL as a surrogate biomarker, up to 18 weeks following this “shock and kill” strategy compared with ART-only in the RIVER trial. Based on these findings, significant neuronal injury was unlikely following this “shock and kill” strategy in this cohort of individuals, supporting the central nervous system safety of this strategy. My findings add to the literature supporting the central nervous system safety of HIV “shock and kill”, however continued monitoring for central nervous system adverse events remains an important aspect of HIV-remission research as alternative and more potent HIV “shock and kill” approaches may affect the central nervous system differently compared to the approach utilised within the RIVER study.

Chapter 8

**Investigating neuro-axonal
injury following cART initiation
in primary HIV infection,
followed by cART interruption:
the SPARTAC trial**

Chapter 8: Investigating neuro-axonal injury following cART initiation in primary HIV infection, followed by cART interruption: the SPARTAC trial

8.1 Introduction

The introduction of cART has significantly reduced morbidity and mortality for PLWH[133]. However, cART alone cannot cure HIV and following cART interruption, plasma viral load rebound occurs within four to eight weeks in the majority of PLWH[62], with a delay in cerebrospinal fluid viral load rebound by around two weeks[468]. For this reason, research efforts into HIV remission strategies that can confer sustained viral control and normal immune function in the absence of cART have intensified, with various strategies currently under trial globally[59,251]. However, to date, there are no validated assays which can accurately confirm whether a HIV remission intervention has worked and the only true way of verifying this is by performing an analytical antiretroviral treatment interruption (ATI), whereby cART is interrupted and time to viral load rebound following ATI is measured[262].

While the SMART study identified that interrupting cART for long periods of time and using a CD4⁺ count re-initiation criteria was unsafe[137], recent HIV remission research suggests that with strict viral load monitoring and rapid cART re-initiation when viral load rebounds beyond a pre-specified threshold is confirmed, short-term ATI in pre-selected healthy individuals may be safe and the best way of confirming HIV remission[262].

Following ATI, the source of viral rebound is from the pool of latently infected cells, termed the HIV reservoir[469]. The main HIV reservoir is thought to lie within resting memory CD4⁺ T-cells in circulation and lymphoid tissue[470]. However, other potential tissue HIV reservoir compartments such as bone marrow, gut-associated lymphoid tissue and central nervous system have recently been identified[64,79,266,402,449,450]. Increasingly convincing evidence demonstrates that from early on in the course of HIV infection, cells within the central nervous system such as microglia, macrophages and astrocytes are infected with

HIV[450], and can become latently infected with HIV[79]. Thus, central nervous system may represent an important source of rebounding virus during ATI[79,266].

It is well-documented that primary HIV infection can be associated with neurological consequences such as aseptic meningitis and transverse myelitis, which often resolves with cART initiation[471]. An important concern with ATI is that the central nervous system may once again be vulnerable to the clinical effects of viral resurgence[472], with potentially serious neurological consequences for participants undergoing ATI as part of a study. The neurological consequences can be caused by direct neuronal injury (HIV viral proteins such as Tat and gp120 are thought to be directly neurotoxic[473–475]) or via indirect neuronal injury caused by the immune activation and neuro-inflammation associated with uncontrolled viral replication within the central nervous system in the absence of cART [36,266,290].

Observational cohort studies have demonstrated that prolonged periods of untreated HIV with active HIV viral replication are associated with neuronal biomarkers of injury[276] but the reasons for stopping cART may be a potential bias. On the other hand, very short periods of protocol-indicated ATI have not demonstrated detectable neuro-axonal injury nor central nervous system adverse events [472,476].

The **Short Pulse Anti-Retroviral Therapy At SeroConversion** (SPARTAC) trial was a multi-site, randomised controlled trial investigating whether a 12- or 48-week course of cART initiated during primary HIV infection can lengthen the time until patients reach a CD4⁺ T-cell count of <350 cells/mm³ or require long-term cART compared with not receiving immediate cART[140]. Data and samples from participants in the SPARTAC trial provide a unique and valuable resource to answer the question on the impact of prolonged ATI on neuro-axonal injury in people who initiated cART during primary HIV infection, given the protocol-indicated cART cessation, minimising the risk of confounding by indication.

In this chapter, I test both my first hypothesis that lower biomarker concentrations of HIV reservoir size are associated with lower biomarker concentrations of neural activation **and my second hypothesis** that higher biomarker concentrations of HIV viral transcription are associated with higher biomarker concentrations of neural activation.

To test my first hypothesis, I explored the association between a biomarker of neuro-axonal injury (plasma NfL) with total HIV DNA per 10^6 CD4⁺ T-cells (as a direct measure of HIV reservoir size) and duration between HIV seroconversion and cART initiation (as an indirect measure of HIV reservoir size). To test my second hypothesis, I explored the association between plasma NfL and plasma HIV RNA. This work was performed in a cohort of individuals who initiated 48 weeks of cART during primary HIV infection, followed by a period of cART interruption, as part of the SPARTAC trial.

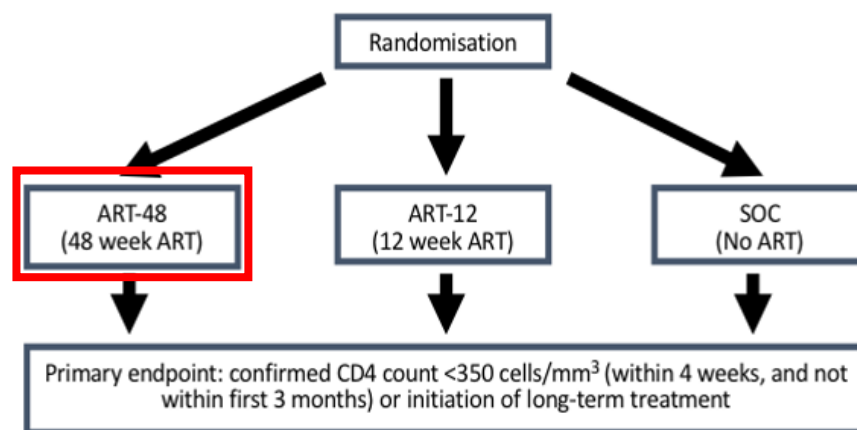
Additionally, I investigated whether stopping cART was associated with a change in neuro-axonal injury and analysed associations between clinical and laboratory parameters with neuro-axonal injury.

8.2 Methods:

8.2.1 Study design and participants

Short Pulse Anti-Retroviral Therapy At SeroConversion (SPARTAC)[477] was a multicentre, open-label, randomised controlled trial comparing 12 weeks cART (ART12) or 48 weeks cART (ART48), with deferred cART (standard of care at the time); amongst participants diagnosed within a maximum of six months of HIV seroconversion (Figure 87). Evidence of seroconversion were categorised according to: 1) a positive HIV-antibody test within 6 months after a negative test, 2) a negative HIV-antibody test with a positive reverse-transcription–polymerase-chain-reaction assay for HIV RNA, 3) a low level of HIV antibodies (optical density units <0.6) according to a serologic testing algorithm for recent infection (subtype B strain only), 4) an equivocal HIV-antibody test with a repeat test within 2 weeks showing an increase in the level of HIV antibodies and 5) clinical manifestations of symptomatic HIV seroconversion illness supported by antigen positivity and less than 4 positive bands on Western blot analysis.

Figure 87: SPARTAC trial schema and primary endpoint



Legend: Trial schema demonstrating the three study arms within the SPARTAC study and the definition of the primary endpoint. Abbreviations: ART = antiretroviral treatment, SOC = standard of care. The red box signifies the cohort included in the analysis in this chapter

I identified participants in the SPARTAC trial randomised to start immediate cART for 48 weeks during primary HIV infection (ART48) (as indicated in the red box in Figure 87) with sufficient stored plasma samples at the following four time points:

- i. Baseline (on the day cART was commenced)
- ii. Week 48 (after 48 weeks of cART)
- iii. Week 60 (12 weeks after stopping cART)
- iv. Week 84 (36 weeks after stopping cART)

Participants on cART at any of these time points were not included in this analysis.

8.2.2 Blood NfL measurement

As part of my PhD, plasma NfL was measured in samples from baseline, week 48 and week 60 at the Clinical Neurochemistry Laboratory at Sahlgrenska University Hospital, Sweden using the NF-Light kit (UmanDiagnostics AB, Sweden), transferred onto the Simoa platform using a homebrew kit (Quanterix™, USA)[293] (Table 43). I measured plasma NfL on samples from week 84 at the UK Dementia Research Institute, University College London, UK using the commercially available NF-light assay on a HD-1 Simoa instrument (Quanterix™, USA)[342] (Table 43).

8.2.3 Other biomarkers measured within the original SPARTAC trial

Blood sample collection was performed at each scheduled study visit with the following tests performed as part of the SPARTAC study:

- i. Full blood count
- ii. Biochemistry (including creatinine)
- iii. CD4⁺ and CD8⁺ T-cell counts
- iv. Plasma HIV RNA
- v. Plasma storage for future research purposes

Plasma D-dimer (Innovance D-dimer (Siemens Healthcare Diagnostics, Tarrytown, New York, USA)) and plasma interleukin-6 (IL-6) (Quantikine HS IL-6 immunoassay (R&D Systems, Minneapolis, Minnesota, USA)) were measured previously in a subset of participants enrolled in Brazil, Australia, Italy and the United Kingdom at baseline, week 48 and week 60[478] (Table 43). Total HIV DNA from CD4⁺ T-cells enriched from peripheral blood mononuclear cells was measured previously in participants with clade B virus only at baseline and week 48[345] (Table 43).

Table 43: Biomarkers analysed in the SPARTAC trial sub-study and time points when the biomarkers were measured

Biomarker	Time points			
	Baseline (on the day cART was commenced)	Week 48 (after 48 weeks of cART)	Week 60 (12 weeks after stopping cART)	Week 84 (36 weeks after stopping cART)
Plasma NfL, pg/mL	✓	✓	✓	✓
Plasma HIV RNA, copies/mL	✓	✓	✓	✓
CD4 ⁺ and CD8 ⁺ T- cell counts, cells/ μ L	✓	✓	✓	✓
Plasma D-dimer	✓	✓	✓	
Plasma IL-6	✓	✓	✓	
Total HIV DNA in CD4 ⁺ T-cells isolated from peripheral blood	✓	✓		

Abbreviations: cART = combination antiretroviral treatment, NfL = neurofilament light chain protein, IL-6 = interleukin-6

8.2.4 Statistical analysis

The sample size calculation for this sub-study was based on the comparison of plasma NfL in patients of the ART48 arm at weeks 48 and week 60, using a two-sample paired-means test with plasma NfL on a log₁₀-scale and a two-sided α of 0.05 for the null hypothesis that there is no difference between the two time-points.

I assumed that after stopping ART, plasma NfL would increase by 17.5%. On a log₁₀-scale, this corresponds to a difference of 0.07. Further, I assumed a standard deviation of 0.25 for a single measurement at both time-points based on Gisslen et al[293], and a correlation coefficient of 0.6 between the week 48 and week 60 measurements. Then, a sample size of 82 individuals would provide 80% power to detect this difference.

Descriptive statistics were described using median (interquartile range), geometric mean (95% confidence interval) and total (percentage), as required. Baseline characteristics between participants in the ART48 arm included in this analysis versus those excluded and baseline characteristics between male and female participants in the ART48 arm included in this analysis were compared using the Mann-Whitney test, two-sample t-test with equal variances, chi-square and Fisher's exact test, as appropriate. Where data demonstrated Normal distribution, parametric tests were employed, whereas not Normally distributed data were analysed using non-parametric tests.

Plasma NfL, plasma HIV RNA, plasma D-dimer, plasma IL-6 and total HIV DNA in CD4⁺ T-cells isolated from peripheral blood were log₁₀-transformed, where appropriate, to reduce data skewness. Paired-samples T-test (a parametric statistical test) was used to assess changes in plasma NfL, plasma HIV RNA, plasma IL-6, plasma D-dimer and total HIV DNA from baseline to week 48, week 48 to week 60 and week 48 to week 84 (where available), given the Normal distribution of the data. Plasma NfL concentrations greater than 13pg/mL were considered abnormal, based on the publication by Gisslén *et al*[293]. The lower limit of quantification for plasma HIV RNA was <50 copies/mL, except in Africa, where it was <400 copies/mL. As per the publication by Williams *et al*[345], total HIV DNA $\geq 3.88 \log_{10}$ total HIV DNA copies/mL was categorised as 'high' and total HIV DNA $<3.88 \log_{10}$ total HIV DNA copies/mL was categorised as 'low'.

Correlations between plasma NfL and laboratory parameters were assessed using Pearson's correlation. At weeks 48, 60 and 84, a two-sample T-test of equal variance was performed to investigate whether plasma NfL differed significantly in those with detectable versus undetectable plasma HIV RNA. These parametric statistical tests were used given that the data were Normally distributed.

The following factors: gender, age, plasma HIV RNA, CD4⁺ count, CD8⁺ count, CD4⁺/CD8⁺ ratio, duration between seroconversion and randomisation, serum creatinine, site of recruitment, weight and body mass index, and in subgroups with plasma D-dimer, plasma IL-6 and total HIV DNA data were included in univariate regression analyses at the four time points; factors significantly associated with plasma NfL were included in multivariable linear regression models.

Sample size calculations and the statistical analysis for this project was performed by myself, under the supervision of Professor Abdel Babiker and Dr Wolfgang Stohr, the SPARTAC trial biostatisticians based at the MRC Clinical Trials Unit at University College London.

8.3 Results:

Of 123 participants randomised to ART48, 83 had stored plasma available from at least two of the timepoints and were included in this analysis. Baseline characteristics of the participants included in this analysis were similar to the participants in the ART48 arm[140].

8.3.1 Baseline characteristics of the participants included in this analysis

Of the 83 participants included, 50 were male and 33 were female. Overall median age of the participants was 34 years, with male participants being older (median 36 years) compared to the female participants (median 27 years) (Table 44). Participants were mainly recruited in the UK (40%) and South Africa (29%), with smaller numbers of participants recruited in Australia, Italy, Uganda, Spain and Brazil (Table 44). The majority of female participants were recruited in Africa (91%), whereas no male participants were recruited in Africa (Table 44). Male participants mainly acquired HIV via sex between men and harboured clade B virus, whereas all women acquired HIV via heterosexual intercourse, and most harboured clade C virus (Table 44).

Median overall time from HIV seroconversion to randomisation was 13 weeks, but shorter in male participants (11 weeks) compared to female participants (14 weeks) (Table 44). Median plasma HIV RNA at randomisation in the overall cohort was 4.6 log₁₀ copies/mL, but higher in the male participants (median 4.8 log₁₀ copies/mL) compared to the female participants (median 4.3 log₁₀ copies/mL) and 12% of women had plasma HIV RNA <400 copies/mL at baseline, compared to 4% of men (Table 44). Overall median CD4⁺ T-cell count was 612 cells/mm³, median CD8⁺ T-cell count was 1032 cells/mm³ and CD4⁺/CD8⁺ T-cell count ratio was 0.6, with no significant difference by gender (Table 44).

Table 44: Baseline characteristics of participants in the 48-week ART arm with plasma NfL measured at any time point

Values are median (IQR) or total (%)	All participants n = 83	Male n = 50	Female n = 33	p-value ^a
Age, years	34 (27, 41)	36 (31, 46)	27 (22, 37)	<0.001
Country				<0.001
UK	33 (40.0)	31 (62.0)	2 (6.1)	
South Africa	24 (28.9)	0	24 (72.7)	
Australia	9 (10.8)	9 (18.0)	0	
Italy	7 (8.4)	6 (12.0)	1 (3.0)	
Uganda	6 (7.2)	0	6 (18.2)	
Spain	2 (2.4)	2 (4.0)	0	
Brazil	2 (2.4)	2 (4.0)	0	
Route of HIV acquisition				<0.001
Sex between men	46 (55.4)	46 (92)	0	
Heterosexual intercourse	36 (43.4)	3 (6)	33 (100)	
Unknown	1 (1.2)	1 (2)	0	
Virus subtype				<0.001
B	44 (53.0)	43 (86.0)	1 (3.0)	
C	26 (31.3)	1 (2.0)	25 (75.8)	
Other	13 (15.7)	6 (12.0)	7 (21.2)	
Time from seroconversion to randomisation, weeks	13 (9, 15)	11 (7, 13)	14 (12, 17)	<0.001
Seroconversion within the previous 12 weeks	7 (8.4)	5 (10.0)	2 (6.1)	0.53
Evidence of seroconversion ^b				<0.001
Criterion 1 or 2	56 (67.5)	24 (48.0)	32 (97)	
Criterion 3 or 4 or 5	27 (32.5)	26 (52.0)	1 (3)	
Weight, kg	73 (65, 82)	75 (68, 83)	65 (54, 79)	<0.001
Body mass index, kg/m ²	25 (22, 27)	24 (22, 26)	26 (22, 32)	0.008
Serum creatinine, µmol/L	79 (70, 88)	85 (78, 95)	70 (63, 76)	<0.001
Plasma HIV RNA, lg ₁₀ cp/mL	4.6 (4.0, 5.2)	4.8 (4.2, 5.3)	4.3 (3.6, 4.9)	0.004
Plasma HIV RNA <400 cp/mL	6 (7.2)	2 (4.0)	4 (12.1)	0.16
CD4 ⁺ T-cell count, cells/mm ³	612 (471, 760)	577 (424, 755)	625 (484, 763)	0.45
CD8 ⁺ T-cell count, cells/mm ³	1032 (785, 1394)	1101 (868, 1486)	949 (752, 1271)	0.07
CD4 ⁺ /CD8 ⁺ ratio	0.6 (0.4, 0.8)	0.5 (0.3, 0.7)	0.6 (0.4, 1.0)	0.08

^a p-value refers to differences by gender, analysed using the Mann-Whitney test, two-sample t-test with equal variances, chi-square and Fisher's exact test, as appropriate

^b Evidence of seroconversion criterion: Criterion 1: a positive HIV-antibody test within 6 months after a negative test, criterion 2: a negative HIV-antibody test with a positive reverse-transcription-polymerase-chain-reaction assay for HIV RNA, criterion 3: a low level of HIV antibodies (optical density units <0.6) according to a serologic testing algorithm for recent infection (subtype B strain only), criterion 4: an equivocal HIV-antibody test with a repeat test within 2 weeks showing an increase in the level of HIV antibodies and criterion 5: clinical manifestations of symptomatic HIV seroconversion illness supported by antigen positivity and less than 4 positive bands on Western blot analysis.

Abbreviations: ART = antiretroviral treatment, NfL = neurofilament light chain protein.

8.3.2 Longitudinal biomarker parameter results

The longitudinal measurements of the research biomarker parameters included in this sub-study are detailed below in Table 45.

Table 45: Laboratory parameter trends

	Baseline: Before starting ART N = 82	Week 48: After 48 weeks of ART N = 71	Week 60: 12 weeks after stopping ART N = 83	Week 84: 36 weeks after stopping ART N = 65
Plasma NfL, pg/mL ^a	10.5 (8.8, 12.6)	9.1 (7.4, 11.2)	8.8 (7.5, 10.5)	9.9 (8.6, 11.3)
Plasma HIV RNA, log ₁₀ copies/mL	4.6 (4.0, 5.2)	1.7 (1.7, 2.6)	3.7 (2.6, 4.5)	3.9 (3.1, 4.6)
CD4 ⁺ T-cell count, cells/μL	612 (471, 760)	794 (611, 999)	714 (480, 868)	613 (464, 800)
CD8 ⁺ T-cell count, cells/μL	1032 (785, 1394)	780 (618, 969)	994 (739, 1285)	970 (701, 1257)
CD4: CD8 T-cell ratio	0.55 (0.41, 0.82)	0.98 (0.73, 1.32)	0.70 (0.49, 1.00)	0.61 (0.46, 0.98)
Subgroup analysis				
Plasma D-dimer, lg ₁₀ mg/L ^a	N=45 0.35 (0.29, 0.43)	N=35 0.28 (0.24, 0.32)	N=47 0.30 (0.25, 0.37)	n/a
Plasma IL-6, lg ₁₀ pg/mL ^a	N=46 1.38 (1.09, 1.75)	N=37 1.39 (1.07, 1.80)	N=47 1.45 (1.21, 1.74)	n/a
Total HIV DNA, log ₁₀ copies/10 ⁶ CD4 ⁺ T-cells	N=45 3.8 (3.5, 4.0)	N=51 3.3 (3.1, 3.4)	n/a	n/a

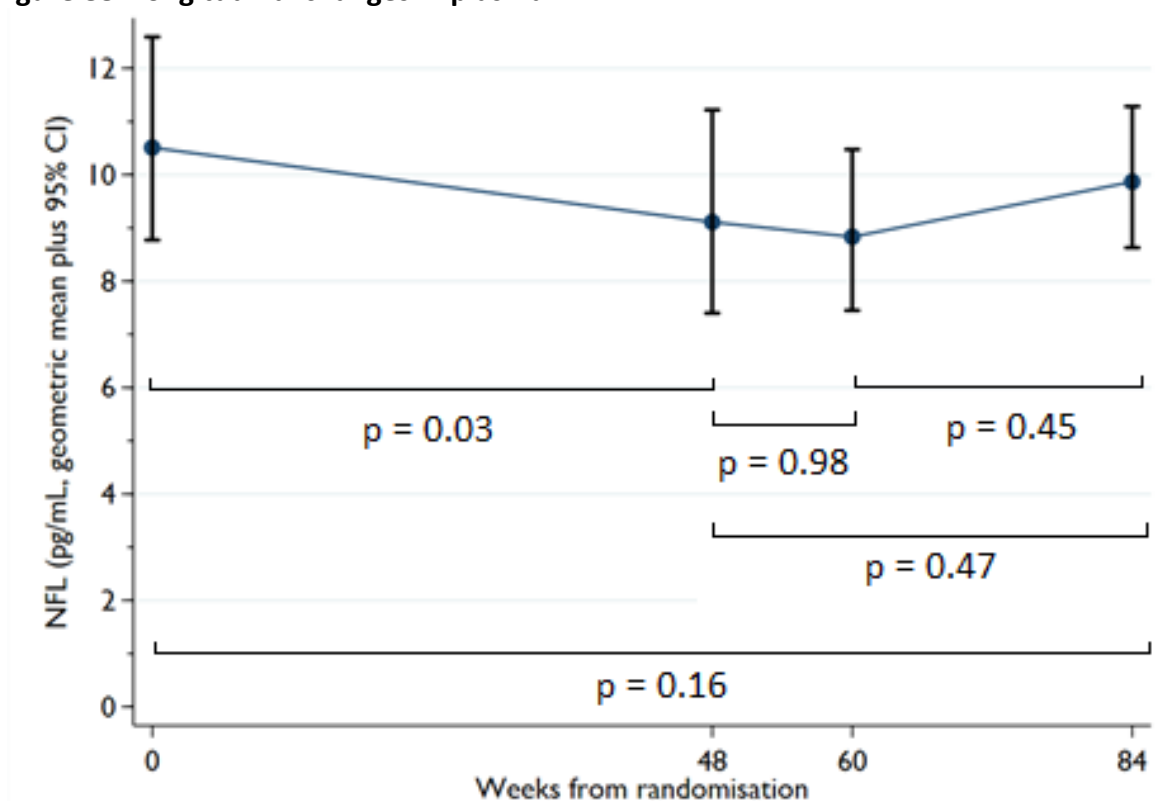
Abbreviations: ART = antiretroviral treatment, NfL = neurofilament light chain protein, IL-6 = interleukin-6.

Legend: Values are median (IQR) unless stated otherwise. ^a Values are geometric mean (95% confidence interval). n/a: not assessed at these timepoints.

8.3.3 Longitudinal trends in plasma NfL

Geometric mean plasma NfL decreased from 10.5 (95% confidence intervals (CI) 8.8, 12.6) at baseline to 9.1pg/mL (95% CI 7.4, 11.2) after 48 weeks ART, $p=0.03$ (Table 45 and Figure 88). ART was then stopped and geometric mean plasma NfL was 8.8 pg/mL (95% CI 7.5, 10.5) at 12 weeks after stopping ART (week 60), and 9.9 pg/mL (95% CI 8.6, 11.3) at 36 weeks after stopping ART (week 84) (Table 45 and Figure 88). No significant change in plasma NfL was seen between weeks 48 and 60 ($p=0.98$) or weeks 48 and 84 ($p=0.47$) (Figure 88) despite plasma viral rebound in most participants during this time period (Table 45 and Figure 89).

Figure 88: Longitudinal changes in plasma NfL



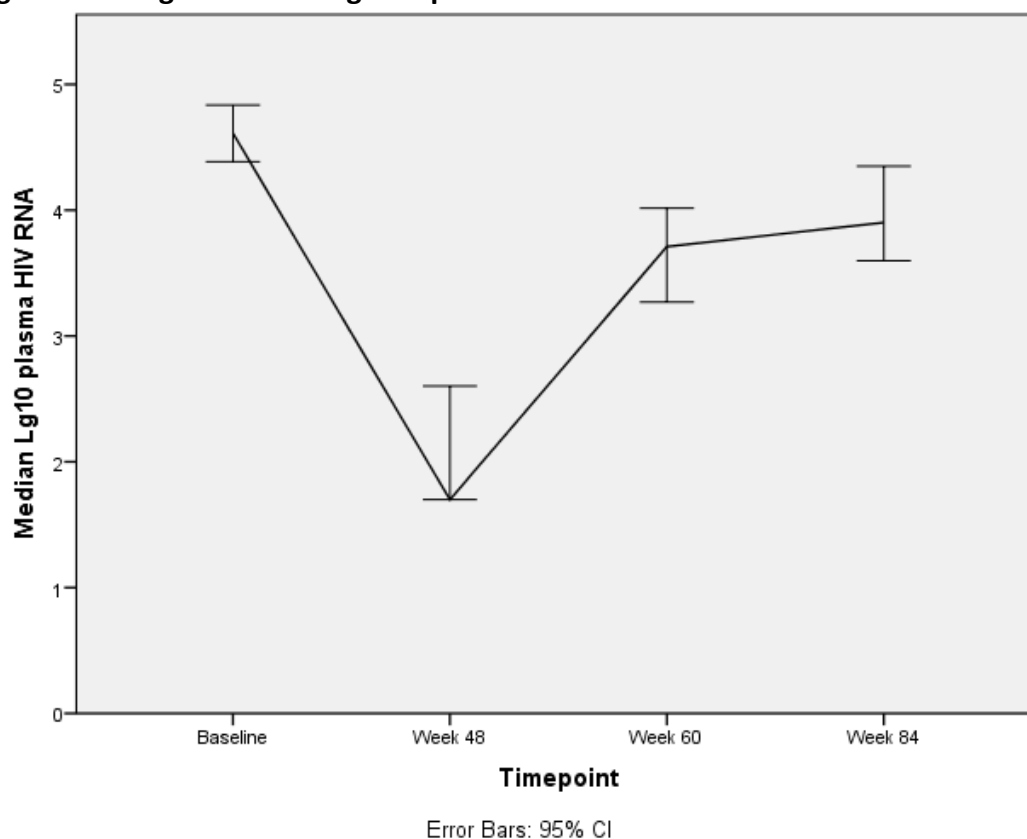
Abbreviations: NfL = neurofilament light chain protein, CI = confidence intervals

Legend: Line graph with error bars denoting 95% CIs demonstrating the longitudinal changes in plasma NfL. P-values were analysed using paired-samples T-test. Number of participants with plasma NfL results available at baseline ($n = 82$), week 48 ($n = 71$), week 60 ($n = 83$) and week 84 ($n = 65$).

8.3.4 Longitudinal trends in plasma HIV RNA

Of the four time points included in this analysis, median plasma HIV RNA was highest at baseline (during primary HIV infection, prior to initiating cART) at 4.6 HIV RNA log₁₀ copies/mL (interquartile range (IQR) 4.0, 5.2) (Table 45 and Figure 89), with 6 (7.2%) participants having plasma HIV RNA <400 copies/mL. At week 48, median (IQR) plasma HIV RNA was 1.7 HIV RNA log₁₀ copies/mL (1.7, 2.6) (Figure 89 and Table 45), with 59 (71%) of participants having plasma HIV RNA <400 copies/mL. Median (IQR) plasma HIV RNA then rose to 3.7 HIV RNA log₁₀ copies/mL (2.6, 4.5) and 3.9 HIV RNA log₁₀ copies/mL (3.1, 4.6) at weeks 60 and 84, respectively (Figure 89 and Table 45), with 21 (25%) and 10 (12%) participants having plasma HIV RNA <400 copies/mL at weeks 60 and 84, respectively.

Figure 89: Longitudinal changes in plasma HIV RNA



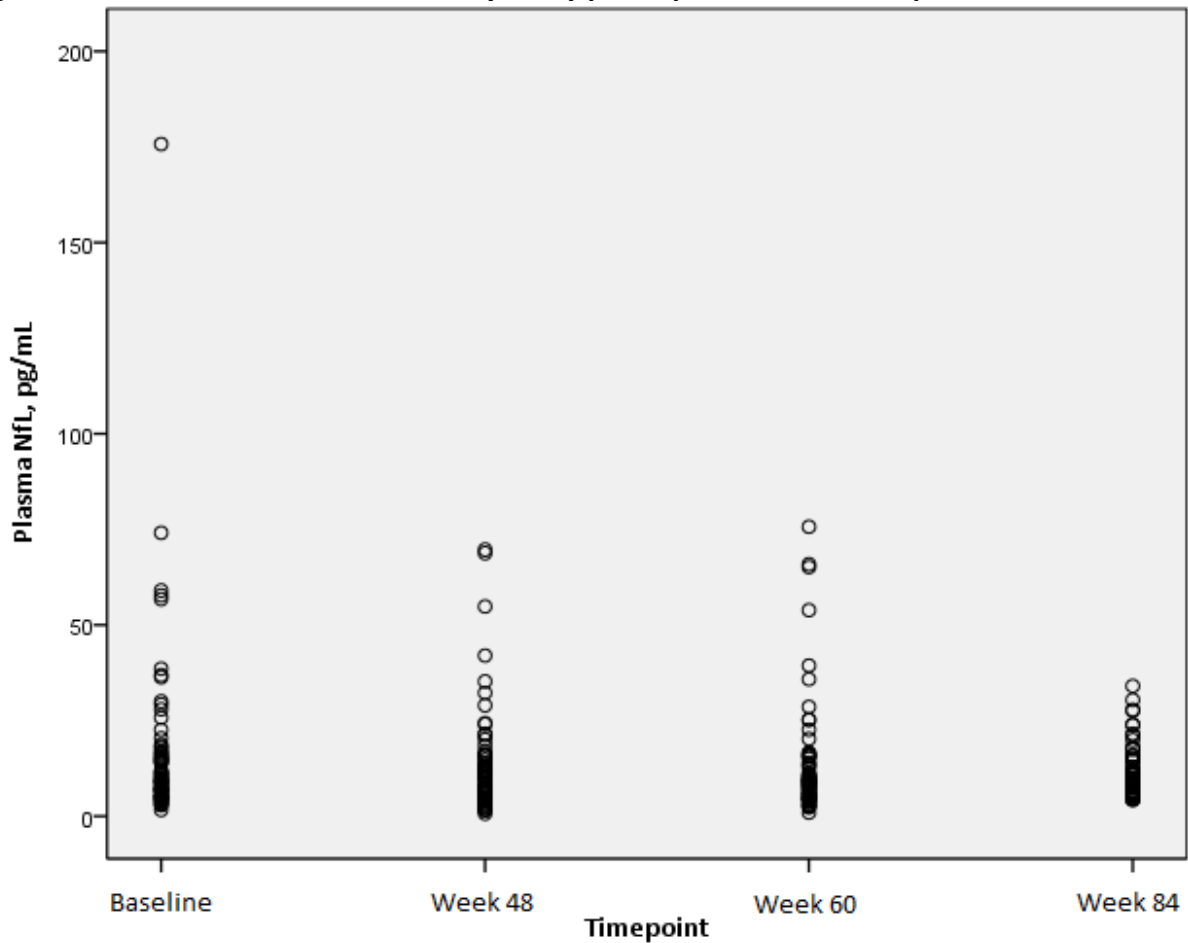
Abbreviations: CI = confidence intervals

Legend: Line graph with 95% CIs demonstrating the longitudinal changes in plasma HIV RNA. Number of participants with plasma HIV RNA results available at baseline (n = 82), week 48 (n = 71), week 60 (n = 83) and week 84 (n = 65)

8.3.5 Participant(s) with outlying plasma NfL measurement(s)

One participant was an outlier with plasma NfL measuring 175.8 pg/mL at baseline (Figure 90). This participant did not have a week 48 plasma sample available for this analysis, but plasma NfL measured at week 60 and week 84 were 4.7 pg/mL and 6.3 pg/mL, respectively.

Figure 90: Plasma NfL measurements by study participant at each time point

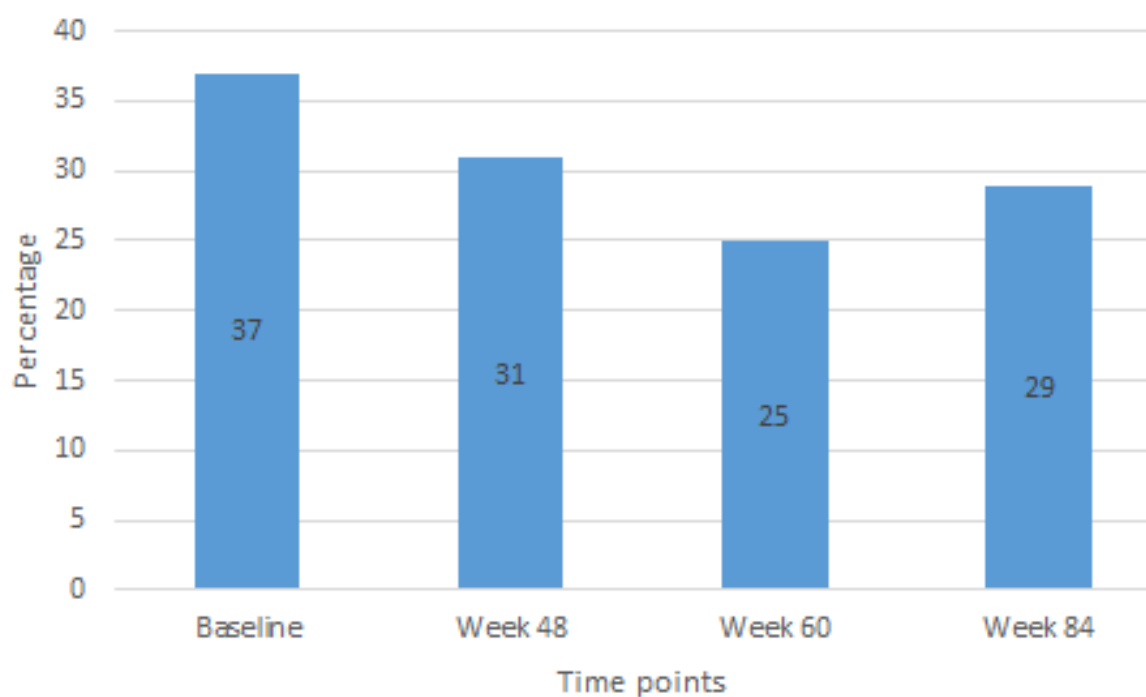


*Legend: Scatter plot demonstrating plasma NfL distribution by study participant, at each time point.
Abbreviations: NfL = neurofilament light chain protein*

8.3.6 Analysis of participants with plasma NfL above and below 13 pg/mL

The proportion of participants with NfL ≥ 13 pg/mL was 37% at baseline, and 31%, 25%, and 29% at weeks 48, 60, and 84, respectively ($p=0.47$) (Figure 91).

Figure 91: Participants with plasma NfL ≥ 13 pg/mL at each time point

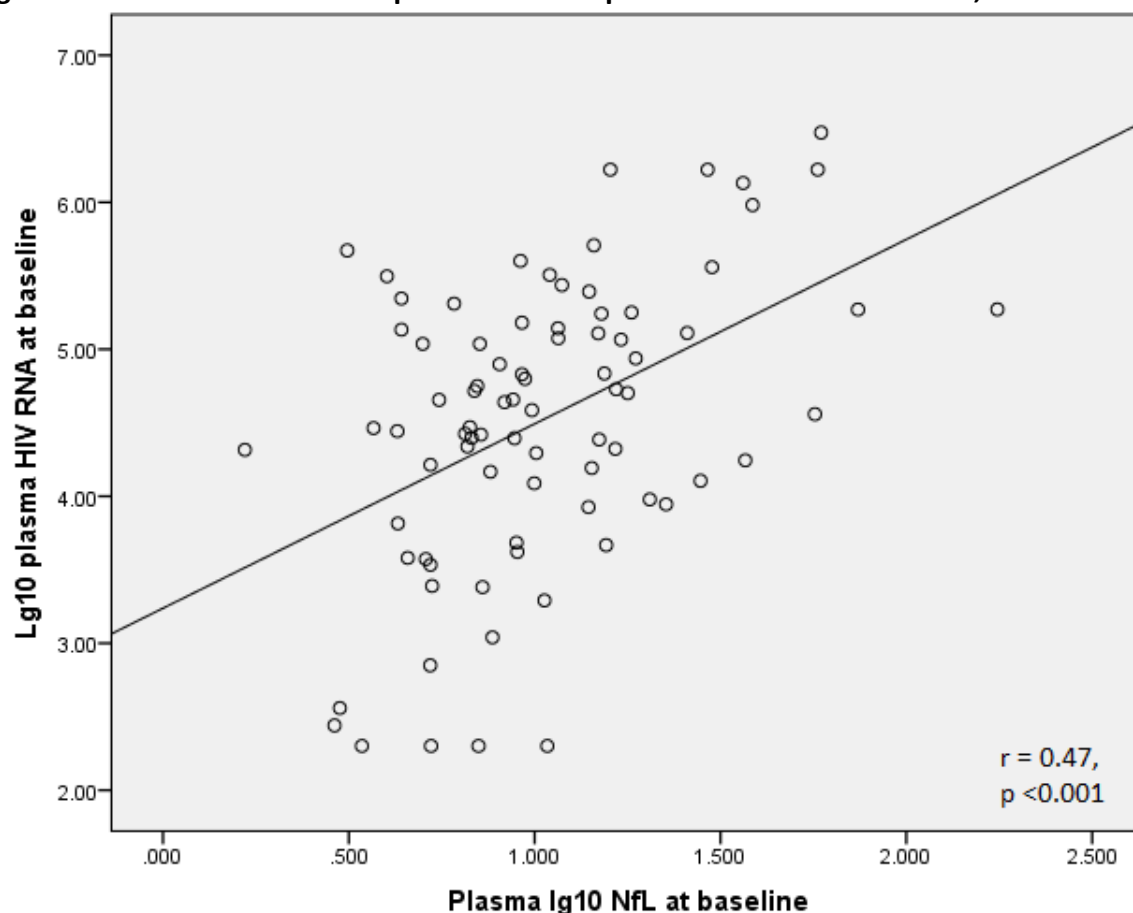


Legend: Bar chart demonstrating the percentages of participants with plasma NfL ≥ 13 pg/mL at each time point

8.3.7 Plasma NfL and plasma HIV RNA at baseline

A significant positive correlation was seen between plasma HIV RNA and plasma NfL at baseline ($r = 0.47$, $p < 0.001$) (Figure 92).

Figure 92: Correlation between plasma NfL and plasma HIV RNA at baseline, $n = 82$



Abbreviation: NfL = neurofilament light chain protein

Legend: Correlation between plasma neurofilament light chain protein and plasma HIV RNA at baseline was analysed using Pearson's correlation.

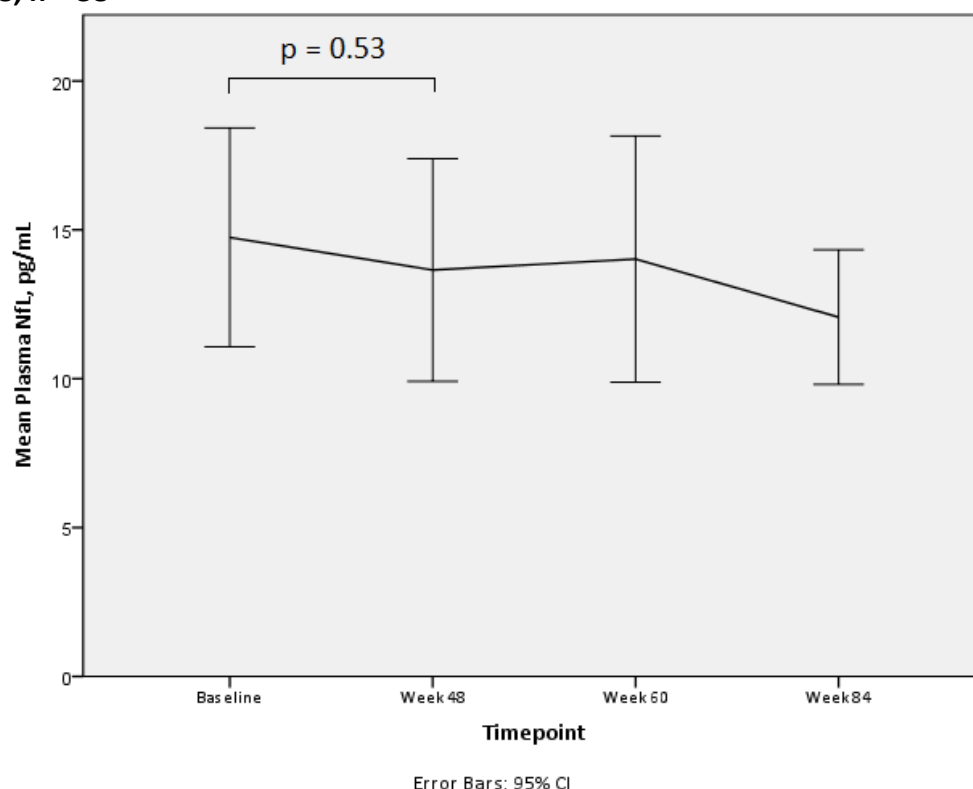
However, no significant correlation was seen for change in plasma HIV RNA and change in plasma NfL between the following time points: baseline and week 48 ($r = 0.03$, $p = 0.80$), weeks 48 and 60 ($r = 0.00$, $p = 0.99$), weeks 48 and 84 ($r = -0.60$, $p = 0.67$), weeks 60 and 84 ($r = 0.05$, $p = 0.71$) and baseline and week 84 ($r = 0.22$, $p = 0.08$) (figures not shown).

8.3.8 Analysis of plasma NfL according to whether plasma HIV RNA was less than and more than 400 copies/mL

Plasma HIV RNA was <400 copies/mL in 7%, 86%, 25%, and 16% of all participants at baseline and weeks 48, 60 and 84, respectively. There was no significant difference in plasma NfL between participants with plasma HIV RNA <400 copies/mL and $\geq$ 400 copies/mL at weeks 48, 60 and 84 ($p=0.52$, $p=0.55$, and $p=0.24$, respectively).

In participants with plasma HIV RNA <400 copies/mL at week 48 ($n = 58$), mean plasma NfL decreased numerically between baseline and week 48, but this change was not statistically significant (14.7 (95% CI 11.1, 18.4) and 13.7 (95% CI 9.9, 17.4) pg/mL, respectively, $p = 0.53$) (Figure 93).

Figure 93: Plasma NfL trends in participants with plasma HIV RNA <400 copies/mL at week 48, $n = 58$

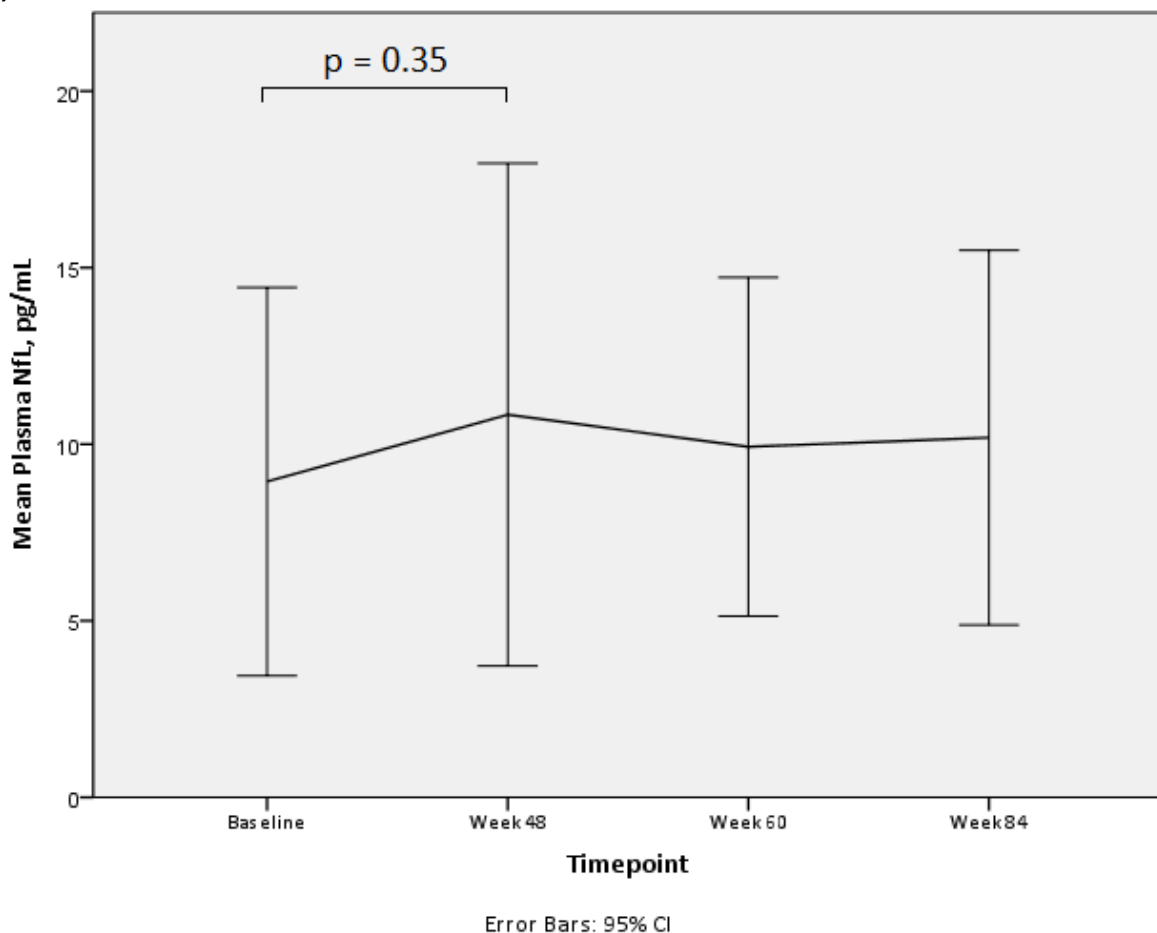


Abbreviations: NfL = neurofilament light chain protein

Legend: Line chart demonstrating plasma NfL trends in participants with plasma HIV RNA <400 copies/mL at week 48. P-value was analysed using paired-samples T-test

In participants with plasma HIV RNA ≥ 400 copies/mL at week 48 (n=10), mean plasma NfL increased between baseline and week 48, but once again, this difference was not statistically significant (8.9 (95% CI 3.4, 14.4) and 10.8 (95% CI 3.7, 18.0) pg/mL, $p = 0.35$) (Figure 94).

Figure 94: Plasma NfL trends in participants with plasma HIV RNA ≥ 400 copies/mL at week 48, n = 10



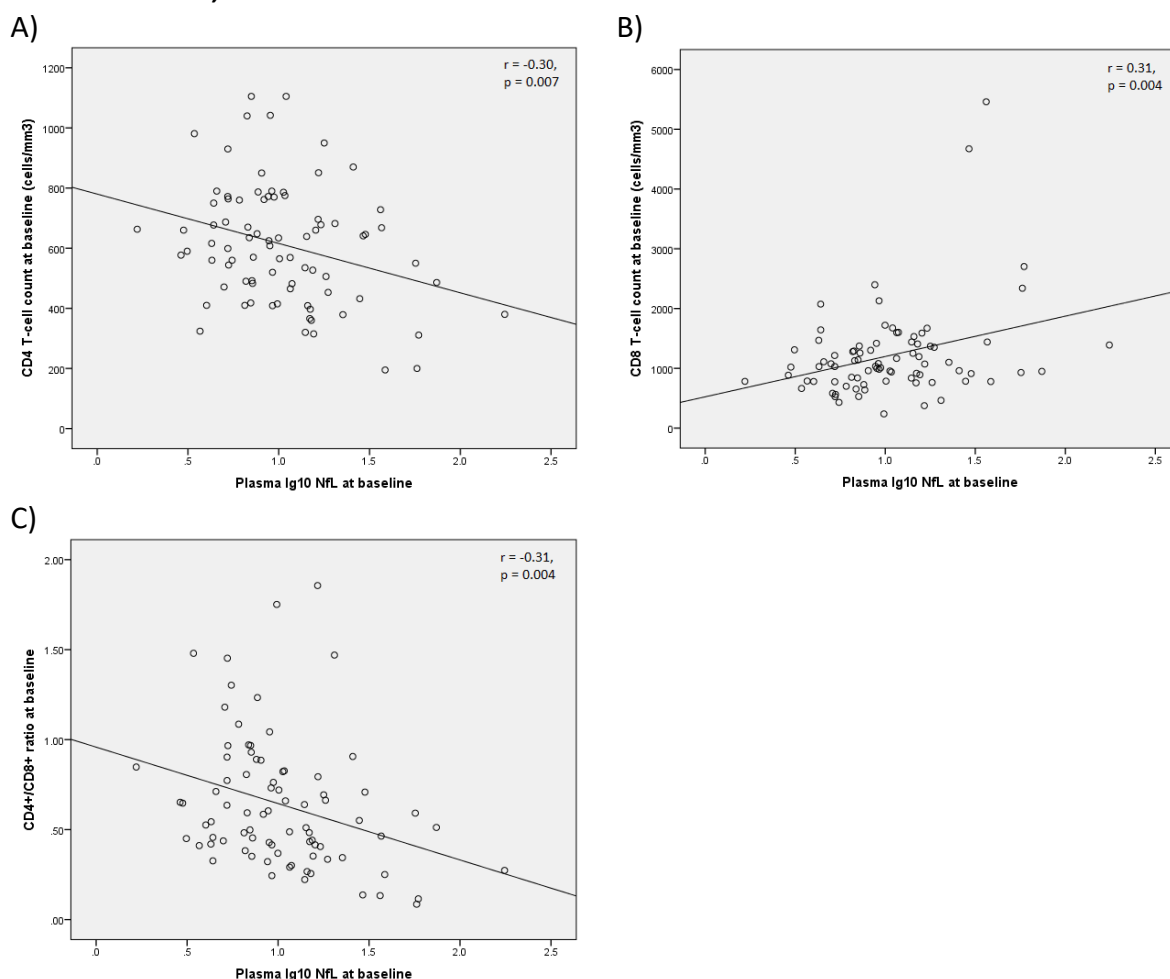
Abbreviations: NfL = neurofilament light chain protein

Legend: Line chart demonstrating plasma NfL trends in participants with plasma HIV RNA ≥ 400 copies/mL at week 48. P-value was analysed using paired-samples T-test

8.3.9 Plasma NfL and CD4⁺ count, CD8⁺ count and CD4⁺:CD8⁺ ratio

At baseline, significant correlations were seen between plasma NfL and CD4⁺ count ($r = -0.30$, $p = 0.007$) (Figure 95A), CD8⁺ count ($r = 0.31$, $p = 0.004$) (Figure 95B) and CD4⁺/CD8⁺ ratio ($r = -0.31$, $p = 0.004$) (Figure 95C). Lower CD4⁺ count and lower CD4⁺/CD8⁺ ratio were associated with higher plasma NfL, and higher CD8⁺ count was associated with higher plasma NfL (Figure 95). No significant associations were seen between plasma NfL and CD4⁺ count, CD8⁺ count and CD4⁺/CD8⁺ ratio at weeks 48, 60 and 84 (results not shown).

Figure 95: Correlation between plasma NfL and CD4⁺ count, CD8⁺ count and CD4⁺: CD8⁺ ratio at baseline, n = 82



Abbreviation: NfL = neurofilament light chain protein

Legend: Correlations between plasma neurofilament light chain protein and CD4⁺ T-cell count, CD8⁺ T-cell count and CD4⁺/CD8⁺ ratio at baseline were analysed using Pearson correlation.

8.3.10 Factors associated with baseline NfL

In multivariate regression analysis, higher baseline NfL was independently associated with older age (0.16 log₁₀ NfL higher per 10 years increase in age, $p < 0.001$) and CD8⁺ count (0.012 log₁₀ plasma NfL higher per 100 CD8⁺ count increase, $p = 0.048$) (Table 46). No significant independent associations were seen between baseline NfL and baseline plasma HIV RNA ($p = 0.09$), baseline CD4⁺ count ($p = 0.10$), baseline CD4⁺/CD8⁺ ratio ($p = 0.30$) and duration between seroconversion and randomisation ($p = 0.67$) (Table 46).

Table 46: Multivariable regression analysis for factors associated with plasma log₁₀ NfL at baseline, $n = 82$

Variable	Univariable analysis		Multivariable analysis	
	Change (95% CI)	p-value	Parameter estimate	p-value
Female vs male	-0.07 (-0.23, 0.09)	0.40		
Age (per 10 years)	0.19 (0.13, 0.26)	<0.001	0.16 (0.09, 0.22)	<0.001*
Lg ₁₀ plasma HIV RNA	0.17 (0.10, 0.24)	<0.001	0.07 (-0.01, 0.15)	0.09
CD4 ⁺ (per 100 cells/μL)	-0.05 (-0.09, -0.01)	0.01	-0.03 (-0.07, 0.01)	0.10
CD4 ⁺ :CD8 ⁺ ratio	-0.30 (-0.51, -0.09)	0.01	0.14 (-0.13, 0.41)	0.30
CD8 ⁺ (per 100 cells/μL)	0.01 (0.00, 0.02)	0.004	0.01 (0.00, 0.02)	0.048*
Time between seroconversion and baseline, weeks	-0.02 (-0.04, 0.00)	0.03	0.00 (-0.01, 0.02)	0.67
Serum creatinine (per 10 μmol/L)	0.05 (0.00, 0.11)	0.08		
Recruited in Africa	-0.15 (-0.31, 0.01)	0.07		
Weight (per 10 kg)	0.03 (-0.03, 0.09)	0.41		
Body mass index (per 10kg/m ²)	0.01 (-0.15, 0.18)	0.87		

Abbreviation: NfL = neurofilament light chain protein.

¹ Parameter estimates reflect the associated impact of each independent variable in the model on plasma NfL at baseline.

* denotes statistically significant results in multivariable regression analysis.

8.3.11 Factors associated with plasma NfL at week 48

At week 48, higher plasma NfL remained independently associated with older age (0.16 log₁₀ NfL higher per 10 years increase in age, $p < 0.001$) and serum creatinine emerged as a novel factor independently associated with plasma NfL (0.08 log₁₀ plasma NfL higher per 10 µmol/L serum creatinine increase, $p = 0.01$) (Table 47).

Table 47: Multivariable regression analysis of factors associated with plasma log₁₀ NfL at week 48, n = 71

Variable	Univariable analysis		Multivariable analysis	
	Change (95% CI)	p-value	Parameter estimate	p-value
Female vs male	-0.19 (-0.37, -0.01)	0.04	0.41 (-0.06, 0.88)	0.09
Age (per 10 years)	0.19 (0.12, 0.26)	<0.001	0.16 (0.08, 0.23)	<0.001*
Lg ₁₀ plasma HIV RNA	-0.08 (-0.18, 0.03)	0.14		
Plasma HIV RNA <400 copies/mL	0.08 (-0.18, 0.35)	0.54		
CD4 ⁺ (per 100 cells/µL)	0.00 (-0.04, 0.02)	0.55		
CD4 ⁺ :CD8 ⁺ ratio	0.03 (-0.15, 0.20)	0.77		
CD8 ⁺ (per 100 cells/µL)	0.00 (-0.03, 0.02)	0.56		
Time between seroconversion and baseline, weeks	-0.01 (-0.03, 0.01)	0.27		
Serum creatinine (per 10 µmol/L)	0.11 (0.05, 0.16)	<0.001	0.08 (0.02, 0.15)	0.01*
Recruited in Africa	-0.24 (-0.41, -0.06)	0.01	-0.32 (-0.78, 0.14)	0.17

Abbreviation: NfL = neurofilament light chain protein.

¹ Parameter estimates reflect the associated impact of each independent variable (measured at week 48, where applicable) in the model on plasma NfL at week 48.

* denotes statistically significant results in multivariable regression analysis.

8.3.12 Factors associated with plasma NfL at weeks 60 and 84

At weeks 60 and 84, higher plasma NfL was independently associated with older age only (0.16 log₁₀ NfL higher per 10 years increase in age, p <0.001 at week 60) (Table 48) and 0.11 log₁₀ NfL higher per 10 years increase in age, p <0.001 at week 84 (Table 49), respectively).

Table 48: Multivariable regression analysis of factors associated with plasma log₁₀ NfL at week 60, n = 83

Variable	Univariable analysis		Multivariable analysis	
	Change (95% CI)	p-value	Parameter estimate	p-value
Female vs male	-0.04 (-0.19, 0.12)	0.65		
Age (per 10 years)	0.17 (0.11, 0.23)	<0.001	0.16 (0.10, 0.23)	<0.001*
Lg ₁₀ plasma HIV RNA	0.05 (-0.02, 0.13)	0.15		
Plasma HIV RNA <400 copies/mL	-0.05 (-0.23, 0.12)	0.55		
CD4 ⁺ (per 100 cells/μL)	-0.02 (-0.05, 0.01)	0.16		
CD4 ⁺ :CD8 ⁺ ratio	-0.10 (-0.28, 0.09)	0.31		
CD8 ⁺ (per 100 cells/μL)	0.00 (-0.02, 0.02)	0.91		
Time between seroconversion and baseline, weeks	-0.02 (-0.03, 0.00)	0.06		
Serum creatinine (per 10 μmol/L)	0.01 (0.00, 0.01)	0.04	0.02 (-0.02, 0.07)	0.27
Recruited in Africa	-0.08 (-0.23, 0.07)	0.30		

Abbreviation: NfL = neurofilament light chain protein.

¹ Parameter estimates reflect the associated impact of each independent variable (measured at week 60, where applicable) in the model on plasma NfL at week 60.

* denotes statistically significant results in multivariable regression analysis.

Table 49: Multivariable regression analysis of factors associated with plasma log₁₀ NfL at week 84, n = 65

Variable	Univariable analysis		Multivariable analysis	
	Change (95% CI)	p-value	Parameter estimate	p-value
Female vs male	-0.17 (-0.28, -0.06)	0.004	0.16 (-0.06, 0.38)	0.14
Age (per 10 years)	0.14 (0.09, 0.19)	<0.001	0.11 (0.05, 0.17)	<0.001*
Lg ₁₀ plasma HIV RNA	0.03 (-0.04, 0.09)	0.43		
Plasma HIV RNA <400 copies/mL	-0.09 (-0.24, 0.07)	0.27		
CD4 ⁺ (per 100 cells/μL)	-0.01 (-0.04, 0.01)	0.33		
CD4 ⁺ :CD8 ⁺ ratio	-0.07 (-0.23, 0.08)	0.36		
CD8 ⁺ (per 100 cells/μL)	0.00 (-0.02, 0.02)	0.82		
Time between seroconversion and baseline, weeks	-0.01 (-0.02, 0.01)	0.33		
Serum creatinine (per 10 μmol/L)	0.08 (0.05, 0.11)	<0.001	0.04 (0.00, 0.08)	0.07
Recruited in Africa	-0.21 (-0.32, -0.10)	<0.001	-0.15 (-0.38, 0.07)	0.19

Abbreviation: NfL = neurofilament light chain protein.

¹ Parameter estimates reflect the associated impact of each independent variable (measured at week 84, where applicable) in the model on plasma NfL at week 84.

* denotes statistically significant results in multivariable regression analysis

8.3.13 Summary of the multivariate linear regression analyses results for factors independently significantly associated with plasma NfL at the different time points

The table below summarises the multivariate linear regression analyses results for factors independently associated with plasma NfL at the various time points. While age was consistently observed to be an independent factor associated with plasma NfL at all four time points, higher CD8⁺ count was independently associated with higher NfL at baseline only and higher serum creatinine was independently associated with higher NfL at week 48 only (Table 50). No other factors were identified to be independently associated with plasma NfL at any other time point.

Table 50: Summary of the factors independently significantly associated with plasma NfL at the different time points¹

Variable	Baseline N = 82	Week 48 N = 71	Week 60 N = 83	Week 84 N = 65
Female vs male				
Age (per 10 years)	0.16 (0.09, 0.22) p <0.001	0.16 (0.08, 0.23) p <0.001	0.16 (0.10, 0.23) p <0.001	0.11 (0.05, 0.17) p <0.001
Lg ₁₀ plasma HIV RNA	NS	n/d	n/d	n/d
Plasma HIV RNA <400 copies/mL	NS	NS	NS	NS
CD4 ⁺ (per 100 cells/μL)	NS	NS	NS	NS
CD4 ⁺ :CD8 ⁺ ratio	NS	NS	NS	NS
CD8 ⁺ (per 100 cells/μL)	0.01 (0.00, 0.02) p = 0.048	NS	NS	NS
Time between seroconversion and baseline, weeks	NS	NS	NS	NS
Serum creatinine (per 10 μmol/L)	NS	0.08 (0.02, 0.15) p = 0.01	NS	NS
Recruited in Africa	NS	NS	NS	NS

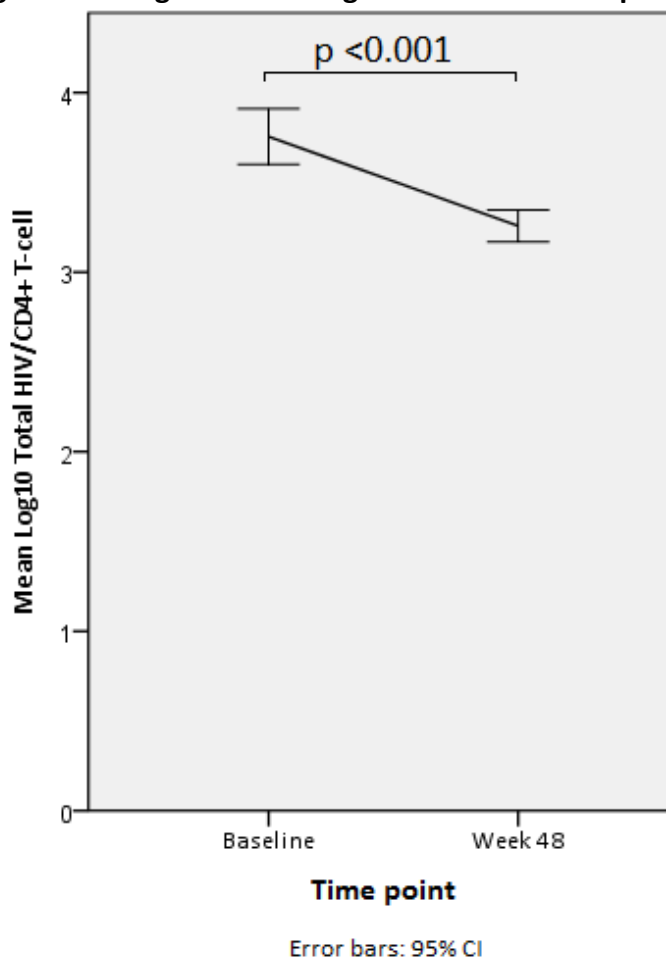
Abbreviations: NfL = neurofilament light chain protein, NS = not significantly associated, n/d = not done. ¹ Results are reported as parameter estimate (95% confidence interval), reflecting the associated impact of each independent variable in the model on plasma NfL.

8.3.14 Subgroup analysis

8.3.14.1 Total HIV DNA per CD4⁺ T-cell

Total HIV DNA per CD4⁺ T-cell results were available in 51/83 participants, as described in Table 45. Total HIV DNA decreased significantly between baseline and week 48 ($p < 0.001$), as illustrated in Figure 96.

Figure 96: Longitudinal changes in total HIV DNA per CD4⁺ T-cell, n = 45



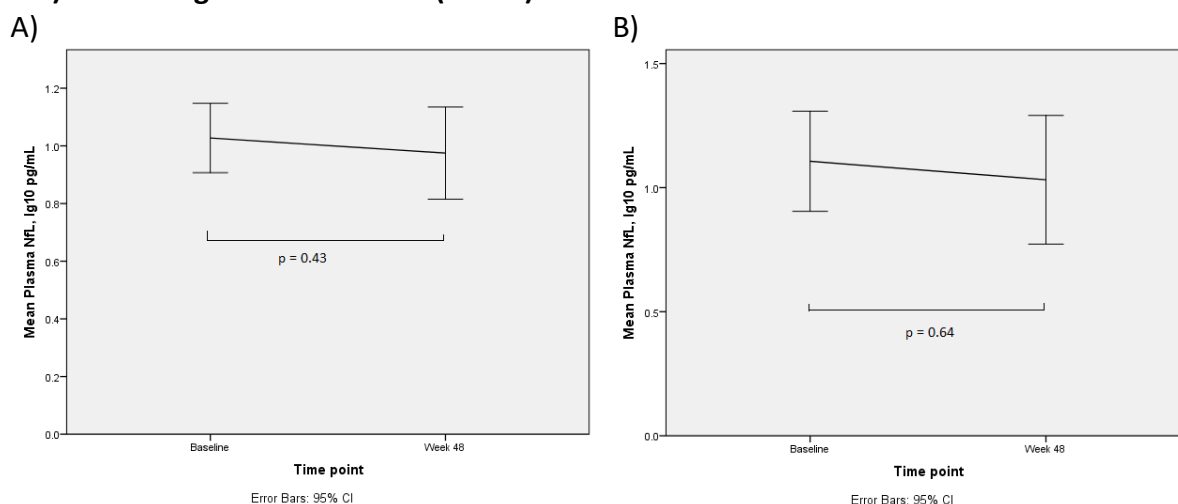
Legend: Line graph demonstrating the longitudinal changes in total HIV DNA per CD4⁺ T-cell with error bars demonstrating 95% confidence intervals. P-value was analysed using paired-samples T-test.

However, there was no significant association between plasma NfL and total HIV DNA per CD4⁺ T-cell at baseline ($r=0.07$, $p=0.63$) or week 48 ($r=0.22$, $p=0.16$), or when analysing the two variables as change from baseline ($r=0.28$, $p=0.08$) (figures not shown).

When the participants were stratified according to whether they had 'low' versus 'high' total HIV DNA, no significant change in plasma NfL was seen between baseline and week 48 in participants considered to have 'low' total HIV DNA (mean (standard deviation (SD)) 1.03

(0.30) vs 0.98 (0.39) $\log_{10}$ total HIV DNA copies/mL, $p = 0.43$ (Figure 97A)) or in participants considered to have 'high' total HIV DNA (mean (SD) 1.11 (0.41) vs 1.03 (0.43) $\log_{10}$ total HIV DNA copies/mL, $p = 0.64$ (Figure 97B)).

Figure 97: Longitudinal change in plasma NfL, stratified according to 'low' total HIV DNA (n = 27) versus 'high' total HIV DNA (n = 18) at baseline



Abbreviations: NfL = neurofilament light chain protein

Legend: Line graph demonstrating the longitudinal changes in plasma NfL when the participants are stratified according to A) 'low' total HIV DNA per CD4⁺ T-cell at baseline and B) 'high' total HIV DNA per CD4⁺ T-cell at baseline. P-value was analysed using paired-samples T-test. Error bars demonstrating 95% confidence intervals.

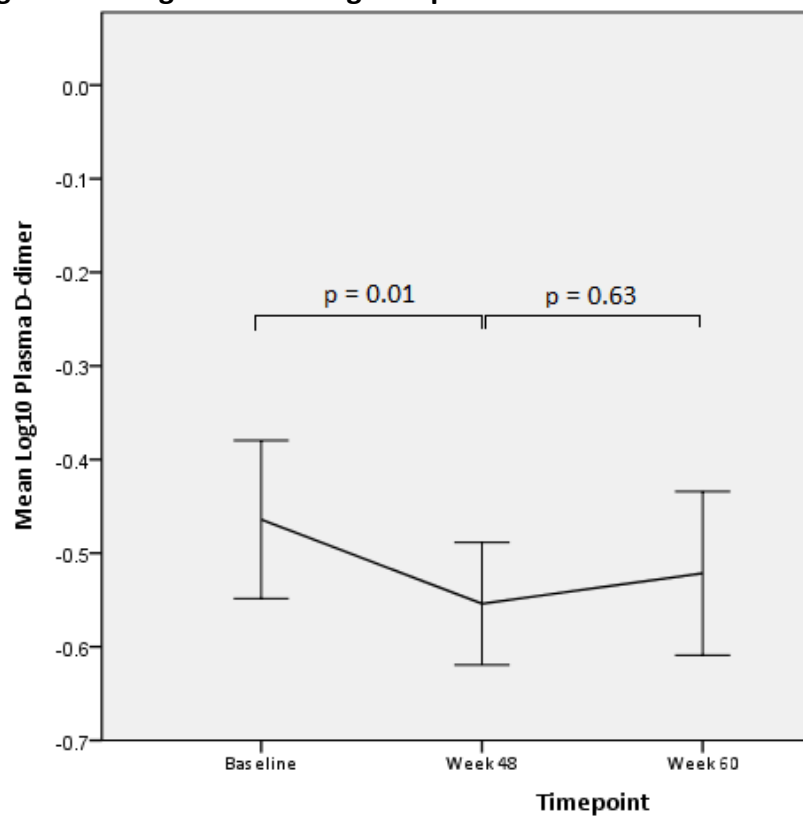
8.3.14.2 Plasma IL-6

Plasma IL-6 results were available in 47/83 participants, as described in Table 45. No significant change in plasma IL-6 was noted between baseline, week 48 and week 60 (Table 45). Furthermore, no significant correlation between plasma NfL and plasma IL-6 was observed at any timepoint (results not shown).

8.3.14.3 Plasma D-dimer

Plasma D-dimer were available in 47/83 participants as described in Table 45. Plasma D-dimer decreased significantly between baseline and week 48 (mean -0.44 to -0.55 $\log_{10}$, $p=0.01$), with no significant change between weeks 48 and 60 ($p = 0.63$) (Figure 98).

Figure 98: Longitudinal changes in plasma D-dimer

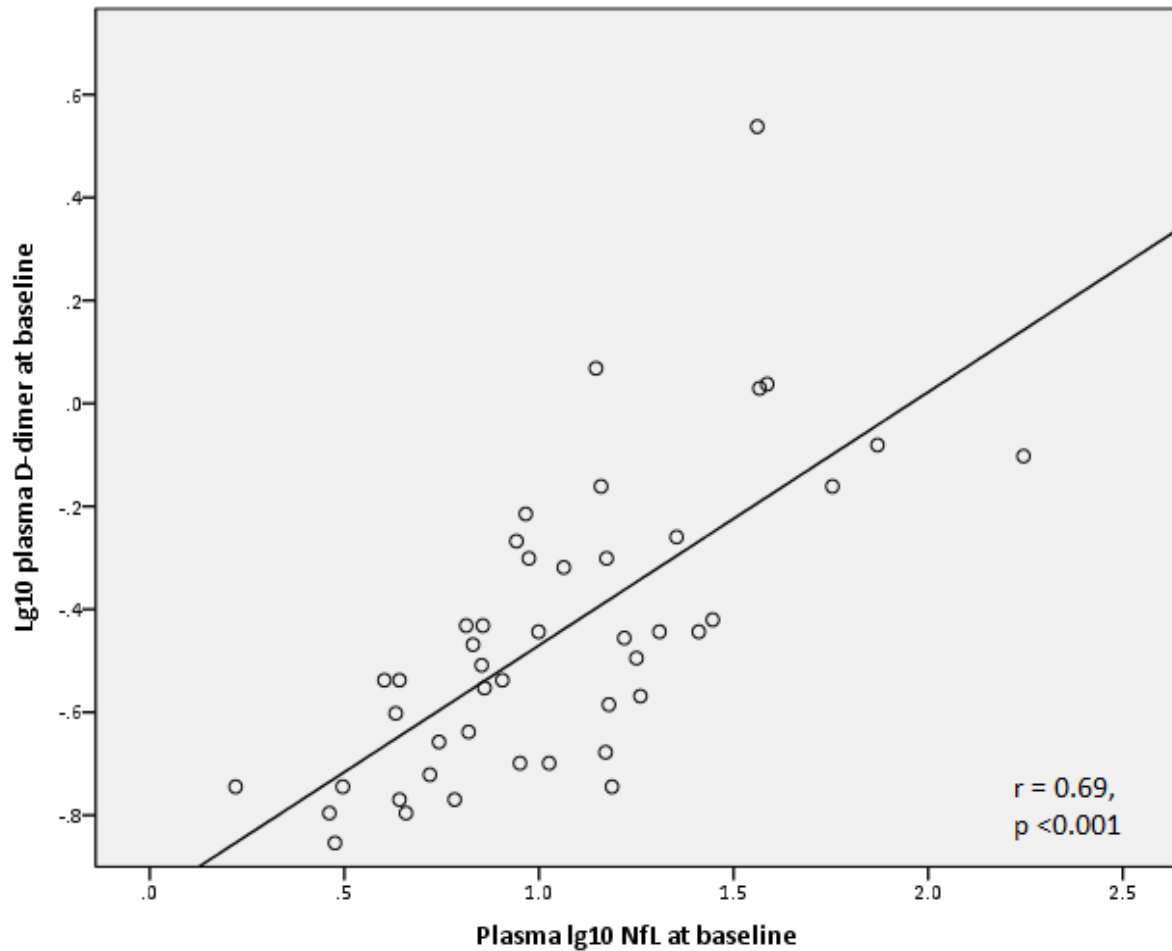


Error Bars: 95% CI

Legend: Line graph demonstrating the longitudinal changes in plasma D-dimer with 95% confidence intervals. Number of participants with plasma D-dimer results available at baseline (n = 45), week 48 (n = 35) and week 60 (n = 47). P-values were analysed using paired-samples T-test

Plasma D-dimer significantly correlated with NfL at baseline ($r=0.69$, $p < 0.001$) (Figure 99), week 48 ($r=0.40$, $p=0.018$) and week 60 ($r=0.46$, $p=0.001$).

Figure 99: Correlation between plasma NfL and plasma D-dimer at baseline, $n = 45$



Abbreviation: NfL = neurofilament light chain protein

Legend: Correlation between plasma neurofilament light chain protein and plasma D-dimer at baseline was analysed using Pearson's correlation.

Upon adjusting for baseline D-dimer in the multivariate linear regression analysis, only higher baseline plasma D-dimer remained significantly associated with baseline plasma NfL (1.01 log₁₀ plasma NfL higher per 1 log₁₀ rise in plasma D-dimer; p<0.001) (Table 51).

Table 51: Multivariable regression sub-analysis including plasma D-dimer for factors associated with plasma log₁₀ NfL at baseline, n = 45

Variable	Univariable analysis		Multivariable analysis	
	Change (95% CI)	p-value	Parameter estimate	p-value
Female vs male	-0.07 (-0.23, 0.09)	0.40		
Age (per 10 years)	0.19 (0.13, 0.26)	<0.001	0.08 (-0.03, 0.19)	0.14
Lg ₁₀ plasma HIV RNA	0.17 (0.10, 0.24)	<0.001	0.02 (-0.11, 0.14)	0.77
CD4 ⁺ (per 100 cells/μL)	-0.05 (-0.09, -0.01)	0.01	0.02 (-0.06, 0.10)	0.61
CD4 ⁺ :CD8 ⁺ ratio	-0.30 (-0.51, -0.09)	0.01	-0.17 (-0.68, 0.35)	0.52
CD8 ⁺ (per 100 cells/μL)	0.01 (0.00, 0.02)	0.004	-0.02 (-0.04, 0.00)	0.07
Time between seroconversion and baseline, weeks	-0.02 (-0.04, 0.00)	0.03	-0.01 (-0.04, 0.01)	0.37
Serum creatinine (per 10 μmol/L)	0.05 (0.00, 0.11)	0.08		
Recruited in Africa	-0.15 (-0.31, 0.01)	0.07		
Weight (per 10 kg)	0.03 (-0.03, 0.09)	0.41		
Body mass index (per 10kg/m ²)	0.01 (-0.15, 0.18)	0.87		
Plasma D-dimer	0.98 (0.67, 1.29)	<0.001	1.01 (0.49, 1.53)	<0.001*

Abbreviation: NfL = neurofilament light chain protein

*Legend:¹ Parameter estimates reflect the associated impact of each independent variable in the model on plasma NfL at baseline. * denotes statistically significant results in multivariable regression analysis*

Upon adjusting for plasma D-dimer at weeks 48 and 60 in the multivariate linear regression analyses, plasma D-dimer was not independently associated with plasma NfL at either of these time points (results not shown).

8.4 Discussion

In order to investigate my **first hypothesis of this thesis**, I investigated the association between plasma NfL, a biomarker of neuro-axonal injury, and 1) total HIV DNA per 10^6 CD4⁺ T-cells (as a direct measure of HIV reservoir size) and 2) duration between HIV seroconversion and cART initiation (as an indirect measure of HIV reservoir size). The results of this study demonstrated **the lack of association between plasma NfL and total HIV DNA per 10^6 CD4⁺ T-cells, and between plasma NfL and duration between HIV seroconversion to cART initiation**. Furthermore, when I stratified the participants according to low versus high total HIV DNA per CD4⁺ T-cells at baseline, I did not identify a significant change in plasma NfL between baseline (during untreated primary HIV infection) and week 48 (following 48 weeks continuous cART).

Taken together, **I could not confirm the first hypothesis of my thesis** that lower biomarker concentrations of HIV reservoir size are associated with lower biomarker concentrations of neural activation.

With regards to investigating the second hypothesis of this thesis, this study set out to investigate the association between plasma NfL and plasma HIV RNA (as a direct measure of HIV viral transcription). **In untreated primary HIV infection, I observed a positive association between plasma NfL and plasma HIV RNA, thus confirming my second hypothesis.**

My observation that plasma HIV RNA is associated with plasma NfL is in keeping with previously published findings[293]. At the time points subsequent to untreated primary HIV infection (following 48 weeks cART initiated during primary HIV infection and 12- and 36-weeks post-ATI), many participants had plasma HIV RNA <400 copies/mL at each of these time points, thus preventing a reliable direct correlation analysis between plasma HIV RNA and plasma NfL at these time points. Instead, I categorised plasma HIV RNA according to <400 HIV RNA copies/mL or ≥ 400 HIV RNA copies/mL, then associations between plasma HIV RNA in these categories with plasma NfL were analysed. While not statistically significant, individuals with plasma HIV RNA ≥ 400 copies/mL after 48 weeks cART had a numerical increase in mean plasma NfL concentration between baseline and week 48,

whereas individuals with an undetectable plasma HIV RNA after 48 weeks of cART had a numerical decrease in mean plasma NfL between baseline and week 48. This could imply that individuals with ongoing viral replication despite cART, experienced increased neuro-axonal injury compared to individuals virally suppressed on cART and the lack of statistical significance may be due to the study not being powered to investigate this specifically. However, the lack of statistical significance in the change of plasma NfL between baseline and week 48 in either group (plasma HIV RNA <400 copies/mL or ≥400 copies/mL) may also indicate there not being a true difference in plasma NfL trajectories in the two groups, and the numerical changes seen were due to physiological variations in plasma NfL. Of note, nearly one third of participants in this analysis had plasma HIV RNA >400 copies/mL at week 48 (after 48 weeks cART), which may reflect slow viral decay (the participants were on a protease inhibitor-based cART regimen) or incomplete adherence to cART.

The lower limit of quantification of plasma HIV RNA in the SPARTAC study was <50 HIV RNA copies/mL in participants from sites in Europe, Brazil and Australia, and <400 HIV RNA copies/mL in participants from sites in Africa. More sensitive methods of quantifying HIV RNA have since been developed, such as the single-copy assay or cell-associated HIV RNA in CD4⁺ T-cells measured using qPCR, which if used in this study, may have proven my hypothesis at the time points following the baseline visit, when overall plasma HIV RNA was lower.

The association between plasma NfL with a marker of HIV viral transcription but not a marker of HIV reservoir size implies that while active virus production is positively associated with neuro-axonal injury, the size of the latent HIV reservoir does not appear to correlate with neuro-axonal injury. This finding is interesting given that while the SPARTAC trial previously demonstrated that HIV DNA predicted time to plasma virus rebound[345], my findings suggest it is the magnitude of plasma virus load that triggers neuro-axonal injury. My findings are consistent with the cumulative results from four HIV remission studies with cART interruption, where no change in cerebrospinal fluid immune activation or brain diffusion tensor imaging markers were seen during closely monitored analytical treatment interruption[479].

Ideally, I would have liked to have performed serial plasma NfL measurements in participants randomised to the control (standard of care) arm as well, to compare the plasma NfL trajectories between the two groups. This would have aided the interpretation of the NfL changes seen in the intervention arm, in light of the natural trajectory of plasma NfL during untreated primary HIV infection in this setting. Unfortunately, due to funding constraints, I was unable to measure plasma NfL in the control arm.

My results demonstrated that CD4⁺ count and CD4⁺/CD8⁺ ratio (measures of immune system function) and CD8⁺ count (a measure of cytotoxic T-cell activity) were significantly associated with plasma NfL during untreated primary HIV infection. However, of these, only higher CD8⁺ count was independently associated with higher plasma NfL in multivariate regression analysis. Published data suggests that peripheral HIV-specific CD8⁺ T-cells (at peak plasma viral load during acute HIV infection) have an enhanced capacity to kill HIV-infected cells and are associated with viral load decline and reduced seeding of the HIV reservoir[480]. Thus, it could be hypothesised that the killing of HIV-infected cells by CD8⁺ T-cells in the central nervous system during primary HIV infection and consequential neuro-inflammation and immune activation, may lead to neuro-axonal injury.

While I observed a positive association between plasma NfL and plasma D-dimer (a biomarker of pro-coagulation), I saw no evidence to support an association between plasma NfL with plasma IL-6 (a pro-inflammatory cytokine). Data from the SPARTAC trial demonstrated that within four weeks of interrupting cART initiated during primary HIV infection, 78% of participants had detectable plasma HIV RNA ≥ 400 copies/mL[62], plasma biomarkers of inflammation (IL-6 and D-dimer) which decreased during cART had returned to pre-cART levels[478] and plasma HIV RNA strongly correlated with plasma D-dimer[481]. These findings suggest a plausible biological explanation by which viral transcription might lead to a pro-coagulation and pro-inflammatory milieu, resulting in neuro-axonal injury.

From this unique, large, international cohort of individuals who were randomly allocated to interrupt cART initiated during PHI, I also observed that despite evidence of increased neuro-axonal injury during untreated PHI prior to cART initiation, I saw no evidence supporting a recurrence in neuro-axonal injury up to 36 weeks following cART interruption. Plasma HIV RNA rebounded following cART cessation at week 48, however did not reach the

concentrations seen at baseline, prior to cART initiation. One hypothesis could be that during primary HIV infection, an individual's immune system is naïve to HIV, thus generates an ineffective immune response towards HIV. This results in poorly controlled HIV replication and thus, very high plasma HIV RNA, which can then be neurotoxic and cause neuro-axonal injury. On the other hand, despite 48 weeks cART and plasma HIV RNA <400 copies/mL in the majority of individuals adherent to cART, the individual's immune system would still have been continually exposed and primed to HIV virions, HIV-related proteins and HIV-related antibodies throughout the period on cART, albeit at low concentrations. Thus, upon cART cessation, the immune system generates a more controlled immune response to the presence of HIV virions, leading to improved viral replication control compared to during primary HIV infection, and thus causing less neuro-axonal injury.

Data to date suggest that during early HIV infection, neuronal injury is delayed compared to the viral and inflammatory changes that are frequently reported[482] but increases in biomarker concentration of neuronal injury are more likely to be observed with longer durations of ATI, as CD4⁺ T-cell count decreases[269]. The precise mechanisms underlying this phenomenon are not clearly defined. In prospective observational studies, no detectable rise in cerebrospinal fluid NfL was seen in neuro-asymptomatic participants with hyperacute HIV infection (median duration since exposure of 18 days)[287] whereas, another cohort demonstrated that cerebrospinal fluid NfL was elevated in nearly half of individuals several months after acquiring HIV[483], when 9% of participants were had acute HIV infection-related neurological symptoms. The cohort included in my analysis lies partway between both these trials, given the median time from seroconversion to baseline for the participants in my analysis was 13 weeks, with 7/83 participants (8.4%) diagnosed <12 weeks since seroconversion. Another retrospective study of eight individuals on suppressive cART initiated during chronic infection who interrupted cART demonstrated that while none developed neurological symptoms, three experienced significant increases in cerebrospinal fluid NfL, measured up to 148 days post-ATI[290]. Recently, a prospective ATI intervention study of 11 individuals with chronic HIV disease on cART who underwent ATI, reported no evidence of increased cerebrospinal fluid NfL at the first point of viral load rebound (plasma HIV RNA>1000 copies/mL or >200 copies/mL on two consecutive measurements); median time to viral load rebound was 21 days in this ATI study[476].

Taken together, my results and data in the literature suggests that when closely monitored, ATIs are safe from a neurological perspective, and neuronal injury is unlikely to happen with short periods of cART interruption. The SPARTAC study design was much more permissive in terms of cART restart criteria than the current ATI guidelines would allow, and even then, neuronal injury was not observed in the SPARTAC participants up to 36 weeks post-ATI.

My observation that plasma NfL is positively associated with age is in keeping with published data[293], however the exact mechanism for this remains unclear. It could be postulated that with advancing age, the neuronal-axonal complex becomes more fragile and vulnerable to damage, which could be immune- or inflammatory-mediated. My finding that serum creatinine is independently associated with plasma NfL in individuals on suppressive cART was replicated in my results from the COBRA cohort, as described in Chapter 4. This suggests that renal function would need to be considered when interpreting plasma NfL. It could be hypothesised that plasma NfL relies on adequate renal function for excretion, however, the molecular weight of NfL (68kDa) and the abundantly negative charged amino acid in its sequence both hypothetically decrease the ability of plasma NfL to cross the glomerular filtration barrier for renal excretion.

The strengths of this study include the protocol-indicated cART interruption in participants with PHI, which enabled me to assess the impact of ATI on the biomarkers without risk of bias through confounding by indication. Furthermore, the SPARTAC trial remains the largest trial of individuals diagnosed during primary HIV infection randomised to standard of care or 12-week or 48-week cART, and stored samples from these participants can give us valuable insight into the impact of ATI, especially since ATI studies of this type and size will unlikely be carried out in the present day.

Limitations include the relatively short follow-up period after stopping ART and not all participants had D-dimer, IL-6, and total HIV DNA results available at each of the time points. Total HIV DNA measurements were performed over seven years ago, and HIV reservoir quantitative assays have advanced significantly since then. Ideally, I would like to measure HIV reservoir size using one of the more novel assays such as IPDA or Q4PCR but the number of participants with stored PBMC samples remaining is limited, and given the

age of the stored samples, cell viability may now be poor, which will negatively impact the results.

Potential unmeasured confounders such as genetic differences, environmental factors, lifestyle factors, concomitant drug use and chronic co-infections may be responsible for the associations seen with plasma NfL, such as CD8⁺ count and serum creatinine. Of note, all the male participants were recruited from Europe, Brazil or Australia, mainly acquired HIV via sex between men and harboured clade B virus, whereas almost all the women were recruited from sites in Africa, acquired HIV via heterosexual intercourse and harboured clade C virus. The discrepancy between the men and women in their demographics (apart from gender) and HIV-related characteristics may have had an impact on my findings, given the non-homogeneity of our participant population. It has been previously demonstrated that women with HIV have higher CD4⁺ T-cell counts[484], lower plasma HIV RNA[485] and are more likely to have sustained virological remission following treatment interruption[486] compared to men with HIV, reinforcing the notion that these differences in biological characteristics may have an impact on disease course.

The lack of concurrent cerebrospinal fluid samples restricts our knowledge about the parallel trends in cerebrospinal fluid NfL during this time period, however my findings in Chapter 3 and Chapter 4 suggests an overall moderate correlation between cerebrospinal fluid and blood NfL[293,368,369,372]. Importantly, while age-related reference values for cerebrospinal fluid NfL have been defined[285], similar reference values for plasma NfL are not yet available. In cohorts where the age range of the participants is broad, this may have a significant impact on the blood NfL interpretation and analysis. Plasma NfL measurements in samples from baseline, week 48 and week 60 were performed using a manual method whereas the week 84 plasma NfL measurements were performed using the novel validated automated method. In order to make the plasma NfL measurements from the four time points truly comparable, plasma NfL measurements from baseline, week 48 and week 60 would have to be repeated using the automated NfL method, which was not available at the time when the samples were first analysed.

Overall, these results are reassuring, but it is unclear whether they can be extrapolated to other populations, such as those undergoing HIV eradication strategies followed by ATI, and those with low nadir CD4⁺ counts and/or chronic HIV infection.

8.5 Conclusion

HIV viral transcription was observed to be positively associated with neuro-axonal injury, but no significant association was seen between HIV reservoir size and neuro-axonal injury. Of the viral and immune markers, only plasma D-dimer was independently associated with neuro-axonal injury, during untreated primary HIV infection.

Using plasma NfL as a surrogate marker, I observed a decrease in neuro-axonal injury in a cohort of participants following ART initiation during PHI, with no evidence of neuro-axonal injury rebound following ART interruption for up to 36 weeks, despite the majority of participants having a rebound in plasma HIV RNA by this point. This would suggest that ATIs for up to 36 weeks after early cART initiation may be considered safe from a neurological perspective. The effect of ATI on plasma NfL concentration beyond 36 weeks after stopping cART is unknown.

Chapter 9

Discussion

Chapter 9: Discussion

It has been proposed that the HIV reservoir and low-level HIV viral transcription may contribute to the persistence of immune activation and inflammation[487], leading to the development of HIV-associated brain disease in PLWH despite suppressive cART[146].

My PhD thesis sought to examine the following two hypotheses:

- 1. Lower biomarker concentrations of HIV reservoir size are associated with lower biomarker concentrations of neural activation**
- 2. Higher biomarker concentrations of HIV viral transcription are associated with higher biomarker concentrations of neural activation**

9.1 First Hypothesis

Lower biomarker concentrations of HIV reservoir size are associated with lower biomarker concentrations of neural activation

I aimed to test this hypothesis in four clinical studies.

In Chapters 5 and 6, I planned to test this hypothesis in the 'PET in AHI' and 'PET in CHI' studies, assessing *in vivo* microglial activation via brain [^{11}C] PBR28 binding on PET imaging in neuro-asymptomatic individuals living with HIV on stable cART, compared to control participants. Participants enrolled into the 'PET in AHI' study initiated cART during acute HIV infection, while participants enrolled into the 'PET in CHI' study initiated cART during chronic HIV infection.

In Chapter 5, I observed no significant differences in brain [^{11}C] PBR28 binding (as a proxy for neural activation) across all the brain regions assessed between control participants (who by definition, have no HIV reservoir) versus participants who initiated cART during acute HIV infection (who presumably have smaller HIV reservoir size) versus participants who initiated cART during chronic HIV infection (who presumably have larger HIV reservoir size). Thus, I had insufficient evidence to confirm my first hypothesis that lower HIV reservoir size is associated with lower neural activation. An important point to note is that the small sample

size of participants in the three groups may have hindered the detection of significant regional differences in brain [^{11}C] PBR28 binding between the three groups of participants. The analyses presented at this stage are exploratory hence, adjustments for multiple testing was not performed, which may conceal subtle but significant signals.

In contrast to my results, previous work by Garvey *et al* demonstrated increased TSPO binding at certain brain regions in neuro-asymptomatic people living with HIV on stable cART compared to control participants, using a less sensitive TSPO radioligand, [^{11}C] PK11195[405]. Furthermore, Vera *et al* demonstrated increased [^{11}C] PBR28 binding in certain brain regions and decreased [^{11}C] PBR28 binding in others[210] compared to control participants, using a different methodology to quantify brain [^{11}C] PBR28 binding. Moving forwards, further studies are warranted to validate the ideal methodology for standardising the quantification of brain [^{11}C] PBR28 binding in people living with treated HIV.

Due to the COVID-19 pandemic, I was unable to complete my planned laboratory work to measure total HIV DNA in CD4⁺ T-cells, thus could not investigate the associations between total HIV DNA (as a surrogate marker of larger HIV reservoir size) with measures of neural activation (regional brain [^{11}C] PBR28 binding, cerebrospinal fluid and plasma NfL, and cognitive performance), which would have addressed my first PhD hypothesis. When permitted, I plan to measure total HIV DNA concentrations in all the HIV-positive participants included in the PET in AHI and PET in CHI studies, which will allow me to test my first hypothesis.

Future studies comparing the longitudinal changes in microglial activation in people who initiated cART during acute versus chronic HIV infection would also be interesting, to investigate how early cART initiation impacts the development of long-term microglial activation. Choice of cART agents may also impact on microglial activation through neurotoxicity; participants who initiated cART during chronic HIV infection were more likely to be on NNRTI and PI-based regimens, whereas participants who initiated cART during primary HIV infection were more likely to be on INSTI-based regimes. Future studies recruiting participants on similar cART regimens would be essential to account for the impact of cART agents on microglial activation.

With regards to NfL (as a marker of neuro-axonal injury and for the purposes of my PhD, a proxy marker of neural activation), in Chapter 5, I observed significantly lower plasma NfL concentrations in people who initiated cART during acute HIV infection (who presumably have smaller HIV reservoirs) compared to people who initiated cART during chronic HIV infection (who presumably have larger HIV reservoirs), which corroborated my first hypothesis that lower measures of neural activation are associated with lower HIV reservoir size.

In order to evaluate the impact of HIV reservoir size on cognitive performance, participants enrolled into the 'PET in AHI' and 'PET in CHI' studies underwent cognitive testing. For the purposes of my PhD, reduced cognitive performance is considered a proxy for neural activation. In Chapter 5, I observed higher global composite cognitive T-scores in people who initiated cART during acute HIV infection (who presumably have smaller HIV reservoirs) compared to those who initiated cART during chronic HIV infection (who presumably have larger HIV reservoirs), thus corroborating my first hypothesis that lower markers of neural activation are associated with lower HIV reservoir size.

I also tested my first hypothesis in Chapter 7, utilising blood samples from participants enrolled into a randomised controlled HIV "shock and kill" trial involving vorinostat (a latency reversing agent) and HIV-specific vaccination, in people who initiated cART during primary HIV infection. Here, this trial demonstrated no significant change in two surrogate measures of HIV reservoir size (total HIV DNA and integrated HIV DNA in CD4⁺ T-cells isolated from PBMCs) and in parallel with these findings, I did not observe any change in plasma NfL (as a marker of neural activation), by study arm at each of the time points measured or longitudinally. Furthermore, I found no significant associations between total HIV DNA and integrated HIV DNA in CD4⁺ T-cells isolated from PBMCs with plasma NfL at any of the time points when these markers were measured in all the participants enrolled, before and after HIV "shock and kill" in the intervention arm. In this setting, I was unable to confirm my first hypothesis that lower biomarker concentrations of HIV reservoir size are associated with lower biomarker concentrations of neural activation. My findings suggest that this HIV "shock and kill" intervention did not alter peripheral HIV reservoir size nor affect neuro-axonal injury using the biomarkers mentioned above, and measures of HIV

reservoir size and neuro-axonal injury did not significantly correlate, both before and after this HIV “shock and kill” intervention.

Similarly, I investigated my first hypothesis in Chapter 8, amongst individuals who initiated cART during primary HIV infection who were then randomised to initiate cART for 48 weeks, followed by a protocol-indicated treatment interruption. In this setting, I found that despite a reduction in total HIV DNA per 10^6 CD4⁺ T-cells (as a proxy for HIV reservoir size) following 48 weeks cART initiated during primary HIV infection, I observed no significant associations between plasma NfL (a proxy marker of neural activation) with total HIV DNA per 10^6 CD4⁺ T-cells before and after cART initiation nor with duration between HIV seroconversion and cART initiation (as an indirect measure of HIV reservoir size, with longer duration between HIV seroconversion and cART initiation being associated with increased HIV reservoir size). In this instance, I was again unable to confirm the first hypothesis of my PhD thesis that lower biomarker concentrations of HIV reservoir size are associated with lower biomarker concentrations of neural activation. Using the biomarkers mentioned above, my observations suggest the lack of an impact of peripheral HIV reservoir size on neuro-axonal injury during untreated primary HIV infection and following 48 weeks cART.

While the biomarkers utilised in Chapters 7 and 8 are valid and widely used as research tools, they may be insufficiently sensitive to detect signal changes in these specific settings, or they may have been measured at the wrong timepoints to detect signals following the interventions.

9.2 Second Hypothesis

Higher biomarker concentrations of HIV viral transcription are associated with higher biomarker concentrations of neural activation

I examined the second hypothesis of this thesis in Chapter 7, where I analysed the associations between cell-associated unspliced HIV RNA in CD4⁺ T-cells isolated from PBMCs and ultra-sensitive plasma HIV RNA (as surrogate measures of HIV viral transcription) with plasma NfL (a measure of neuro-axonal injury, and proxy for neural activation) at sequential

time points in all participants enrolled into the RIVER trial, a randomised controlled HIV “shock and kill” cure study in individuals initiating cART during primary HIV infection. Here, I observed no significant change in cell-associated unspliced HIV RNA in CD4⁺ T-cells isolated from PBMCs, ultra-sensitive plasma HIV RNA or plasma NfL, both before and after the HIV “shock and kill” intervention by study arm and longitudinally. Additionally, I observed no significant associations between cell-associated unspliced HIV RNA in CD4⁺ T-cells isolated from PBMCs or ultra-sensitive plasma HIV RNA with plasma NfL at any of the time points when these markers were measured, both before and after “shock and kill” in the intervention arm. Thus, in this setting, I could not prove my second hypothesis that higher biomarker concentrations of HIV viral transcription are associated with higher biomarker concentrations of neural activation. My observations may reflect the lack of effect of this HIV “shock and kill” intervention on HIV viral transcription and hence, neuro-axonal injury.

Secondly, in Chapter 8, I measured plasma HIV RNA (as a surrogate measure of HIV viral transcription) and plasma NfL at four time points in participants enrolled into SPARTAC, a treatment interruption study. In this setting, I observed significant reductions in both plasma HIV RNA and plasma NfL following 48 weeks cART initiated during primary HIV infection. Following cART interruption, while plasma HIV RNA load rebounded, a similarly significant rebound in plasma NfL was not detected. Furthermore, during untreated primary HIV infection when viral replication was uncontrolled, I noted a significant positive association between plasma NfL and plasma HIV RNA, thus confirming my second hypothesis. However, at the subsequent time points following cART initiation and cART interruption in the SPARTAC study, I observed no significant association between plasma HIV RNA and plasma NfL and was unable to maintain confirmation of my second hypothesis. If further serial plasma samples were measured for NfL, it is possible that in time, plasma NfL may reach pre-cART concentrations. Alternatively, the initiation of cART may provide some form of protection of uncertain duration against neuro-axonal injury despite subsequent treatment interruption, or the primary HIV infection milieu may be particularly toxic to the neuro-axonal bundle, at a level not seen during chronic HIV infection with well-maintained CD4⁺ T-cell counts[293]. Ideally, I would like to measure plasma HIV RNA in these participants using an ultra-sensitive assay, to investigate associations between plasma HIV RNA and plasma NfL when plasma HIV RNA is undetectable using conventional

methods, and to continue measuring the association between plasma HIV RNA and plasma NfL during cART interruption at further time points to assess whether associations between these biomarkers reappear when the biomarkers reach pre-cART concentrations.

Once again, important limitations to consider are that the biomarkers used in Chapters 7 and 8 may not have been sensitive enough to detect deviations in this context or I may have missed detecting a change in biomarkers by measuring at the wrong time points.

I was denied access to the laboratory to complete my planned laboratory work to measure ultra-sensitive HIV RNA in participants enrolled into the PET in HIV studies, thus could not investigate the associations between ultra-sensitive HIV RNA (as a surrogate marker of HIV viral transcription) with measures of neural activation (regional brain [^{11}C] PBR28 binding, cerebrospinal fluid and plasma NfL, and cognitive performance), which would have addressed my second PhD hypothesis in Chapter 6. When permitted, I plan to measure cerebrospinal fluid and plasma ultra-sensitive HIV RNA in all the HIV-positive participants included in the PET in AHI and PET in CHI studies, which will allow me to test my second hypothesis.

9.3 Summary of additional findings

Cerebrospinal fluid NfL is well-established as a marker of central nervous system neuro-axonal injury[279] but the invasive nature of cerebrospinal fluid collection limits its widespread use. Data is emerging to support the use of blood NfL as a surrogate measure of cerebrospinal fluid NfL, with studies reporting moderate to strong correlations between cerebrospinal fluid NfL and blood NfL depending on the neurological condition under investigation[358,378].

As part of my PhD thesis, I performed a systematic review and meta-analysis to determine the pooled correlation coefficient estimate between cerebrospinal fluid and blood NfL, to elucidate the overall association between cerebrospinal fluid and blood NfL and to understand how the association varies under different conditions. My analysis demonstrated that overall, cerebrospinal fluid and blood NfL correlated moderately across different disease states and physiological conditions, supporting its use as a surrogate marker of cerebrospinal fluid NfL. However, cerebrospinal fluid and blood NfL did not

correlate perfectly in all conditions and additional factors such as choice of blood NfL assay and blood NfL concentration range need to be accounted for when interpreting blood NfL measurements. Simoa is the current preferred blood NfL assay especially at low or physiological concentrations due to its low detection limit[291,294] and my findings support its utility as the current most advanced surrogate measure of cerebrospinal fluid NfL.

In the context of plasma NfL in PLWH under various conditions, preliminary studies suggest that cerebrospinal fluid strongly correlates with blood NfL in a cohort of mainly untreated people with HIV[293], but whether this strong correlation persists in people with HIV on virally suppressive cART is unknown. Using stored samples from the observational COBRA cohort, I compared the correlation between cerebrospinal fluid and plasma NfL in people living with treated HIV and in lifestyle-similar HIV-negative individuals and determined the factors associated with cerebrospinal fluid and plasma NfL in people living with treated HIV and lifestyle-similar HIV-negative individuals.

I observed that the correlation between cerebrospinal fluid and plasma NfL in PLWH on suppressive cART is moderate and similar to that observed in a cohort of lifestyle-similar HIV-negative controls, supporting the use of plasma NfL as a reliable proxy marker of cerebrospinal fluid NfL in the context of people living with HIV on suppressive cART. Furthermore, my observations that renal function and body composition may affect the interpretation of plasma NfL warrants further investigation in larger scale and diverse cohorts.

9.4 Overall conclusions and clinical implications

Milder forms of cognitive impairment affecting people living with HIV on suppressive cART have become increasingly apparent in recent years[157], but the precise underlying mechanisms for its development remain unknown. The HIV reservoir and persistent low-level HIV viral transcription have been postulated to be associated with central nervous system immune activation and neuroinflammation[487], which can lead to neuronal injury and result in neurological sequelae such as cognitive impairment[270].

In Chapters 5 and 6, I report the novel use of an *in vivo* TSPO PET brain imaging tool to investigate differences in neuroinflammation between people who initiated cART during acute HIV infection versus chronic HIV infection. **My results were inconclusive as I did not observe differences in brain [¹¹C] PBR28 binding on PET imaging (as a proxy marker of neuroinflammation) between control participants, people who initiated cART during acute HIV infection and people who initiated cART during chronic HIV infection.**

Further studies with larger sample size are warranted to investigate the pathogenic mechanisms leading to the development of HIV-associated cognitive impairment, which would then allow the discovery of novel objective biomarkers to identify and assess cognitive impairment and the development of therapeutics for the management of cognitive impairment.

My results in Chapters 7 and 8 demonstrated no evidence of associations between measures of HIV reservoir size and HIV viral transcription with a marker of neuronal injury following two interventions: a randomised controlled HIV “shock and kill” strategy and a treatment interruption study. **My observations suggest that HIV “shock and kill” using this combination of vorinostat and HIV-specific vaccination and treatment interruption, which are increasingly being explored in the context of HIV-cure research, may be safe from a central nervous system perspective but further research is warranted to explore whether current HIV-cure strategies (which mainly target peripheral HIV reservoirs) have a discernible impact on central nervous system HIV reservoirs and continued central nervous system monitoring is essential to investigate whether more potent HIV-cure strategies may have adverse central nervous system effects.** With numerous therapeutics in the pipeline for HIV-management and HIV-remission research studies, assessment of central nervous system safety remains a vital aspect, especially those targeting central nervous system reservoirs. My observations demonstrate that implementing a blood-based monitoring system for neuro-axonal injury within a HIV remission trial and ATI trial is feasible and can be easily embedded into future HIV remission and analytical treatment interruption trials.

In Chapters 3 and 4, I investigated associations between cerebrospinal fluid and plasma NfL in greater detail. My results showed that the correlation between cerebrospinal fluid and

plasma NfL was moderate in PLWH on virologically suppressive cART and similar to that seen in lifestyle-similar HIV-negative individuals but renal function and body composition may affect plasma NfL concentration.

At this stage, plasma NfL is likely not sensitive enough as the sole screening tool for central nervous system safety monitoring, and future intervention studies should still include a combination of blood and cerebrospinal fluid analysis and neuroimaging for central nervous system surveillance, which ideally can be incorporated into a predictive algorithm to identify those at highest risk of developing central nervous system complications.

9.5 Future considerations

Most of the study participants that contributed data for the analyses included in my PhD thesis were on modern cART in the form of triple or quadruple antiretroviral therapy. However, the landscape is rapidly changing and in the next five to ten years, it is likely that oral dual therapy cART and alternative antiretroviral drug delivery forms such as long-acting injectables or antiretroviral drug implants will be used more frequently in routine clinical practice. The benefits may include less drug toxicities and improved medication adherence, but the adequacies of central nervous system penetration of these novel therapeutics remain unclear. Early data suggests adequate cerebrospinal fluid concentrations of injectable cabotegravir and rilpivirine[488], however whether this translates into adequate brain parenchymal concentrations is unknown. Reduced central nervous system antiretroviral drug exposure risks compartmentalised HIV viral replication and increased immune activation and neuroinflammation, which could then lead to neuronal injury and neurological sequelae.

It is also becoming increasingly clear that to achieve HIV remission, HIV reservoirs in the central nervous system cannot be ignored. Many current remission technologies poorly cross the blood-brain barrier and much work is being undertaken to develop novel technologies to improve the central nervous system penetration of HIV remission agents, such as by increasing lipophilicity and utilising nanotechnology. Under these circumstances, close central nervous system monitoring would be even more crucial if higher central nervous system penetration and impact is expected.

I plan to continue investigating the central nervous system safety of HIV remission strategies, starting with the RIO study. RIO (ClinicalTrials.gov Identifier: NCT04319367) is a randomised trial investigating the effect of dual long-acting broadly neutralising antibodies in individuals with virologically suppressed HIV, followed by a period of closely monitored ATI. I am part of a collaboration devising a sub-study within RIO to include real-time central nervous system monitoring with neuroimaging and detailed cerebrospinal fluid and blood biomarker measurements of neuroinflammation and neuronal injury throughout the study. Broadly neutralising antibodies are a promising new candidate for HIV remission, but the impact of this strategy on the central nervous system is yet to be determined.

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11 List of Communications

Data presented in this thesis have been submitted to scientific journals or presented at scientific conferences as listed below:

Publications:

1. Correlation between CSF and blood neurofilament light chain protein: A systematic review and meta-analysis (*revisions submitted, awaiting editorial decision*)
2. Correlation between cerebrospinal fluid and plasma neurofilament light protein in treated HIV infection: results from the COBRA study (*revisions submitted, awaiting editorial decision*)
3. No evidence of neuronal damage as measured by neurofilament light chain in a HIV cure study utilising a kick-and-kill approach: the RIVER trial (*under peer review*)
4. Neuronal injury marker plasma neurofilament light following short-course ART initiated during primary HIV-1 infection: the SPARTAC trial (*under peer review*)

Scientific conferences: Oral presentations

1. Investigating neuro-axonal injury and T-cell immune responses following a HIV cure strategy
 - 6th HIV CNS UK meeting, London, UK (March 2019)
 - Awarded prize for best research presentation
2. Investigating neuro-axonal injury following a HIV cure strategy
 - 12th HIV Neuropsychiatry Symposium, Barcelona, Spain (June 2019)
3. Correlation between cerebrospinal fluid and plasma NfL in people with HIV
 - 8th NeuroHIV meeting, Rome, Italy (Oct 2019)

4. Correlation between cerebrospinal fluid and plasma NfL in people with HIV and lifestyle-similar controls

- 17th European AIDS Clinical Society conference, Basel, Switzerland (November 2019)

Scientific conferences: Poster presentations as the presenting author

1. Investigating plasma neurofilament light (NFL), a marker of neuronal integrity, following ART interruption in primary infection

- 22nd International AIDS Conference (World AIDS 2018), Amsterdam, The Netherlands (July 2018)

2. No evidence of neuronal injury following HIV cure: the RIVER trial

- 25th British HIV Association conference, Bournemouth, UK (April 2019)
- 10th International AIDS Society conference, Mexico City, Mexico (July 2019)

3. Correlation between cerebrospinal fluid and plasma NfL in people with HIV and lifestyle-similar controls

- 21st International Workshop on Co-morbidities and Adverse Drug Reactions in HIV , Basel, Switzerland (November 2019)

Data from academic work undertaken during this PhD published in scientific journals

or presented at scientific conferences:

Publications:

1. **Alagaratnam J**, Dilly-Penchala S, Challenger E, et al. Cerebrospinal fluid exposure of cenicriviroc in HIV-positive individuals with cognitive impairment. *Br J Clin Pharmacol*. 2019. doi:10.1111/bcp.13878
2. Vera JH, Bracchi M, **Alagaratnam J**, et al. Improved central nervous system symptoms in people with HIV without objective neuropsychiatric complaints switching from efavirenz to rilpivirine containing cART. *Brain Sci*. 2019. doi:10.3390/brainsci9080195
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4. **Alagaratnam J**, Peters H, Francis K, et al. An observational study of initial HIV RNA decay following initiation of combination antiretroviral treatment during pregnancy. *AIDS Res Ther*. 2020. doi:10.1186/s12981-020-00297-w.
5. Dilly Penchala S, **Alagaratnam J**, Challenger E, et al. The development and validation of a novel LC–MS/MS method for the quantification of cenicriviroc in human plasma and cerebrospinal fluid. *Biomed Chromatogr*. 2020. doi:10.1002/bmc.4711
6. Toniolo S, Cercignani M, Mora-Peris B, Underwood J, **Alagaratnam J**, et al. Changes in functional connectivity in people with HIV switching antiretroviral therapy. *J Neurovirol*. 2020. doi: 10.1007/s13365-020-00853-0.
7. Beanland A, **Alagaratnam J**, Goffe C et al. Objective and subjective rapid frailty screening tools in people with HIV. *HIV Med*. 2021. doi:10.1111/hiv.12988
8. The use of antiretroviral agents in persons with HIV with relative contraindications (*under peer review*)

Scientific conferences: Oral presentations

1. Indeterminate HIV test results in the context of HIV pre-exposure prophylaxis: a case presentation
 - 25th British HIV Association conference, Bournemouth, UK (April 2019)
2. Varicella zoster virus vasculitis: a complex clinical case presentation
 - 12th HIV Neuropsychiatry Symposium, Barcelona, Spain (June 2019)

Scientific conferences: Poster presentations as the presenting author

1. Initiating cART in Pregnancy: Impact on HIV RNA Decay
 - Conference on Retroviruses and Opportunistic Infections (CROI), Boston, USA (Feb 2016)
2. Cerebrospinal fluid exposure of cenicriviroc in HIV-positive individuals with cognitive impairment
 - HIV Glasgow congress, Glasgow, UK (Nov 2018)
3. ART in persons with HIV with relative contraindications: Are we prescribing according to BHIVA guidelines?
 - 25th British HIV Association conference, Bournemouth, UK (April 2019)

12 Appendix

Quality Assessment Tool for Observational Cohort and Cross Sectional Studies[355]

Criteria	Yes	No	Other
1. Was the research question or objective in this paper clearly stated?			
2. Was the study population clearly specified and defined?			
3. Was the participation rate of eligible persons at least 50%?			
4. Were all the subjects selected or recruited from the same or similar populations (including the same time period)? Were inclusion and exclusion criteria for being in the study prespecified and applied uniformly to all participants?			
5. Was a sample size justification, power description, or variance and effect estimates provided?			
6. For the analyses in this paper, were the exposure(s) of interest measured prior to the outcome(s) being measured?			
7. Was the timeframe sufficient so that one could reasonably expect to see an association between exposure and outcome if it existed?			
8. For exposures that can vary in amount or level, did the study examine different levels of the exposure as related to the outcome (e.g., categories of exposure, or exposure measured as continuous variable)?			
9. Were the exposure measures (independent variables) clearly defined, valid, reliable, and implemented consistently across all study participants?			
10. Was the exposure(s) assessed more than once over time?			
11. Were the outcome measures (dependent variables) clearly defined, valid, reliable, and implemented consistently across all study participants?			
12. Were the outcome assessors blinded to the exposure status of participants?			
13. Was loss to follow-up after baseline 20% or less?			
14. Were key potential confounding variables measured and adjusted statistically for their impact on the relationship between exposure(s) and outcome(s)?			

Quality Rating (Good, Fair, or Poor)
Rater #1 initials:
Rater #2 initials:
Additional Comments (If POOR, please state why):

*CD, cannot determine; NA, not applicable;
NR, not reported

Quality assessment tool for before-after (pre-post) studies with no control group^[355]

Criteria	Yes	No	Other
1. Was the study question or objective clearly stated?			
2. Were eligibility/selection criteria for the study population prespecified and clearly described?			
3. Were the participants in the study representative of those who would be eligible for the test/service/intervention in the general or clinical population of interest?			
4. Were all eligible participants that met the prespecified entry criteria enrolled?			
5. Was the sample size sufficiently large to provide confidence in the findings?			
6. Was the test/service/intervention clearly described and delivered consistently across the study population?			
7. Were the outcome measures prespecified, clearly defined, valid, reliable, and assessed consistently across all study participants?			
8. Were the people assessing the outcomes blinded to the participants' exposures/interventions?			
9. Was the loss to follow-up after baseline 20% or less? Were those lost to follow-up accounted for in the analysis?			
10. Did the statistical methods examine changes in outcome measures from before to after the intervention? Were statistical tests done that provided p values for the pre-to-post changes?			
11. Were outcome measures of interest taken multiple times before the intervention and multiple times after the intervention (i.e., did they use an interrupted time-series design)?			
12. If the intervention was conducted at a group level (e.g., a whole hospital, a community, etc.) did the statistical analysis take into account the use of individual-level data to determine effects at the group level?			

Quality Rating (Good, Fair, or Poor) (see guidance)
Rater #1 Initials:
Rater #2 Initials:
Additional Comments (If POOR, please state why):

*CD, cannot determine; NA, not applicable; NR, not reported

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Page no. in thesis	Type of work:	Name of work in journal article	Source of work	Copyright holder and contact	Permission requested on	I have permission yes /no	License Number
36	Figure	Figure 2. A schematic, semi-quantitative display of the progression of HIV markers.	Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection: Fiebig et al (2003)[14]	Wolters Kluwer Health, Inc.	14.4.2021	Yes	5047360452838
37	Figure	Figure 1. Change in HIV viral load and CD4 count following infection	Natural history of HIV and AIDS: O'Cofaigh et al (2013)[20]	Elsevier	14.4.2021	Yes	5047321232281
39	Figure	Figure 3: Neuropathogenic mechanisms that contribute to HIV-associated neurocognitive disorders.	HIV-associated neurocognitive disorder — pathogenesis and prospects for treatment: Saylor (2016)[35]	Springer Nature	23.3.2021	Yes	5034880096732
40	Figure	Figure 1. M1/M2 polarization of microglia and their immunoregulatory functions.	Role of Microglial M1/M2 Polarization in Relapse and Remission of Psychiatric Disorders and Diseases: Nakagawa (2014)[47]	MDPI		Yes	No special permission is required to reuse all, or part of article published by MDPI, including figures and tables

43	Figure	Figure 1. Key Figure: Latent HIV Is a Multifactorial Challenge	HIV-1 Reservoirs During Suppressive Therapy: Barton et al (2016)[78]	Elsevier	23.3.2021	Yes	5034870446999
46	Table	Table 2: Characteristics of assays.	Comparative Analysis of Measures of Viral Reservoirs in HIV-1 Eradication Studies: Eriksson (2013)[104]	Plos		Yes	Articles and accompanying materials published by PLOS on the PLOS Sites, including peer reviews, are licensed by the respective authors for use and distribution by you subject to citation of the original source in accordance with the Creative Commons Attribution (CC BY) license.
48	Figure	Box 4: The fraction of intact HIV genomes in virally suppressed individuals	International AIDS Society global scientific strategy: towards an HIV cure 2016: Deeks (2016)[111]	Springer Nature	26.3.2021	Yes	5036540207245
54	Figure	Figure 2: Immune activation sustains HIV persistence during antiretroviral therapy.	Immune activation and HIV persistence: implications for curative approaches to HIV infection: Klatt (2013)[152]	John Wiley and Sons	13.4.2021	Yes	5047250898424
72	Table	Table 1. Comparison of different strategies for curing HIV	A cure for HIV: is it in sight?: Pace (2014)[251]	Taylor & Francis		Yes	Taylor & Francis is pleased to offer reuses of its content for a thesis or

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73	Figure	Figure 1: Shock and Kill Strategy to Eliminate HIV Latently Infected Cells	Getting the “Kill” into “Shock and Kill”: Strategies to Eliminate Latent HIV: Kim (2018)[255]	Elsevier	23.3.2021	Yes	5034921370072
77	Table	Table 1: Examples of defined CSF biomarkers examined in our studies of HIV	Approach to Cerebrospinal Fluid (CSF) Biomarker Discovery and Evaluation in HIV Infection: Price et al (2013)[273]	Springer Link		Yes	This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.
78	Figure	Figure 1: Overview of the structure of neurofilaments and neurofilament light chain.	Neurofilament light chain as a biomarker in neurological disorders: Gaetani (2019)[281]	BMJ Publishing Group Ltd.	24.3.2021	Yes	5035401263667
83	Figure	Figure 1: Representative standard uptake value ratio image of [¹¹ C]-PBR28 positron emission tomography (PET) scan	Molecular Imaging of Neuroinflammation in HIV: Boerwinkle (2019)[318]	Springer Nature	25.3.2021	Yes	5036020349557

86	Table	Table 1: Summary of PET studies using TSPO radioligands to measure neuroinflammation in PLWH	Molecular Imaging of Neuroinflammation in HIV: Boerwinkle (2019)[306]	Springer Nature	25.3.2021	Yes	5036020349557
90	Figure	Figure 2: PET/CT results from a chronically infected macaque, before and at 5 weeks of ART.	Whole-body immunoPET reveals active SIV dynamics in viremic and antiretroviral therapy-treated macaques: Santangelo (2015)[108]	Springer Nature	13.4.2021	Yes	5047370987464

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