

Exploring microglial activation in treated HIV-infected individuals

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Thesis submitted in fulfillment of the requirements for the
degree of Doctor of Philosophy of Imperial College London

2014

Declaration

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Abstract

The introduction of combination antiretroviral therapy (cART) has resulted in a dramatic improvement in the life-expectancy for people living with HIV-infection. Although AIDS-defining illnesses such as HIV-dementia are now rare, mild cognitive disorders are still prevalent in a substantial proportion of patients on effective cART and suppressed HIV plasma viraemia.

Although some clinic risk factors for HIV-associated neurocognitive impairment (NCI) such as low nadir CD4 T-cell count and age have been described, the pathological processes underlying this cognitive impairment remain elusive. A major factor that might be contributing to the development of HIV-associated NCI is chronic activation of macrophages and astrocytes resident in the brain microglia. I hypothesise that *in vivo* microglial activation is both present in effectively treated HIV-infected individuals and is associated with disturbances in cognitive function and biomarkers of magnetic resonance imaging (functional, structural and chemical) and inflammation

To address these hypotheses I used cerebral positron emission tomography with [^{11}C] PBR28 to measure translocator protein 18kDa (TSPO), a putative marker for microglial activation. To examine the impact of inflammation on brain structure, function and chemistry, neurologically asymptomatic HIV-infected individuals stable on cART were evaluated using several magnetic resonance imaging methods, cognitive testing and measurements of inflammatory biomarker in plasma and cerebrospinal fluid.

I identified the presence of *in vivo* microglial activation in several brain regions in HIV-infected individuals compared to HIV-negative controls. Microglial activation was associated with disturbances in cognitive function (verbal and learning memory), brain activation (reduced task-related brain activation) and white matter integrity (increased mean diffusivity), suggesting a deleterious effect of neuroinflammation on brain structure and function. Microglial activation, across several anatomical locations correlated with increased concentrations of plasma ribosomal 16s, a marker of microbial translocation. This relationship between a marker of impaired integrity of the

gut-blood barrier, and microglial activation, suggests a communication route is present between peripheral inflammation and the CNS in HIV-infected individuals.

In vivo detection of microglial activation, and systemic inflammatory markers could provide indices to guide risk assessment and treatments for HIV-associated brain disease.

Acknowledgements

I would like to thank the Wellcome Trust and GlaxoSmithKline for funding this project. I am also grateful to all the volunteers for their time and commitment to this research. I would also like to thank the staff from the Clinical Trials Centre, the HIV department at St Mary's Hospital and the Imanova for all their help and in particular to Dr. James Cole and Dr. Qi Guo for their support and patient teaching me and helping me with the imaging analysis. I am extremely grateful to my supervisors Dr. Alan Winston and Professor Paul Matthews for the constant support, and encouragement provided during the last 3 years.

To my family, Caroline, Sophie, and Charlie: I could not have done this without you. Thank you for your patience, and support.

Finalmente a mi madre, Maria Edith quien me ha apoyado durante este tiempo aunque se encuentre lejos.

Table of Contents

Declaration	2
Abstract.....	5
Acknowledgements	7
Abbreviations.....	10
Chapter 1: General Introduction.....	13
Chapter 2: Materials and Methods	52
Chapter 3: Neuroinflammation in HIV-infected individuals stable on cART: a [¹¹C] PBR28 PET CT study	67
Chapter 4: Cross-sectional analysis of the relationship between microglial activation and functional, structural and metabolic MRI imaging parameters in HIV-infected individuals	103
Chapter 5: Cross-sectional analysis of the relationship between microglial activation and blood and CSF inflammatory biomarkers in HIV-infected individuals on cART	128
Chapter 6: Cerebral inflammatory chemokines in cynomolgus macaques infected with SIVmac251	141
Chapter 7: Overall conclusions	155
List of communications	163
References	166

Abbreviations

AIDS	Acquired immunodeficiency syndrome
cART	Combination antiretroviral therapy
BBB	Blood brain barrier
CSF	Cerebrospinal fluid
CNS	Central nervous system
Cho	Choline
Cr	Creatine
[¹¹ C]	Radioisotope carbon 11
CT	Computarised tomography
DTI	Diffusion tensor imaging
DMN	Default mode network
FA	Fractional anisotropy
fMRI	Functional magnetic resonance imaging
FWM	Frontal white matter
FGM	Frontal grey matter
HAD	HIV-associated dementia
HIV	Human immunodeficiency virus-1
IL-8	Interleukin-8
IP-10	Interferon protein ten
LP	Lumbar puncture
MCH-II	Major histocompatibility complex II
MD	Mean diffusivity
¹ H-MRS	Proton magnetic resonance spectroscopy
MRI	Magnetic resonance imaging
ml	Myoinositol
MIP-1β	Macrophage inflammatory protein 1 beta
MCP-1	Monocyte chemoattractant protein 1
NFL	Neurofilament light
NHP	Non-human primate model
NCI	Neurocognitive impairment
PBR28	(N-(2-methoxybenzyl)-N-(4-phenoxy pyridin-3-yl)acetamide)

PK11195	(1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-2-isoquinoline
PK	Pharmacokinetics
R-fMRI	Resting functional magnetic resonance imaging
RNA	Ribonucleic acid
RBG	Right basal ganglia
SDF-1 α	Stromal cell derived factor 1
sCD14	Soluble CD14
sCD163	Soluble CD163
SDF-1 α	Stromal cell derived factor 1
TSPO	Translocator protein 18 Kda
V _T	Volume of distribution

Chapter 1

General Introduction

Chapter 1: General Introduction

1.1 Hypotheses of the thesis	14
1.2 Central nervous system manifestations of HIV-infection	14
1.2.1 Clinical manifestations of HIV-infection in the CNS prior to the advent of cART ..	14
1.2.1.1 Neuropathology prior to the advent of cART	15
1.2.2 HIV-infection of the CNS since the advent of cART	16
1.2.2.1 Neuropathology since the advent of cART	17
1.2.3 Simian immunodeficiency virus models of HIV-associated brain disease	18
1.3 Pathogenesis of HIV-related CNS injury	20
1.3.1 HIV invasion of the brain	20
1.3.2 The role of microglia	21
1.3.3 The role of astrocytes	24
1.3.4 Chemokines/cytokines in HIV-related CNS injury	24
1.4 Mechanisms of HIV-related CNS injury in the setting of cART	25
1.4.1 Early impact of HIV-infection in the CNS	25
1.4.2 Persistent CNS viral HIV replication	26
1.4.3 Ongoing CNS immuneactivation	27
1.4.4 Reduced ART exposure or toxicity within the CNS	29
1.4.5 Neurodegeneration	31
1.4.6 Patient factors	32
1.4.7 Rationale for studying microglial activation	33
1.5 Neuroimaging in HIV disease	33
1.5.1 Brain magnetic resonance spectroscopy findings in HIV-associated NCI	33
1.5.2 BOLD functional magnetic resonance imaging in HIV-associated brain disease ..	36
1.5.2.1 fMRI findings in HIV-NCI	36
1.5.2.2 Effects of cART on brain function evaluated with fMRI	38
1.5.2.3 Resting fMRI findings in HIV-associated NCI	39
1.5.3 Diffusion tensor imaging findings in HIV-associated NCI	40
1.5.4 <i>In vivo</i> imaging of neuroinflammation	41
1.5.4.1 Positron emission tomography (PET) imaging of the 18kDA translocator protein (TSPO)	41
1.5.4.2 Imaging of TSPO in HIV-associated NCI	42
1.5.4.3 [¹¹ C] PBR28 TSPO radioligand	42

1.1 Hypotheses of the thesis

The following hypotheses will be examined in this thesis:

- 1. Microglial activation will be greater in neuroasymptomatic HIV-infected individuals stable on cART compared to HIV-negative controls.**
- 2. Microglial activation in neuroasymptomatic HIV-infected individuals stable on cART will be associated with poorer cognitive function, increase biomarkers of inflammation and abnormalities of functional, structural, and metabolic imaging parameters on cerebral MRI.**
- 3. Microglial activation in neuroasymptomatic HIV-infected individuals stable on cART will be greater in subjects with low nadir CD4 counts compared to those with high nadir CD4 counts.**

1.2 Central nervous system manifestations of HIV-infection

1.2.1 Clinical manifestations of HIV-infection in the CNS prior to the advent of cART

Early in the HIV/AIDS epidemic the deleterious consequences of HIV-infection in the central and peripheral nervous system were described (1). They were predominantly attributable to either CNS opportunistic infections, due to severe immunosuppression and depletion of CD4 T-cell blood counts or a spectrum of HIV-associated NCI, the most severe form being HIV-associated dementia (HAD).

HAD is a subcortical dementia that typically occurs at the later stages of HIV-infection when there is advanced immunosuppression (CD4 T-cell counts below 200 cells/mL). HAD develops in approximately 20 to 30% of HIV-infected individuals without cART. Clinically HAD is characterised by a group of severe cognitive (mental slowing, attention and memory deficits), behavioral (apathy, social withdrawal, and mood disturbances) and motor symptoms (loss of balance and slowness) (2). HAD in its more severe forms could lead to paraplegia, vegetative state and dead (3). The onset of HAD is associated with several risk factors including low CD4 T-cell count and

high plasma HIV RNA, which accounts for the strong effects of cART on reducing its incidence (4).

1.2.1.1 Neuropathology prior to the advent of cART

HIV-encephalitis is the neuropathological correlate of HAD, and it was consistently observed in brain tissue of HIV-infected individuals throughout the early HIV-epidemic (5-7)

The histopathology of the brains of HIV-infected individuals with encephalitis shows characteristic changes. Widespread microglial activation is prominent usually accentuated by the presence of microglial nodules (8, 9). Multinucleated giant cells, although not pathognomonic of HIV-encephalitis are a prominent feature in both the white and grey matter. Reactive astrocytosis is also commonly seen often around small blood vessels in the central white matter along with activated microglia (9, 10) (8). The white matter pathology observed in HIV-encephalitis is characterized by myelin damage, axonal damage, and the presence of white matter varicosities and axon bulbs. These changes are also known as leucoencephalopathy (11).

HIV-encephalitis can be present in any area of the brain, but it has been shown to have a predilection for subcortical regions particularly the basal ganglia. Kure et al. looked at the distribution of HIV-infection in the brain of 100 individuals with AIDS with histological evidence of HIV-encephalitis, using immune-staining with gp-41 antibodies. Microglial and multinucleated cells were the groups of brain cells with the greatest Gp-41 positivity. They were detected and most abundant in the globus pallidus, ventral midbrain and dentate nucleus. Gp-41 cells were rarely seen in the cerebral cortex, medulla or leptomeninges. These data suggest that the HIV virus might have a tropism for deep grey matter structures such as the basal ganglia and the dentate nucleus (12).

Only a small number of studies have reported the neuropathology of untreated HIV-infected individuals before the onset of AIDS. Most of these studies consisted of HIV-infected drug users who died of drug related accidents or haemophiliacs (13-15). In these individuals, a low grade CD8 lymphocytic infiltrate involving the leptomeninges,

and the perivascular spaces of the white matter was observed, along with astrogliosis and microglial activation (14, 15).

The presence of HIV-encephalitis correlates with the clinical severity of HAD. The pathological features most closely associated with the clinical signs of HAD include: the level of microglial activation in the brain (16), evidence of excitotoxins (17) and selective neuronal loss (18). HAD has been associated with neuronal apoptosis (19, 20) with the distribution of neuronal apoptosis closely related with structural atrophy and microglial activation markers, especially within subcortical brain matter structures (21). In addition, macrophage staining for microglial activation in the basal ganglia has also been found to correlate with HAD (16).

1.2.2 HIV-infection of the CNS since the advent of cART

The effect of cART on the reduction of incidence of CNS opportunistic infections and HAD had been dramatic (22). However, less severe forms of cognitive deficits affecting HIV-infected subjects have become increasingly apparent in recent years, despite access to cART (23).

Rates and clinical features of milder forms of NCI in a range of clinical and geographical settings have demonstrated consistently higher prevalence rates (15 to 50%) of milder forms of NCI in effectively treated HIV-infected individuals compared to the general population (24-29).

Changes in the clinical manifestations of HIV-associated NCI since the introduction of cART are summarised in Table 1.1. Clinically, most HIV-infected patients with these milder forms of NCI complain of difficulty concentrating, memory problems and fatigue when required to engage in demanding mental tasks. The cognitive deficits are frequently seen in the domains of learning, attention/working memory, speed of information processing and executive function (30, 31). NCI is of clinical relevance with consequences for reduced quality of life (32), reduced functioning on activities of daily living (33) and even increased mortality (34). Furthermore, HIV-associated NCI may undermine sustained adherence to lifelong cART, affecting both the long-term effectiveness of treatment for the individual and the prevention of onward HIV-transmission.

Although, the CD4 T-cell count remains an important clinical risk factor for HIV-associated NCI, it is the nadir CD4 T-cell count (the lowest recorded CD4 T-cell count) that is more predictive of milder forms of NCI in the post cART era (35). With the loss of current CD4 T-cell count and plasma HIV RNA, as disease predictors of HIV-associated NCI, there is a lack of clinical laboratory or imaging indicators to predict milder forms of NCI in treated HIV-infected individuals.

1.2.2.1 Neuropathology since the advent of cART

The introduction of cART has changed the neuropathology of HIV-infection in the CNS. Several studies have documented the neuropathology of HIV-infected individuals post-cART (36-39). All have reported a decline in CNS opportunistic infections associated with HIV-infection. Although there has also been a decline in autopsy-proven HIV-encephalitis, it appears that HIV-encephalitis is still common with prevalence rates of up to 25% in some cohort studies (38). Data from the Edinburgh cohort have shown a 50% decrease in the incidence of CMV and a 68% decrease in HIV-encephalitis (40).

The type of HIV-encephalitis described in patients on cART appears to be different from the one observed in HAD of the pre-cART era. Gray et al. studied brain autopsies of cART experienced HIV-infected individuals. The study found evidence of HIV-encephalitis; however, the type of pathological features differed greatly from the ones observed in patients with HAD. Instead of multinucleated giant cells and microglial nodules, the predominant features identified were myelin pallor, astrocytosis, and microglial activation (41).

Consistent with the findings described above, Antony et al. Investigated brain autopsies of effectively treated HIV-infected individuals who died from causes independent of CNS disease in Edinburgh. In this study, high levels of microglial activation evidenced by high expression of CD68 and MHC II in the basal ganglia and hippocampus were found in effectively treated HIV-infected individuals. The levels of microglial activation were comparable or in some cases exceeded those seen in cases of HIV-encephalitis associated with HAD (40).

The success of cART has limited the opportunities for re-evaluating neuropathological findings as the number of fatalities among stable patients on cART is low, and generally confined to subjects that have died from other conditions such as drug overdoses and co-infection with viral hepatitis etc. Although neuropathological studies in such cases revealed ongoing neuroinflammation without productive HIV-infection, the contribution of drug abuse, comorbidities and co-infections requires further study to provide a better understanding of the effects of HIV-infection within the CNS in stable patients on cART.

1.2.3 Simian immunodeficiency virus models of HIV-associated brain disease

The simian immunodeficiency virus (SIV) and HIV virus are primate lentiviruses that have genomic, structural, and virological similarities (42). SIV viruses have been isolated from different genera of non-human primates, and are designated by subscripts indicating the type of primate from which they were originally isolated, for example, SIVmac for rhesus monkeys; SIVsmm from sooty mangabeys etc. SIV-infection of macaques accurately replicates many features of HIV-infection, including the development of histopathological changes typical of HIV-encephalitis, such as astrogliosis, multinucleated giant cells and microglial proliferation (43, 44). The development of SIV animal models that replicate key clinical and pathological features of HIV-infection in the CNS could allow the study of HIV-associated brain disease pathogenesis, as well as the longitudinal evaluation of immune and inflammatory parameters in the CNS, and the evaluation of novel therapeutics.

SIV-infection of rhesus and pig-tailed macaques is the most common non-human primate model of HIV-disease. Initiation of SIV-infection is achieved by using selected strains or clones. Some of these strains have been developed to be highly neurovirulent such as SIV17e-fr and SIVDeltaB670. Inoculation with any of these combinations of viruses results in rapid, acute SIV-viral replication in both the peripheral blood and the CNS. High plasma and CSF viral SIV RNA levels are detected as early as 3 days post-inoculation and peaked 7 to 10 days after inoculation (45). After acute infection CSF SIV RNA declines by 2 to 3 logs, however within 2 to 3 weeks the SIV RNA levels increase again in those animals that develop moderate to severe neurological disease. The continued loss of CD4 T-cells begins

during the acute infection stage and by day 84 post-inoculation most animals will have AIDS and CNS disease (45).

Models of SIV using pigtailed macaques develop immunosuppression, and higher frequency of CNS lesions more rapidly than rhesus macaques of cynomolgus macaques inoculated with the same viruses (44). These accelerated models of SIV-brain disease have been used to study several aspects of HIV-associated brain disease including: the mechanisms of viral and immune damage in the CNS, the influence of the innate immune system on the development of CNS disease, the identification of host genetic resistance to CNS disease and the effect of cART on CNS-disease (46-50).

It has become clear that SIV infects the CNS very early during the disease process and that innate immune responses are induced acutely in response. In the accelerated SIV-macaque model using either SIV17e-fr or SIVDeltaB670 neurovirulent strains greater levels of SIV RNA is present in the CSF by 4 days post-inoculation, regardless of whether those animals ultimately develop neurological lesions (47). SIV replication can also be detected in CD14⁺ microglia, usually accompanied by widespread innate immune responses (47). Using CD8 depletion in the SIV rhesus macaque models, it has been demonstrated that perivascular macrophages and not resident microglia are the major cell population productively infected with SIV during acute infection and among macaques with encephalitis (51).

The innate immune response triggered by SIV-infection is characterised by activation of microglia and astrocytes (52, 53). Microglial activation causes induction of cytokines/chemokines such as CCL2, IL-6, IL-10, TNF- α and INF- γ that leading to neuroinflammation and neuropathological changes associated with encephalitis such as multinucleated giant cells, astrocytosis and neurological disease (54).

Since the CNS represent a distinct HIV compartment and most cART do not cross the blood brain barrier to any significant extend (55), SIV macaque models have been developed where animals are treated with a combination of nucleotide reverse transcriptase inhibitors, protease inhibitors, and integrase inhibitors (56, 57). In these models plasma and CSF SIV RNA declined rapidly with therapy. However, although brain viral SIV RNA levels were undetectable in treated animals, the levels of viral

DNA were not different from viral DNA levels of untreated SIV-infected macaques. CNS inflammation was reduced; however, levels of CD68, TNF- α and INF- γ were not significantly lower compared to controls, indicating continued CSN inflammation, despite controlled CNS SIV replication (56).

Recently Fergusson et al. Described the presence of persistent neuroinflammation in a cynomolgus model of CNS infection using a SIVrtTA, a doxycycline (DOX) dependent, conditional-SIV virus, where viral replication can only occur in the presence of the antibiotic doxycycline. The study compared animals on daily doxycycline for 40 weeks with those where doxycycline was removed after acute infection was achieved. Chronic neuroinflammatory responses (astrocytosis and microgliosis) were detected by immunohistochemical analysis of brain sections in all SIVrtTA infected macaques, even 40 weeks after stopping viral replication (when doxycycline was stopped). The results of this study indicate that despite absence of viral replication, neuroinflammatory responses (microglial activation) persisted after many weeks, suggesting a legacy effect of acute SIV-infection (58)

1.3 Pathogenesis of HIV-related CNS injury

1.3.1 HIV invasion of the brain

During primary HIV-infection, HIV enters the central nervous system (CNS) very early during the course of systemic HIV-infection (59, 60) through its presence in infected macrophages that migrate across the blood brain barrier to replace infected perivascular macrophages. This mechanism is known as the “Trojan horse hypothesis” first described by Hasse et al (61), that demonstrated how HIV-1 and other lentivirus enter the CNS as passengers in cells that are trafficking through the brain. After entry, activated monocytes, perivascular macrophages and microglia infected with HIV act as both the main targets of HIV-replication and source of neurotoxins such as neurotoxic reactive oxygen species, prostaglandin E2 and nitric oxide etc, that lead to further disruption of the blood brain barrier, facilitating brain infection (62). HIV-infection within the CNS can be detected by measurements of viral RNA in the CSF, and CSF HIV RNA has been correlated with brain HIV RNA levels (63) and the degree of cognitive dysfunction in patients with HAD (64, 65).

It is well established that microglia, and perivascular macrophages are the cells that most contribute to viral replication within the CNS and are key players in the neuropathogenesis of HIV-associated brain disease. Microglia and macrophages are the only CNS cells that have CD4 and CCR5 receptors. The HIV virus can also infect astrocytes, however without CD4 receptors, astrocytes are unlikely to contribute to persistent viral replication in the CNS (66, 67).

1.3.2 The role of microglia

Macrophages in the CNS contain several subtypes including perivascular macrophages, meningeal macrophage, choroid plexus macrophages and microglia. Microglia are specialised macrophages of the CNS that constitute approximately 5 to 20% of all glial cells in the human adult brain (68). The resident microglia originate from yolk sac macrophages that migrate to the CNS during early embryogenesis (69), and therefore the origins of these cells are different from other glial cells such as astrocytes and oligodendrocytes. Microglia are essential for the maintenance of CNS homeostasis and the defense against brain injury. They are involved in phagocytosis, antigen presentation, and production of biological substances that regulate inflammation, cell death, and regeneration processes (70).

One characteristic aspect of microglia are the capacity to modify their phenotype in response to brain injury. Based on pathophysiological studies it is understood that, microglia show many states of activation, depending on the nature of the brain injury. Some of the described phenotypic states include: (i) resting phenotype characterised by the presence of ramified branches that perform surveillance of the CNS (71). (ii) M1-type microglial activation and (iii) M2-type microglial activation (72, 73) see Figure 1.1. The distinction between M1-type and M2-type activation represents an ideal, which is often simplistic as the range of chemokines and antigens expressed by activated microglia is not specific to the M1 or M2 categories. It is important to understand the plasticity of microglia, where in different pathological states activated microglia might produce a range of different cytokines/chemokines profiles (74). Knowledge about the precise functions of these phenotypic states is essential for the understanding of the pathogenic mechanisms involve in HIV-associated brain disease

M1-type microglial activation

M1 activated macrophages are developed in response to IFN- γ and microbial products such as LPS. Once activated M1 macrophages produces high levels of proinflammatory cytokines such as IFN- γ , TNF- α , IL-1, chemokines, and nitric oxide that promote cell-mediated-immunity and contribute to the clearance of pathogens. In HIV-associated brain disease the M1 phenotype activates when microglia contacts HIV viral proteins such as tat (75) and toll-like receptors 3 and 4 (76). In this scenario prolonged and excessive M1 activation caused by HIV replication results in the production of proinflammatory mediators and neurotoxins by M1 activated microglia that in turn causes dysfunctional activation of astrocytes leading to neuronal dysfunction.

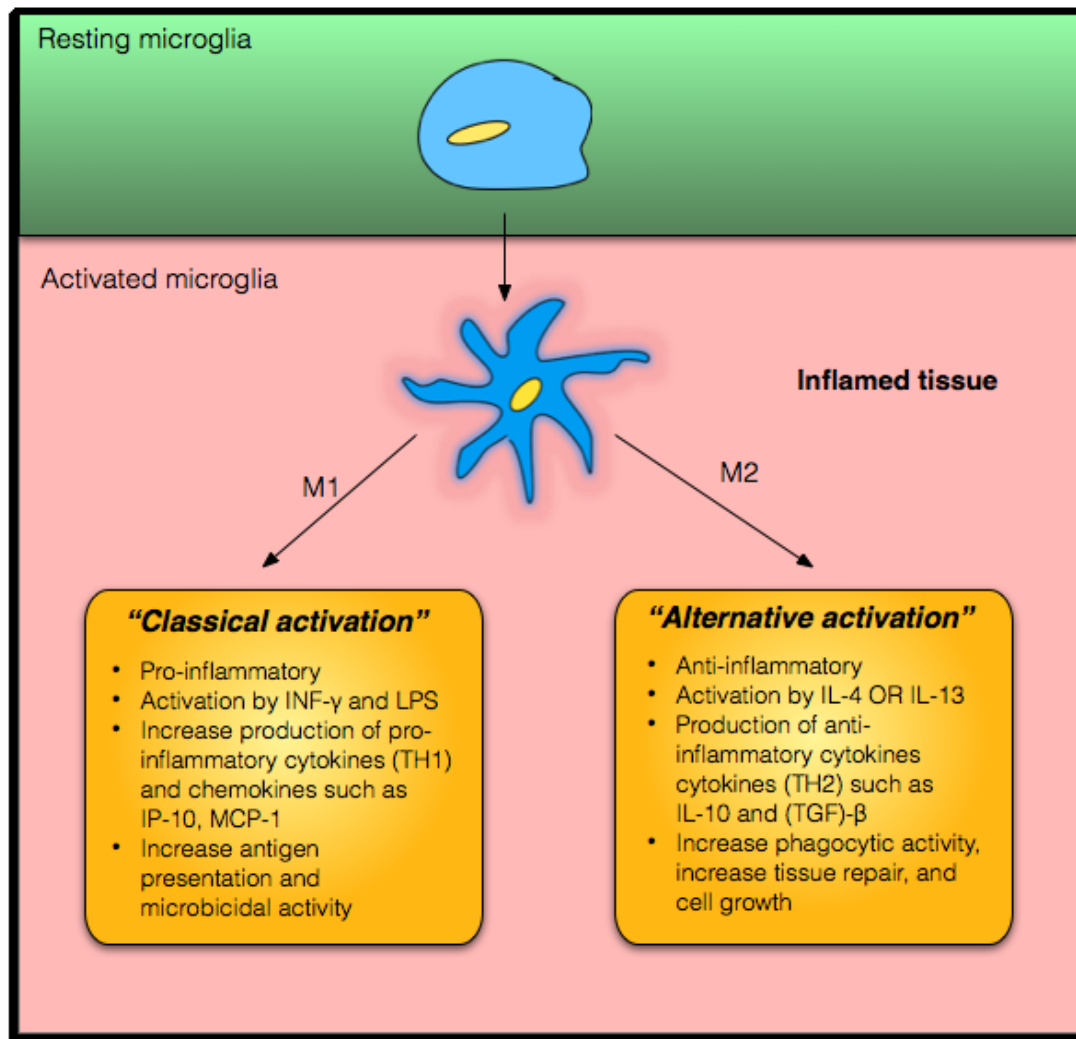
There is evidence that in untreated HIV-infected individuals, M1 microglial activation plays an important role in the pathogenesis of HAD (77). Interestingly, M1 macrophage activation has also been associated with disease states that are driven by low-levels of inflammation such as atherosclerosis (78).

M2-type microglial activation

M2 activated microglia tend to suppress inflammation and play a role in promoting tissue repair (72). M2 activated microglia produce very low levels of inflammatory cytokines such as TNF- α and IL-1 and high levels of anti-inflammatory cytokines such as interleukin-10 (IL-10), interleukin-6 (IL-6), transforming growth factor beta (TGF- β), and reduce expression of major histocompatibility complex (MHC) class II molecules (72, 79, 80).

It is currently unknown whether there are different microglial phenotypes within specific anatomical brain regions, but work by De Hass et al. Showing that microglia from specific regions in the brain express different cell surface proteins suggest that this may be the case (81). Therefore, it is possible that in a disease process such as HIV-associated NCI, different microglial activation phenotypes can be present simultaneously in different brain regions.

Figure 1.1 Diagram representing a model of microglial activation phenotypes in response to brain injury.



[Legend Figure 1.1: M1-type activation is characterised by the production of pro-inflammatory cytokines/chemokines and activation of $\text{INF-}\gamma$. M2-type activation is associated with the release of anti-inflammatory cytokines/chemokines such as IL-10 and TGF- β . In response to changes in the environment is more likely that microglia will move between M1 and M2 phenotypes, with release of a range of pro-inflammatory or anti-inflammatory mediators. Adapted from Gordon et al. (73)]

1.3.3 The role of astrocytes

Astrocytes are abundant within the CNS and play an important role in CNS homeostasis and function (82). They can be infected by HIV inducing reactive astrogliosis a common feature observed in HIV-encephalitis (41). As with microglia, astrocytes respond to any type of brain injury by rapid activation (astrocytosis) comprising the increase in number and size of astrocytes, and the up-regulated expression of cytokines, cell surface molecules, growth factors, and neurotoxic factors (82). Aberrant astrocyte function together with activated microglia, may contribute to the pathogenesis of HIV-associated brain disease. HIV-infection of astrocytes has been demonstrated to disrupt BBB integrity by affecting functional gap junctions (83), and to inhibit glutamate transporters in the astrocytes (84). The disruption of astrocyte glutamate transport and BBB integrity could lead to excitotoxicity and neuronal damage associated with HIV-associated NCI (85).

1.3.4 Chemokines/cytokines in HIV-related CNS injury

Following HIV-infection of the brain, and microglial infiltration, activated microglia within the CNS release a variety of proinflammatory and neurotoxic factors. These include chemokines/cytokines (86), viral proteins (gp120, tat) (17, 87, 88), excitatory aminoacids (glutamate, quinolinate, cysteine) (89, 90), reactive oxygen products (nitric oxide, superoxide anion) (91) and other substances, including platelet activating factor and arachidonic acid (92, 93). Notably, the combination of toxic viral proteins and factors release from activated microglia can act synergistically to promote neuroinflammation even without extensive viral invasion of the CNS (94).

Chemokines are a large family of small proteins (8 to 10 kDa) that are produced in all tissues including the brain. Chemokine receptors are a family of seven transmembrane G-protein coupled receptors and are subdivided in 4 groups: α -chemokine receptors (CXCR2 and CXCR4); β -chemokine receptors (CCR5, CCR4, CCR3 and CCR2); γ -chemokine receptor (XCR1) and δ -chemokine receptors (CX3CR1). Monocytes and microglia are infected with HIV primarily through CCR5 or CCR3 but CXCR4 may also be involved (95, 96).

Increased expression of several α and β -chemokines have been documented in HIV-infected subjects with HAD (summary Table 1.2). Altered expression of α and β -chemokines is commonly seen in HIV-encephalitis (97) and increased expression of

their receptors have been reported in the brains of children and adults with AIDS. Being essential components of brain physiology, chemokines and their receptors may play a crucial role in the balance between neuroprotection and neurotoxicity in HIV-infected individuals (86, 94, 98). For instance, no neurotoxic effects of β -chemokines have been demonstrated *in vivo*, while *in vitro* studies have found that chemokines such as MIP-1 β and RANTES, natural ligands of CCR5 may contribute to protective immunity against HIV-infection (98, 99). Therefore, although there is evident dysfunction in the expression of chemokines and their receptors in HIV-encephalitis and HAD, the positive or negative effect of these in milder forms of NCI has not been elucidated.

1.4 Mechanisms of HIV-related CNS injury in the setting of cART

Although the mechanisms of HIV-related CNS injury associated with HAD have been described, the pathogenesis of milder forms of HIV-associated NCI has not been defined. Several pathogenic mechanisms have been proposed, and many clinical risk factors identified, see Table 1.3

1.4.1 Early impact of HIV-infection in the CNS

HIV invasion of the brain during early stages of HIV-infection is associated with CNS inflammation. However, whether this inflammation within the CNS causes irreversible damage to the brain during early stages of infection prior to initiation of cART is not known. Recently, Peluso et al. reported increased levels of a marker of axonal injury, NFL in HIV neuroasymptomatic subjects within the first year of HIV-infection (100). In this study, concentrations of CSF NFL correlated with increased concentrations of neopterin, a marker of monocyte activation and lower concentrations of cerebral metabolites of neuronal integrity (NAA/Cr). Early initiation of cART in patients from the same cohort of patients had persistently elevated concentrations of NFL and neopterin, suggesting a failure to control inflammation with therapy despite early initiation of cART (101). Whether early initiation of cART could eventually reduce CNS inflammation, is not known. Similarly, it is not clear if reducing CNS inflammation will have a significant effect clinically, for instance reducing the rates of HIV-associated NCI

In the clinical setting most patients are diagnosed with HIV-infection during the chronic phase of HIV-disease, as primary HIV-infection is usually difficult to diagnose because patients with primary HIV-infection rarely present to healthcare settings. Also, most asymptomatic HIV-infected patients start cART when the CD4 T-cell counts are below a value of <350 cells/mL (established as a clinical threshold in the UK) (102). This could mean inflammation in the CNS it is likely to have been present for a significant period of time before cART is initiated.

It remains unclear what is the effect of commencing cART in neurologically asymptomatic subjects with high CD4 T-cell counts. A neurology sub-study with the START study (Strategic Timing of Antiretroviral Therapy; ClinicalTrials.gov Identifier NCT00867048) is assessing the effect of the earlier initiation of cART on cognitive function. In this study subjects with CD4 T-cell counts above 500 cells/uL, are randomised to immediate cART or deferred cART (until CD4 T cell counts falls below 350 cells/uL) with prospective cognitive function assessed during the study period. Results from this study might answer the above conundrum regarding the impact of the earlier initiation of cART on cognitive function.

1.4.2 Persistent CNS viral HIV replication

Following HIV-infection of the brain, HIV RNA remains detectable in the CSF in most of untreated patients during the course of HIV chronic disease (65). Most patients commencing cART achieve plasma and CSF HIV RNA levels below the limit of detection of clinical assays. However, cases of viral escape in the CSF despite suppression of plasma HIV RNA have been reported. Concerns have been raised that the viral replication in the CNS can act as a reservoir for viral persistence and evolution of drug resistance. CSF escape has been reported in 10 to 20% of HIV-infected individuals without neurological symptoms (55, 103). In this group of subjects, CSF escape, has been associated with high levels of intrathecal immune activation, specifically, markers of monocyte activation such as neopterin (103). The clinical significance of CSF HIV RNA escape is unknown, but neurological symptoms with CSF escape have been reported. For example, Canestri et al. described a group of 11 subjects on cART that experienced neurological symptoms and had a CSF HIV RNA of >200 copies/ mL with suppressed plasma HIV RNA (104). This group of patients presented with a combination of acute and sub-acute neurological symptoms

and 7 out of 11 had evidence of resistance-associated mutations in CSF HIV RNA genotypic strains. Conversely, in neuroasymptomatic HIV-infected subjects on stable cART, CSF escape (>50 copies/mL) is present in approximately 10% of subjects, suggesting that CSF escape, might at least in the short-term, be clinically silent (103)

If persistence of low-level viral replication in the CNS is the main driver of intrathecal immuneactivation, treatment intensification could have a significant effect on CNS inflammation. However, to date treatment intensification has shown no significant reduction in levels or intrathecal immuneactivation in patients on effective cART (105).

1.4.3 Ongoing CNS immuneactivation

There is mounting evidence suggesting that microglial activation plays a crucial role in the pathogenesis of milder forms of NCI. First, as described in the neuropathology section earlier, the presence of significant microglial activation in the brains of treatment experienced HIV-infected individuals suggest that neuroinflammation may be a key process in the pathogenesis milder forms of HIV-associated NCI (40). Secondly, there is a consistent association across several studies investigating NCI in treated subjects between low nadir CD4 count (the lowest CD4 count reached by an HIV-infected patient) and cognitive deficit (28, 29, 106, 107). Patients with low nadir CD4 counts have greater levels of ongoing systemic immune activation, and cART does not reverse this effect completely, even after years of effective viral suppression (103, 108). Third, cerebrospinal-fluid (CSF) biomarker studies measuring concentrations of inflammatory markers produced by activated microglia have revealed that despite a reduction in the level of intrathecal immune activation observed with cART, significant on-going inflammation activity is still present (109). Finally, imaging modalities such as [¹H]-MRS (110) have reported significant increases in metabolic markers of inflammation in the basal ganglia and hippocampus of HIV-infected subjects on effective cART with NCI when compared to controls. PET imaging supports these findings with evidence of increased binding of PET-ligands which adhere to activated microglial cells in effectively treated HIV-infected subjects with mild forms of NCI and in those without any evidence of cognitive disease (111). All the above would suggest that sustained levels of immune

activation only partially reversed by therapy might be present for a long time, which could lead to NCI.

An individual's CD4 T-cell count nadir is a marker of historical HIV-disease progression and a well-described risk factor for the presence of cognitive impairment in many cohorts (29, 112-114). As HIV-disease progresses, increased peripheral and CNS immune activation occurs. It is possible this process may persist despite effective cART and be a potential explanation for the strong clinical correlation observed between low nadir CD4 T-cell count and cognitive function, where individuals with lower nadir CD4 T-cell counts are more likely to display poorer cognitive function, despite higher CD4 T-cell counts after effective cART. However, this is a risk factor described within clinical cohorts and although this may be linked to increased neuroinflammation and other pathogenic mechanisms associated with the development of cognitive impairment, cohort biases should also be taken into consideration. For instance, the individual phenotypes of two HIV-infected individuals, one with a very low nadir CD4 T-cell count and one with a high nadir CD4 T-cell count could be quite different. The underlying reason one individual has developed a low nadir CD4 T-cell count may include late HIV-disease presentation, greater risk-taking behavior, and general poorer engagement with healthcare services. Therefore, this individual may inherently have poorer cognitive function instead of this being directly related to HIV disease and as such the earlier initiation of antiretroviral therapy may have little if any impact on cognitive function (115).

Microglial activation is believed to be the pathological substrate of brain immune activation. *In vitro* models of CNS infection have proposed several mechanisms by which activated microglia can lead to neuronal injury. They involve the release of neurotoxic substances that induce astrocyte dysfunction, blood brain barrier dysfunction, and excitotoxicity, and the induction of dysfunctional chemokine and cytokine signalling that can tip the balance towards neurotoxicity instead of neuroprotection (62). The end result of these processes is dendritic damage, synaptic dysfunction, and apoptosis (94). However, *in vitro* models do not take in to account the effects of antiretroviral therapy and other individual patient factors (comorbidities, ageing, lifestyle etc) on host immune responses and chemokine/cytokine signaling within the CNS. The key role microglia play in the CNS

suggests that many of those clinical factors may still have a common pathogenic pathway, in the form of microglial activation, which subsequently results in NCI (Figure 1.2).

Another important factor is the relationship between systemic immune activation, CNS immune activation, and NCI. Ancuta et al. found an association between immune activation induced by microbial translocation and dementia in patients with AIDS (116), suggesting a direct relationship between systemic inflammation and NCI. Microbial translocation caused by the breakdown of the gut mucosa and subsequent translocation of microbial products from the intestinal lumen into the circulation is considered to be the main cause of persistent chronic inflammation in HIV-disease (117).

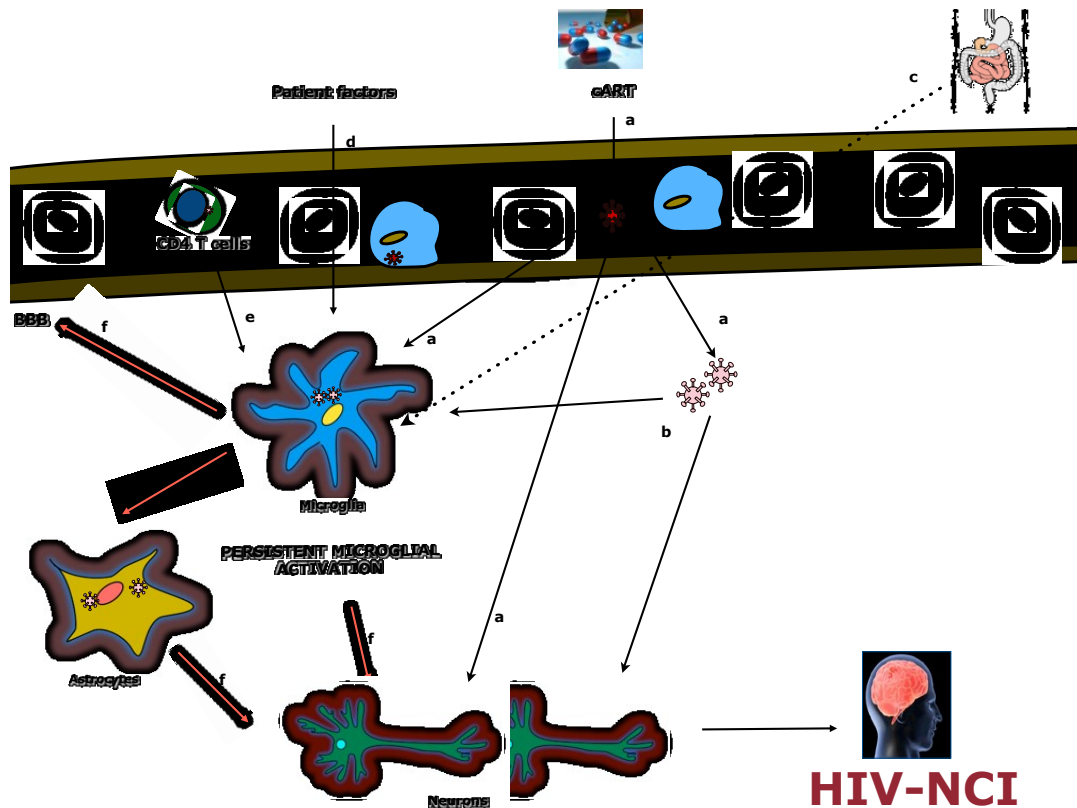
1.4.4 Reduced ART exposure or toxicity within the CNS

Another mechanism that could be responsible for the increasing prevalence of HIV-associated NCI relates to reduced ART exposure within the CNS.

The brain may constitute an important reservoir of latent or replicating HIV-virus in treated HIV-infected individuals. One of the reasons for this may be that some antiretrovirals do not reach effective brain concentrations either because of the blood brain barrier prevents their entry or because the drugs are pumped out of the CNS by ATP-binding membrane transporters (118). There is evidence that poor CNS penetration of ART is associated with detectable CSF HIV RNA (55). However, evidence that better CNS penetrating drugs affect cognitive function is less clear. Studies evaluating the effect of CNS penetrating cART in HIV-associated NCI are conflicting. Some studies described improvements in HIV-associated NCI with better CNS penetrating cART (119, 120), conversely other studies have reported no improvement (121) or even deterioration of cognitive performance (122). Though several studies have demonstrated the beneficial effects of cART for the CNS, there is a concern that ART drug toxicity may contribute to the development of HIV-associated brain disease. An *in vitro* study conducted by Liner et al. demonstrated a considerable loss of neurons on cultured rat cortical neurons challenged with several antiretrovirals within the range of concentrations seen in plasma and CSF with standard clinical dosing (123). This study suggests that several antiretroviral agents

in current clinical use could be associated with neuronal toxicity at concentrations expected in the CNS during general clinical usage.

Figure 1.2 Model of pathogenesis of HIV-NCI in the era of cART:



[Legend Figure 1.2: (a) Despite effective control of plasma viremia, cART could increase microglial activation by two mechanisms: i. Direct effect on microglial causing activation ii. Insufficient concentration of cART in the brain, could lead to low level viral replication. (b) Low level viral replication if present will be responsible for persistent microglial activation and probably direct neuronal injury through the production of neurotoxic viral products. (c) Peripheral immuneactivation, represented by microbial translocation may contribute to persistent microglial activation. (d) Patient factors, such as cerebrovascular disease, age, confectons, and recreational drug use are likely to contribute to the pathogenesis of HIV-NCI. (e) Low nadir CD4 T-cell counts have been associated with an increase in peripheral immuneactivation and HIV-associated NCI. (f) Neuronal injury caused by persistent microglial activation]

Clinical data describing cerebral toxicities from antiretroviral therapy are sparse. Cerebral imaging studies suggest toxicities may be associated with the duration (124) or number (125) of nucleoside-reverse-transcriptase-inhibitor agents used within a cART regimen, which may be markers of mitochondrial toxicity. Within cohort studies efavirenz use has been recently associated with poorer cognitive function (126) and switching from this agent has been associated with improvements in CNS-symptomatology (127).

1.4.5 Neurodegeneration

The longer duration of HIV-disease combined with the increasing life expectancy of HIV-infected patients may play a role in the development of neurodegenerative diseases. HIV-associated brain disease has common pathological and immunological features with Alzheimer's disease and Parkinson's disease.

Several reports have described increase in amyloid plaques in the brains of older patients with AIDS (128). In addition, increases in β -amyloid precursor protein (a marker of axonal degeneration) deposition in HIV-infected individuals on cART have been observed (129). Interestingly, although the data identified amyloid associated neuropathology, amyloid deposition does not appear to be associated with HIV-associated NCI. This could explain why the clinical presentation of severe forms of HIV-associated NCI is that of subcortical dementia, rather than the cortical dementia pattern observed in Alzheimer disease (130)

Movement disorders are not uncommon among HIV-infected individuals. Mirsattari et al. found a 5% incidence of parkinsonism in 155 HIV-infected individuals who fulfilled the United Kingdom Parkinson's Disease Brain Bank criteria for the diagnosis of parkinsonism (131). Another prospective study found that 50% of HIV inpatients with movement disorders had features of parkinsonism (132). More recently, Valcour et al. described an increase in extrapyramidal motor signs in the era of cART (133).

Increased α -synuclein deposition in the substantia nigra in HIV-Infected individuals on cART has been identified (134) and lewy bodies, the morphological hallmark of Parkinson's disease in the substantia nigra is composed of filamentous α -synuclein (135). The presence of lewy bodies is considered a prodrome for Parkinson's

disease and, therefore, the presence of significant amounts of α -synuclein may indicate that affected individuals could be at risk of developing Parkinson's disease, eventually. Indeed, a case series have described HIV-infected individual with young onset parkinsonism in the context of stable cART (136). Further research is needed to clarify the causative link between HIV-infection, cART and Parkinson's disease.

Thus, there is evidence for the existence of neurodegeneration markers in HIV-infected individuals on stable cART. However, neurodegeneration does not appear to be closely linked at present to severe forms of HIV-associated NCI.

1.4.6 Patient factors

Many co-morbidities and lifestyle factors have been associated with higher rates of NCI. Co-morbidities such as cerebrovascular, cardiovascular disease and diabetes (137, 138) are among the strongest predictors of NCI within the SMART study (Strategic Management of ART), one of the largest HIV-treatment studies to date. Such non-communicable co-morbidities are reported to be highly prevalent in treated HIV-infected subjects and may potentially increase prematurely with ageing (139).

Co-infections are also important risk factors for NCI. HIV-infected individuals co-infected with chronic viral hepatitis-C infection have an increased risk of NCI compared to those infected with HIV alone (140). Similarly, poorer cognitive function has been described in HIV-infected individuals with higher cytomegalovirus (CMV) antibody concentrations and those with past history of syphilis infection (141, 142). It is possible that these co-infections are associated with increased neuroinflammatory responses and nervous system injury.

Lifestyle factors such as cigarette smoking, recreational drug and alcohol use are also reported to be highly prevalent in HIV-infected cohorts as are diagnoses of depressive symptoms which may further fuel the incidence of this condition. Not only may these clinical risk factors themselves directly affect cognitive function (for instance recreational drug-use), but they may also contribute to the pathogenesis of other diseases leading to cognitive decline (for instance cardiovascular disease contributing to vascular dementia). Moreover, the presence of these conditions may also lead to increase peripheral inflammatory responses (143) and neuroinflammation (144)

1.4.7 Rationale for studying microglial activation

In HIV-associated brain disease microglial activation is an important factor in the pathogenesis of HAD. However, it is still unclear if the milder HIV-associated NCI, currently seen in treated HIV-infected individuals is the result of persistent microglial activation. Understanding in detail the dichotomous roles of activated microglia as neurotoxic or neuroprotective in HIV-infected individuals and the potential impact of inflammation on brain function is crucial to dissect the pathogenesis of milder forms of NCI. Furthermore, the identification of specific protective pathways and mediators, could pave the way for the development of new therapies that may reverse the negative effects of neuroinflammation.

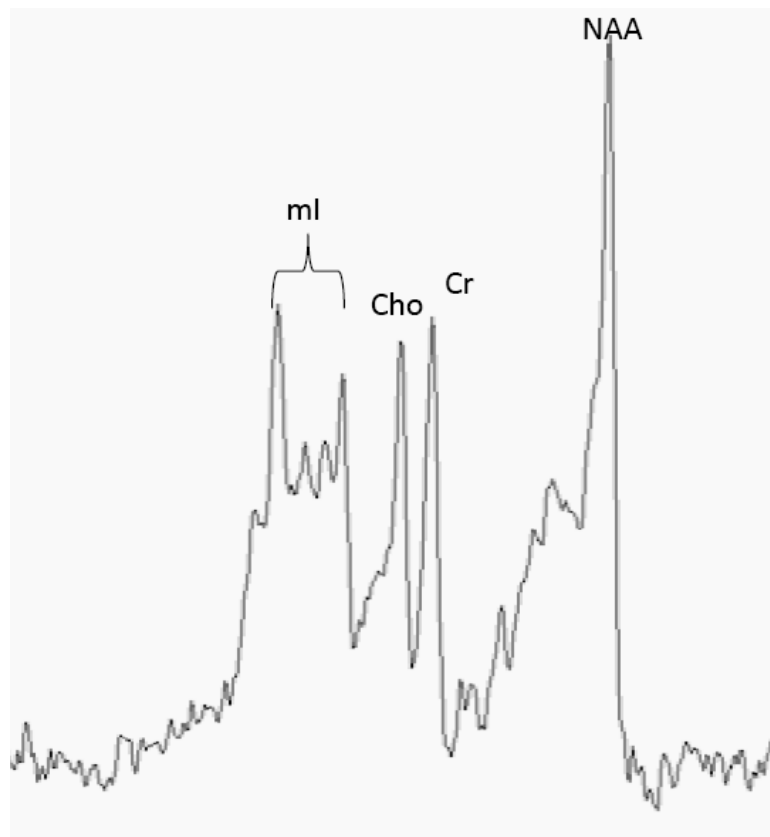
1.5 Neuroimaging in HIV disease

Neuroimaging studies evaluating changes in brain structure, metabolism, and function can improve our understanding of the pathophysiology of HIV-associated brain disease. In this section I will be discussing findings from neuroimaging studies using structural, functional, metabolic, and ionizing radiation neuroimaging techniques in HIV-infected individuals with and without HIV-associated NCI.

1.5.1 Brain magnetic resonance spectroscopy findings in HIV-associated NCI

[¹H]-MRS is a non-invasive tool that detects changes in cerebral metabolites, which reflect disturbances in cerebral function *in vivo*. It uses hydrogen-1 [¹H] isotope to quantify cerebral metabolites. The number of cerebral metabolites that can be detected depends on several technical considerations. Echo time is an important parameter in MRS data acquisition. Short echo times are preferred because they make it possible to detect all major metabolites including NAA, Cr, ml, Cho, glutamate (Glu), glutamine (Gln) and lactate, while long echo time allows only the detection of NAA, Cho and Cr (Figure 1.3). MRS can be performed in a single volume of interest (voxel) at the time or in a group of voxels that can cover a larger brain region, known as MRS imaging. While localized MRS limits spectral acquisition to a few voxels, it has better spectral acquisition and reproducibility than MRS imaging, which makes it a more robust method for the study of cerebral metabolites on HIV-associated brain disease.

Figure 1.3. Example of a cerebral proton magnetic resonance spectroscopy in the frontal white matter



[Legend Figure 1.3: Peaks representing the cerebral metabolites NAA= N-acetyl aspartate; Cr = creatine; Cho = Choline; ml=myo-inositol] (Imaged kindly provided by Lucy Garvey)]

MRS can be performed in a single volume of interest (voxel) at the time or in a group of voxels that can cover a larger brain region, known as MRS imaging. While localized MRS limits spectral acquisition to a few voxels, it has better spectral acquisition and reproducibility than MRS imaging, which make it a more robust method for the study of cerebral metabolites on HIV-associated brain disease.

Interpreting MRS studies is difficult because most studies employ different techniques and parameters to study the brains of HIV-infected individuals (145). Most $[^1\text{H}]$ -MRS studies have been performed using localised, short echo time MRS techniques evaluating between one to six brain regions. NAA, Cho, ml and Cr are the

most studied cerebral metabolites in HIV-disease as MRS data acquisition for these metabolites is easier to obtain without the need of a complex setup.

NAA is located almost exclusively in neurones and is a marker of structural and functional neuronal integrity (146). Cho is a marker of cellular membrane turnover reflecting cellular proliferation and inflammation (147). ml is produced in glial cells and almost exclusively in astrocytes and is a marker of glial proliferation and inflammation (148). Finally, Cr is a marker of energetic systems and intracellular metabolism. Although cerebral metabolites can be expressed as absolute concentrations, they are often reported as ratios. Concentrations of Cr are constant, for this reason Cr is used as an internal reference for calculating metabolite ratios (149). The rationale is that ratios self-correct for imager and localization method differences, regional susceptibility variations, and partial volume effects.

MRS studies pre-cART consistently showed a decrease in NAA (or NAA/Cr), and elevated Cho (or Cho/Cr) and ml (or ml/Cr) (150-152) in patients with HAD. These changes were primarily observed in the frontal gray matter and basal ganglia (153, 154). Localised MRS studies in HIV-infected subjects without NCI, have found decreased levels of NAA in the white matter and the thalamus with no change in Cho or Cho/Cr (155-157). However, Meyerhoff et al. showed a decrease in NAA and increased in Cho throughout the brain using MRS imaging. (158). In patients with milder forms of HIV-NCI, decreases in NAA/Cr, and increases in ml/Cr and Cho/Cr have also been reported, especially in the white matter, basal ganglia and hippocampus of HIV infected subjects when compared to controls (110).

Partial reversal of metabolic abnormalities has been observed in several studies following initiation of cART (159). Chang et al. found partial reversal of Cho and ml in the frontal white matter and basal ganglia of HIV-infected patients before and after cART (160). Winston et al. described improvement in cognitive function along with increases in NAA/Cr ratios in the frontal white matter of HIV-infected patients after 48 weeks of starting cART (125).

The decrease NAA or NAA/Cr has commonly been interpreted as a consequence of neuronal loss in HIV-infected patients. Increases in Cho or Cho/Cr may be

associated with either an increase in cellularity, reflecting recruitment of microglia or an increase in cell membrane breakdown caused by HIV-infection (147). ml does not cross the blood brain barrier and has a role in regulating cellular osmotic environment as well as cell volume (148), therefore, any elevation in ml is believed to represent glial proliferation and activation. This is supported by the fact that ml levels increase with disease severity (161), as observed in patients with HAD, especially in the frontal white matter where glial activation has also been observed in neuropathological studies (162).

1.5.2 BOLD functional magnetic resonance imaging in HIV-associated brain disease

Blood oxygenation level dependent (BOLD) functional magnetic resonance imaging (fMRI) allows the direct observation of brain activation while subjects are performing a cognitive task. fMRI is based on a group of physiological processes that begin with neuronal firing and glycolysis. These biological events lead to increase blood flow and oxygenation in activated brain regions. Changes in blood oxygenation alter the local magnetic properties of the blood, which can then be detected with MRI sequences. The signals changes are small; therefore, higher magnetic fields obtained with 3 or 4 Tesla (or higher) MR scanners are preferred as they yield better contrast to noise ratio, reducing the sample size required for BOLD-MRI studies. BOLD-MRI is an indirect measure of brain activation that correlates with neuronal activity.

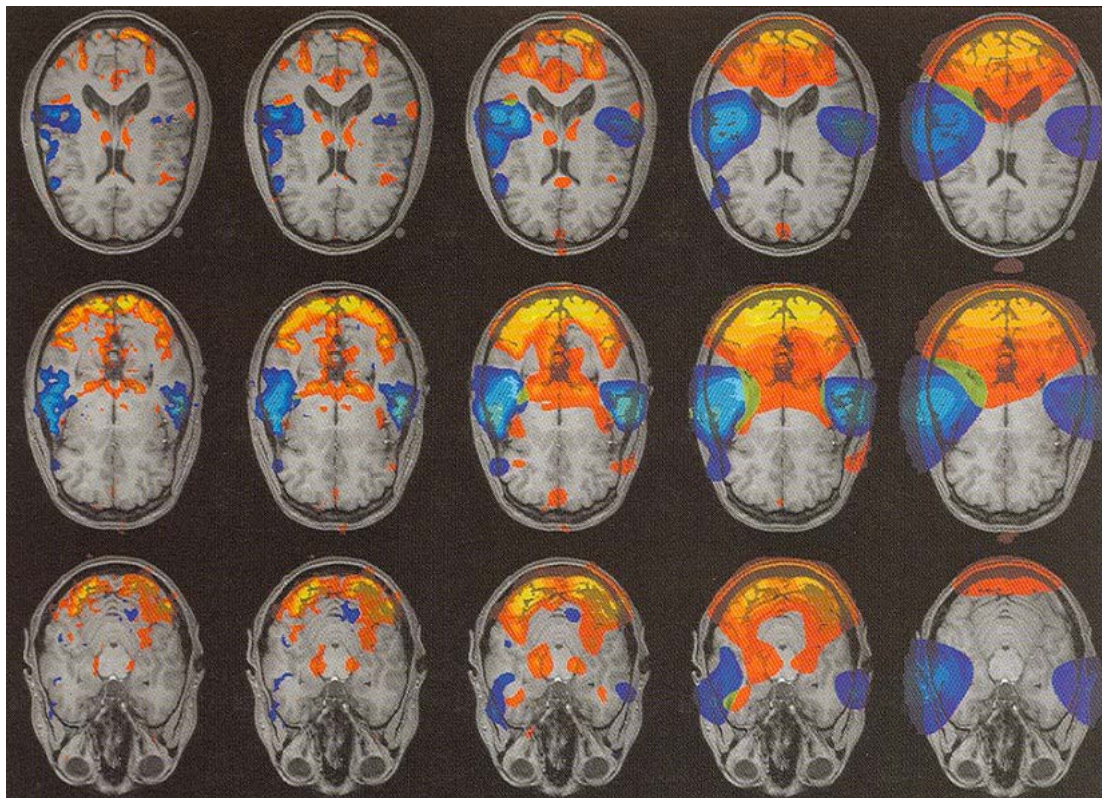
Data from fMRI studies require sophisticated software to perform statistical analyses that determine which voxels are activated by stimulation. What is seen on the screen of a BOLD –fMRI study is not a T1 or T2 MRI intensity view, but the transformed intensity value into a pixel value (linear or non-linear). The fMRI visualization is in the form of a statistical parametric map showing the areas of brain activation resulting from a statistical analysis performed by the software (Figure 1.4).

1.5.2.1 fMRI findings in HIV-NCI

Several fMRI studies have found correlations between changes in brain activation and working memory, attention, and episodic memory deficits in HIV-infected individuals stable on cART (Table 1.4).

Ernst et al. looked at the degree of brain activation in 10 HIV-infected individuals of stable cART with normal performance on a battery neurocognitive tests compared to match controls. Each subject was scanned while performing a set of working memory tasks with various degrees of difficulty. Compared to HIV-negative controls HIV-infected subjects showed greater BOLD activation in the lateral prefrontal cortex, posterior parietal cortex, and the caudate suggesting working memory functional impairments. The fact that patients were cognitively intact also suggests that the increased brain activation observed in the HIV-group, might represent adaptive activity to maintain normal cognitive function (163).

Figure 1.4 Visualization of BOLD-fMRI.



[Legend Figure 1.4 Images are showing a statistical parametric map of brain activation of a group of individuals performing an audiovisual experiment. Red clusters show visual activation; Blue clusters show auditory activation (Image kindly provided by Garyfilididis)]

Another fMRI study used a set of visual attention tasks with increasing levels of attentional load to evaluate efficiency of the normal attention network in 18 HIV-infected individuals with milder cognitive impairment compared to HIV-negative

controls (164). In this study, HIV-infected subjects showed an altered pattern of activation during the task with decreased activation of the visual attention network and increased activation in adjacent brain regions compared to controls. Castelo et al. evaluated episodic memory in neuroasymptomatic HIV-infected subjects, while performing an episodic encoding task (complex visual scenes) repeated stimulus (165). The HIV-infected group showed significantly reduced signal in the right posterior hippocampus, right inferior gyrus and left lingual gyrus, suggesting an alteration in the integrity of the hippocampus.

In summary, the data presented here suggest that HIV-infected individuals have a greater use of brain neuronal reserves to maintain normal cognitive function. BOLD-fMRI has the potential to detect pre-symptomatic abnormalities of brain activation.

1.5.2.2 Effects of cART on brain function evaluated with fMRI

A few studies have investigated the effects of cART on brain activation using fMRI techniques. Chang et al. evaluated the effects of cART (non-nucleoside-reverse transcriptase-inhibitors) in brain activation of HIV-infected individuals on therapy compared to those ART naïve and HIV-negative controls (166). Consistent with some of the studies described earlier, HIV-infected individuals with or without cART, showed greater increase in brain activation in right frontal regions (involved with visual attention) compared to HIV-negative controls. Also, compared to HIV-negative controls HIV-infected subjects on cART showed greater brain activation in bilateral superior frontal regions. The increase in brain activation was load-dependent, which suggests that greater recruitment of neurons was needed as the visual attention task (tracking four balls) became more difficult. The pattern of these abnormal activations suggests that chronic cART might lead to deficiencies in the attentional network. Interestingly, abnormalities found in the frontal lobe are consistent with damage of fronto-striatal structures seen in patients with HAD.

Finally, another fMRI study compared brain activation changes at baseline and after 1 year of cART using a similar visual attention task (tracking balls) (167). The study evaluated 32 neuroasymptomatic HIV-infected patients (all on stable cART) and 31 HIV-negative controls. After 1 year of therapy, HIV-infected subjects had no changes in brain activation. However, the more difficult the tasks were the more brain

activation was observed in the HIV-infected group compared to the negative controls. The brain areas involved were again those related with visual attention. This study concluded that after 1 year of therapy, HIV-infected individuals have decreased attentional network efficiency compared to the HIV-negative controls. This study also suggested that HIV-infected subjects might require more brain activation, to maintain the task performance.

1.5.2.3 Resting fMRI findings in HIV-associated NCI

Brain regions activated during any cognitive process are functionally connected, forming neuronal networks. These functional networks can be examined using fMRI during active conditions (task-positive) or during rest (task-negative) (168, 169). Resting functional brain networks are active during passive states and de-activated during active processing. In a sense, the brain is always active even at rest, and resting networks are in charge of preparing the scene for an active process. One of the most studied resting network is the default mode network (DMN) that comprises functionally connected regions including posterior cingulate cortex, medial prefrontal cortex, lateral and medial temporal lobes and posterior inferior parietal lobule (170). There are clear associations between brain functionality, and resting functional brain networks such as default mode network. Disruption of the default mode network has been associated with other neurological conditions such as brain trauma (171), neuropsychiatric disorders (172) and neurodegenerative disease (173).

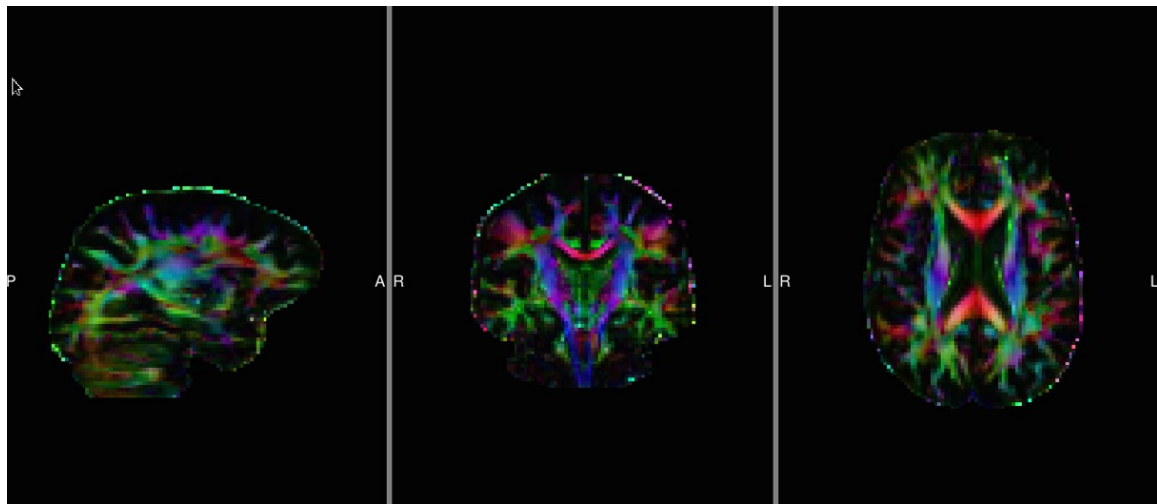
Only a few studies have evaluated the effect of HIV-infection on resting functional brain networks such as default mode network. Wang et al. investigated resting functional connectivity in 15 HIV-infected subjects within the first year of HIV-infection compared to HIV-negative controls (174). The study identified changes in resting functional connectivity of visual networks early in HIV-infection, suggesting an early effect of the HIV virus within the brain. More recently Thomas et al. studied intra and inter-network connectivity among 5 resting functional brain networks in 58 HIV-infected subjects (44% on stable cART) compared to 53 HIV-negative controls (175). The study reported that HIV subjects had disruption of the default mode network compared to HIV-negative negative controls. Interestingly, connectivity of resting functional brain networks was lower in HIV-infected subjects compared to controls, but decreased at the same rate as a function of age for both groups. This suggests

that HIV may lead to patterns of alteration in brain function similar to those associated with older age.

1.5.3 Diffusion tensor imaging findings in HIV-associated NCI

Diffusion tensor imaging is an imaging method for studying white matter integrity *in vivo* (176). DTI measures the diffusion of water molecules in white matter allowing the visualization and characterisation of major white matter fibre tracts (177). Diffusion (movement of water) can be greater along the fiber (longitudinal direction, anisotropic) or can go perpendicular to it, in a radial or transverse direction, see Figure 1.5.

Figure 1.5 Fractional anisotropy DTI map of healthy HIV-negative individual.



[Legend Figure 1.5: The colours represent the direction of white matter tracts: longitudinal direction (green), transverse (red), horizontal (blue). DTI measures the diffusion of water molecules in the white matter allowing the visualisation and characterisation of major white matter fibre tracts]

DTI has been used to study the effects of HIV-infection on white matter integrity. Studies looking at differences between HIV-negative and HIV-infected individuals have reported increases in mean diffusivity and decreases in fractional anisotropy within the corpus callosum and centrum semiovale in HIV-infected subjects (178). Wu et al. found that the reduction in fractional anisotropy within the corpus callosum, and the mean diffusivity was associated with poorer performance in motor speed tasks (179).

Gongvatana et al. looked at fractional anisotropy and mean diffusivity from 29 cerebral white matter regions. The study found that individuals with higher CD4 T-cell counts had higher anisotropy in parietal lobe regions. HIV-infected individuals on cART had higher anisotropy in temporal regions. In contrast, they found age and hepatitis-C co-infection to be associated with white matter injury (decreased fractional anisotropy and increased mean diffusivity) in most of brain regions (180). More recently, Wright et al. investigated the effects of HIV-infection and cART on white matter integrity in 21 naïve HIV-infected subjects, 21 HIV subjects on cART and 21 HIV-negative controls. The study found that HIV-infected naïve subjects had disturbances in white matter integrity when compared to HIV-infected subjects on cART and HIV-negative controls. It also found that HIV-infected subjects on cART had no differences in white matter integrity, and that initiation of cART was associated with increases in mean diffusivity (181).

1.5.4 *In vivo* imaging of neuroinflammation

1.5.4.1 Positron emission tomography (PET) imaging of the 18kDA translocator protein (TSPO)

PET imaging allows *in vivo* quantification of neuroinflammation by measuring the density of the TSPO. As a mitochondrial transmembrane protein, TSPO is highly expressed in microglia and astrocytes (182). Following activation through host responses to cellular injury, microglia and astrocytes increase the expression of TSPO (183) so that it can serve as a proxy for microglial activation and/or peripheral macrophage recruitment. TSPO is widely used as a target for nuclear imaging of neuroinflammation and therefore is an ideal target for monitoring the course of a disease and the effect of treatments.

The functions of TSPO are not known, TSPO has been associated with steroidogenesis (184) and mitochondrial functions. TSPO may act as a sensor for cellular oxygen, mediating protective effects for neurons against damage caused by reactive oxygen products. Also, TSPO may be involved with regulating the mitochondrial permeability pores in response to signals for apoptosis (185). Regarding steroidogenesis, TSPO may play a role in nerve regeneration. Specifically, TSPO may be involved with the transport of cholesterol from the cellular stores across the mitochondrial membrane, so it can be used to produce neurosteroids (186).

In summary, these functions of TSPO may be important in controlling neuronal damage and could be the reason there is increased expression of the receptor during microglial activation.

1.5.4.2 Imaging of TSPO in HIV-associated NCI

Ligands for TSPO can be used to image microglial activation in the human brain *in vivo* using PET (187). [^{11}C] PK11195 is the prototypical TSPO radioligand that has been used to measure neuroinflammation in several diseases including HIV. Studies investigating microglial activation using TSPO CT PET in HIV-associated brain disease are sparse (Table 1.5). Three studies using [^{11}C] PK11195 in humans have been published to date. Hammoud et al. demonstrated greater [^{11}C] PK11195 binding in HIV-infected individuals with dementia compare to HIV-negative controls, with no evidence of binding between HIV-infected subjects without NCI and controls (188). Wiley et al. found no difference in binding between HIV-infected subjects with and without NCI and controls (189). More recently Garvey et al. showed an increase in [^{11}C] PK11195 binding in neuroasymptomatic HIV-infected subjects stable on cART compared to controls (111). The reasons for these contradictory results could be because of methodological problems associated with acquisition and analysis of [^{11}C] PK11195 data. Despite high affinity to the TSPO *in vitro* [^{11}C] PK11195 has a high plasma protein binding and relatively poor penetration of the blood brain barrier, which results in low levels of tracer accumulation in the brain. It also has low specific-to-nonspecific binding ratio (i.e., displaceable and non-displaceable) *in vivo*. Consequently, it has a poor signal to noise ratio (190) that limits accurate quantification of differences in TSPO expression. This has a direct effect on the sensitivity of the ligand to detect mild neuroinflammation.

1.5.4.3 [^{11}C] PBR28 TSPO radioligand

[^{11}C] PBR28 (N-(2-methoxybenzyl)-N-(4-phenoxy pyridin-3-yl)acetamide) is a second generation TSPO radioligand with high affinity for TSPO and significantly better signal-to-noise compared with [^{11}C] PK11195 with greater sensitivity (190). Humans exhibit different affinity to TSPO ligands such as [^{11}C] PBR28. This differential affinity is caused by a single nucleotide polymorphism in the TSPO gene (rs6971) that leads to an amino acid substitution (Ala147Thr), which is associated with *in vitro* affinity of TSPO in platelets (191, 192). Three patterns of TSPO binding phenotypes have been identified in humans: high affinity binders (HABs) who are those subjects without the

polymorphism (HH), low affinity binders (LABs) who are homozygotes (LL) and mixed affinity binders (MABs) who are heterozygotes (HL) expressing both low and high affinity binding to TSPO. HABs and LABs express a single binding site for TSPO with either high or low affinity, whereas mixed affinity binders express equal amounts of high affinity binders and low affinity binders binding sites.

In the case of [^{11}C] PBR28, the difference in affinity between low affinity binders and high affinity binders is twenty-fold, so that LABs have no discernible PET signal in the brain when scanned with [^{11}C] PBR28 (193). The identification of the TSPO affinity phenotype is critical for the proper interpretation of TSPO PET data. Correcting *in vitro* binding data for the rs6971 genotype improves the ability of [^{11}C] PBR28 to distinguish differences in TSPO density in diseased and tissue from controls (194). Genotype analysis in plasma is currently available to characterise TSPO affinity.

Only one study to date has used [^{11}C] PBR28 to study neuroinflammation in HIV-NCI. Kreisl et al. looked at inflammatory brain changes in HIV-infected individuals with mild NCI (195). They compared asymptomatic HIV-infected patients (n=9), with HIV-infected subjects with mild NCI (n=7), and HIV-negative controls (n=8). The study found that patients with mild NCI had 37 to 53% greater binding with [^{11}C] PBR28 than HIV-infected patients without NCI in frontal, parietal, medial temporal, and cingulate cortex, as well as putamen, thalamus and pons. However, there were no differences in binding between HIV-negative controls and any of the HIV-infected groups.

This study had an important methodological problem, which is the lack of information about the affinity status of the patients used in the modeling.

More recently, Coughlin et al. used another second generation TSPO ligand [^{11}C] DPA-713 to investigate regional brain distribution of TSPO in HIV-infected patients compared to HIV-negative controls (196). The study compared 23 HIV-infected individuals with and without HIV-associated NCI with 12 HIV-negative controls. HIV-infected individuals demonstrated significantly higher TSPO binding in the white matter, cingulate cortex, and the supramarginal gyrus. An increase in TSPO binding within the frontal cortex was specifically linked to individuals with HAD.

In summary, second generation TSPO ligands such as [^{11}C] PBR28 and [^{11}C] DPA-713 have been shown to improve specificity and signal-to-noise ratio, which should make them better suited to detect abnormal brain distribution or microglial activation in HIV-infected individuals.

Microglial activation is likely to contribute towards the pathogenesis of HIV-associated brain disease. In this thesis, I studied the role of microglial activation in neuroasymptomatic HIV-infected individuals on stable cART, by examining the relationship between microglial activation and immunological and imaging biomarkers of inflammation.

Table 1.1 The changing manifestations of HIV-associated NCI pre and post-cART

Characteristic	Pre-cART	Post-cART
Clinical syndrome	Subcortical dementia	• Milder forms of cognitive impairment • Subcortical involvement
Risk factors	• Low CD4 T cell count, • High plasma/CSF HIV RNA viral load	• Nadir CD4 T cell count • Undetectable plasma viral load • Detectable CSF RNA viral load
Course of disease	Rapidly progressive	Fluctuating
Clinical presentation	• Cognitive: attention and memory deficits • Behavioural: apathy, social withdrawal • Motor: loss of balance, slowness	• Cognitive: attention, memory and executive function deficits • Behavioural: uncommon • Fine motor symptoms
Pathological findings	• HIV-encephalitis with multinucleated giant cells and microglial nodules • Brain atrophy	• HIV-encephalitis without multinucleated giant cells

Table 1.2 Summary of the role of selected chemokines in HIV-brain disease

Chemokines	Receptor (Location in the brain)	Cellular sources in the brain	Physiological function	Effects in the HIV+ brain
IP-10 (197-199)	CXCR3 and CCR5 (astrocytes, microglia and neurones)	Astrocytes and microglia and brain endothelial cells	Chemotaxis of neutrophils, lymphocytes and modulation of angiogenesis	Increased concentrations in patients with HIV-NCI Stimulates HIV-1 replication in MDM
IL-8 (200, 201)	CXCR1 and CXCR2(astrocytes, microglia and neurones)	Astrocytes and microglia	Proinflammatory chemokine, monocyte chemoattractant	Stimulates HIV -1 replication in monocytes and lymphocytes, HIV-1gp120 upregulates IL-8 in astrocytes
MCP-1(53, 202, 203)	CCR2, CCR5 (astrocytes and neurones)	Astrocytes and microglia	Powerful monocyte chemoattractant, also lymphocytes and basophils	Increased concentration in patients with HIV-NCI
Eotaxin (95, 204)	CCR3, CCR5 (microglia and astrocytes)	Microglia	Powerful eosinophil and monocytes chemoattractant	Upregulates HIV-1 entry into microglia
Fractalkine(205-207)	CX3CR1 (microglia, astrocytes, neurones and endothelial cells)	Microglia	Monocyte recruitment	Downregulated with ART, upregulated in brain tissue and CSF of patients with HIV-NCI
SDF-1 α(208-210)	CCR3, CCR5 (astrocytes, microglia, neurones and endothelial cells)	Microglia, neurones, astrocytes	Chemoattractant of monocytes, involved with astrocyte proliferation	Astrocyte proliferation in response to HIV-infection
MIP-β(99, 211)	CCR5 (astrocytes, microglia and neurones)	Astrocytes and microglia	Monocyte chemoattractant	May decrease infection of macrophages and microglia
RANTES (98, 212, 213)	CCR5, CCR1 and CCR5 (astrocytes, microglia and neurones)	Astrocytes and microglia	Involvement of migration of monocytes, microglia and astrocytes	Protects neurons against apoptosis induced by gp120

Table 1.3 Factors implicated in the pathogenesis of HIV-NCI in the era of cART

Pathogenic factors	Associated clinical risk factors
Persistent immune activation	Nadir CD4 count
Immune-reconstitution	Nadir CD4 count
Antiretroviral toxicity	Type of antiretroviral therapy
Inadequate exposure to ART in the CNS	Type of antiretroviral therapy
Accelerated brain aging	Age
Neurodegeneration	Family history of dementia and other neurodegenerative diseases
Co-infections	• Hepatitis C • Syphilis • CMV
Co-morbidities and lifestyle factors	• Cardiovascular disease • Diabetes • Clinical depression • Drug and alcohol abuse • Smoking

[Legend Table 1.3: ART: antiretroviral therapy, CMV: cytomegalovirus, CNS: central nervous system]

Table 1.4 Summary of selected BOLD fMRI studies in HIV-infected individuals

Author	Study design	HIV-infected subjects	HIV negative controls	Cognitive domain evaluated	Task	Findings in HIV-infected individuals
Ernst et al. (163) 2002	Cross-sectional, BOLD fMRI	10 HIV+ asymptomatic (9 on cART, 1 ART naïve)	10 controls	Working memory	N-back tasks with letters (1.5 Tesla MRI)	Greater activation in the lateral prefrontal cortex compared to controls
Chang et al. (164) 2004	Cross-sectional, BOLD fMRI	18 HIV+ (2 asymptomatic and 16 with NCI); 15 on cART, 3 ART naïve	18 controls	Visual attention	Tracking 2, 3 and 4 balls (4 Tesla MRI)	HIV+ less activation in normal visual attention network and greater activation in adjacent brain regions. HIV+ had increases activation in reserve network regions
Castelo (165) 2006	Cross-sectional, BOLD fMRI	14 HIV+ asymptomatic (10 on cART; 3 ART naïve)	14 controls	Episodic memory	Encoding task (3 Tesla MRI)	HIV+ showed reduced activation in the right posterior hippocampus, right inferior gyrus and left lingual gyrus. Pattern similar to ageing subjects
Chang et al. (166) 2008	Cross-sectional, BOLD fMRI	HIV+ asymptomatic (n=12 on stable cART, n=12 ART naïve)	18 controls	Visual attention	Tracking 2, 3 and 4 balls (4 Tesla MRI)	HIV+ showed greater activation in right frontal regions compared to negative controls. HIV+ on cART had greater activation in bilateral superior frontal regions compared to negative controls
Ernst (167) 2009	Prospective, BOLD fMRI assessment at baseline, 6 months and 1 year	32 HIV+ asymptomatic (all on cART)	31 controls	Visual attention with high attentional load, fMRI	Tracking 2, 3 and 4 balls (4 Tesla MRI)	Declined attention network efficiency after 1 year of follow up. Increase activation in prefrontal and posterior parietal cortices for the more difficult tasks
Wang et al. (174) 2011	Cross-sectional, resting fMRI	15 HIV+ within first year of HIV-1 infection (ART naïve)	15 controls	Resting visual networks	Resting (eyes shut during scanning)	Disruption of connectivity in resting visual networks
Thomas et al. (175) 2013	Cross-sectional, resting fMRI	58 HIV+ (44% on cART)	53 controls	Resting functional brain networks	Resting (eyes shut during scanning)	Disruption of default mode network with patterns of similar to the ones seen in old age

Table 1.5 Summary of studies evaluating TSPO in HIV-infected subjects

Author	Subjects	TSPO ligand used	Brain regions evaluated	cART	Findings in HIV-infected subjects
Wiley et al. 2006 (189)	6 HIV+ with HIV-NCI 6 HIV+ asymptomatic 5 HIV- controls	[¹¹ C] PK11195	10 brain regions	All on cART, 9 out 12 HIV+ with detectable plasma viremia	No differences in ligand binding between HIV+ and controls
Hammoud et al. 2005(188)	5 HIV+ with HIV-NCI (3 with HAD) 5 HIV+ asymptomatic 5 HIV- controls	[¹¹ C] PK11195	8 cortical region one white matter	7 HIV+ on cART, 3 cART naive	Higher ligand binding in: Thalamus, putamen, temporal, frontal and occipital lobe In HIV+ with NCI, and in HIV+ individuals overall when compared to controls
Garvey et al. 2013 (214)	9 HIV+ asymptomatic 8 HIV- controls	[¹¹ C] PK11195	Template derived regions of interest	Not described	Higher ligand binding in: Corpus callosum, anterior, posterior cingulate, frontal and temporal lobe in HIV+ asymptomatic compared to controls
Kreisl et al. 2013, (195)	7 HIV+ asymptomatic 7 HIV+ with HIV-NCI 6 HIV- controls (no genotype information provided)	[¹¹ C] PBR28	119 brain regions	All on cART with undetectable plasma viremia	Higher ligand binding in frontal, parietal and, medial temporal lobe, putamen, pons, thalamus, and cingulate cortex in HIV+ with HIV-NCI vs HIV-asymptomatic. No difference in binding between any HIV+ and controls

Chapter 2

Materials and Methods

Chapter 2: Materials and Methods

2.1 Clinical cohorts.....	53
2.1.1 Subject selection.....	53
2.2 Non-human primate model	53
2.3 Brain imaging.....	55
2.3.1 Positron emission tomography imaging with [¹¹ C] PBR28.....	55
2.3.2 Brain magnetic resonance imaging	56
2.3.2.1 Functional magnetic resonance imaging	56
2.3.2.1.1 Resting functional magnetic resonance imaging	57
2.3.2.1.2 Task functional MRI imaging.....	57
2.3.2.2 Diffusion tensor imaging	58
2.3.2.3 Magnetic resonance imaging spectroscopy (¹ H-MRS)	59
2.4 Neurocognitive testing	60
2.5 Blood and CSF biomarkers	60
2.5.1 Lumbar puncture.....	60
2.5.2 Blood samples and CSF processing	60
2.5.3 Analysis of plasma and CSF biomarkers.....	61
2.5.3.1 Chemokines.....	61
2.5.3.2 Soluble CD14	61
2.5.3.3 CD163	62
2.5.3.4 16s Ribosomal DNA	62
2.6 Statistical analysis.....	63
2.7 Ethical approval.....	63

2.1 Clinical cohorts

2.1.1 Subject selection

Subjects attending the HIV outpatient department at St Mary's Hospital, Imperial College NHS Healthcare were invited to participate in the clinical studies presented in this thesis.

Eligibility criteria required subjects to be HIV antibody positive for at least 2 years, proficient in the English language of male gender between 20 to 50 years of age, receiving stable cART with a plasma HIV RNA viral load of <50 copies/mL at screening. Exclusion criteria included current use of any anti-inflammatories or benzodiazepines, current use of recreational drugs or alcohol abuse and low affinity binder affinity to TSPO on genotype testing. Patients were screened for anxiety and depression using the hospital anxiety and depression scale (Figure 2.1). This scale is unconfounded by the presence of HIV symptomatology and hence is considered a reliable tool to assess anxiety and depression in HIV-infected individuals (215). Subjects with active or past history of neurological, psychiatric disease or evidence of depression and anxiety were also excluded. The age range was selected because there is evidence (unpublished) that age may affect the TSPO ligands volume of distribution, and our patient demographics indicated that approximately 65% of our patient cohort belongs to this age range, which made recruitment easier. For the analysis of [^{11}C] PBR28 data from a control group of 10 male, age-matched, HIV-negative, healthy participants were used. These control groups were obtained from studies conducted at the Imanova centre for imaging sciences, located at the Hammersmith campus, Imperial College London.

2.2 Non-human primate model

Samples from CSF and blood (plasma) from cynomolgus macaques infected with SIVmac251 were provided by the National Institute of Biological Standards and Control (Hertfordshire, UK) in collaboration with Dr Deborah Fergusson and Dr Neil Almond for chemokine analysis. These animals were part of several neuropathology and vaccine studies. Purpose bred juvenile, SRV-negative and STLV-negative cynomolgus macaques originally derived from Mauritius, were used in all these studies. Animals were group housed and maintained in accordance with Home Office guidelines for care and maintenance of non-human primates. Animals were sedated

Figure 2.1 Hospital anxiety and depression scale

Hospital Anxiety and Depression Scale (HADS)

Tick the box beside the reply that is closest to how you have been feeling in the past week.
Don't take too long over you replies: your immediate is best.

D	A		D	A	
		I feel tense or 'wound up':			I feel as if I am slowed down:
	3	Most of the time	3		Nearly all the time
	2	A lot of the time	2		Very often
	1	From time to time, occasionally	1		Sometimes
	0	Not at all	0		Not at all
		I still enjoy the things I used to enjoy:			I get a sort of frightened feeling like 'butterflies' in the stomach:
0		Definitely as much	0		Not at all
1		Not quite so much	1		Occasionally
2		Only a little	2		Quite Often
3		Hardly at all	3		Very Often
		I get a sort of frightened feeling as if something awful is about to happen:			I have lost interest in my appearance:
	3	Very definitely and quite badly	3		Definitely
	2	Yes, but not too badly	2		I don't take as much care as I should
	1	A little, but it doesn't worry me	1		I may not take quite as much care
	0	Not at all	0		I take just as much care as ever
		I can laugh and see the funny side of things:			I feel restless as I have to be on the move:
0		As much as I always could	3		Very much indeed
1		Not quite so much now	2		Quite a lot
2		Definitely not so much now	1		Not very much
3		Not at all	0		Not at all
		Worrying thoughts go through my mind:			I look forward with enjoyment to things:
	3	A great deal of the time	0		As much as I ever did
	2	A lot of the time	1		Rather less than I used to
	1	From time to time, but not too often	2		Definitely less than I used to
	0	Only occasionally	3		Hardly at all
		I feel cheerful:			I get sudden feelings of panic:
3		Not at all	3		Very often indeed
2		Not often	2		Quite often
1		Sometimes	1		Not very often
0		Most of the time	0		Not at all
		I can sit at ease and feel relaxed:			I can enjoy a good book or radio or TV program:
	0	Definitely	0		Often
	1	Usually	1		Sometimes
	2	Not Often	2		Not often
	3	Not at all	3		Very seldom

Please check you have answered all the questions

Scoring:

Total score: Depression (D) _____ Anxiety (A) _____

0-7 = Normal

8-10 = Borderline abnormal (borderline case)

11-21 = Abnormal (case)

with ketamine hydrochloride (Vetalar V, Pharmacia Animal Health Ltd, Corby, UK) before inoculation of virus or sampling of blood by venepuncture. On each of these occasions, sedated macaques were weighed and inspected for general health and signs of disease. At the end of the study, macaques were killed humanely by an overdose of anaesthetic.

2.3 Brain imaging

2.3.1 Positron emission tomography imaging with [^{11}C] PBR28

Radiotracer synthesis

[^{11}C] PBR28 was manufactured at Imanova immediately prior to use according to local Standard Operating Procedures (SOPs). Quality assurance checks were performed prior to injection to ensure the product meet specification as laid down by the local product manufacture specifications.

Plasma sampling

An arterial line was placed in all subjects for the measurement of the arterial plasma input function. All subjects completed pre-arterial line assessment that consisted of an Allen's test to evaluate collateral circulation in both hands. Following administration of local anaesthesia (infiltration of the skin with 1% lidocaine), a cannula was inserted into the radial artery to allow arterial blood sampling during the PET CT scan.

Image generation and processing

For the PET study, the [^{11}C] PBR28 PET scans were acquired using a Biograph 6 PET CT scanner (Siemens Healthcare). Each participant was injected with intravenous bolus of 400MBq of carbon-11 labeled PBR28 over 20 seconds at the start of a 90 minutes 3 dimensional mode dynamic PET acquisition.

PET data were reconstructed using filtered back projection with corrections for attenuation and scatter (based on a low-dose CT acquisition). Dynamic data were binned into 26 frames (durations: 8x15s, 3x1min, 5x2min, 5x5min, 5x10min). Arterial blood data were sampled via the radial artery 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 blood samples were manually withdrawn at 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, and 90 minutes after scan starts to facilitate measurement of whole blood and plasma activity. Samples taken at 5, 10, 15, 20, 30, 50, 70 and 90 minute time points were also analysed using HPLC to determine the fraction of parent radioactivity in arterial plasma. The first three discrete blood samples were used to calibrate the continuous blood data, and then the continuous and discrete data sets 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 form plasma-over-blood (POB) data. A constant POB model was fitted. This POB value was then multiplied by the whole blood curve to generate a total plasma curve. Parent fraction data were fitted to a sigmoid model $f = \left(\left(1 - \frac{t^3}{t^3 + 10^a} \right)^b + c \right) / (1 + c)$ where t is 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 was fitted and applied to the input function to account for any temporal 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 30kDa, Millex, Ireland) in triplicate using Tris buffer (0.1M, pH=7.4) to determine and enable correction for non-specific binding.

2.3.2 Brain magnetic resonance imaging

For the PET study, MRI data acquisition was performed on a Siemens MAGNETOM® Verio 3.0 tesla magnetic resonance (MR) scanner (Siemens, Germany). Functional images were obtained using a T2 weighted echo-planar-imaging (EPI) sequence with whole brain coverage repetition time (TR) of 2000ms, echo time (TE) of 30ms with 35 ascending slices with thickness of 3mm; voxel size 3x3x3mm. T1 weighted whole brain structural images were also obtained in all subjects.

2.3.2.1 Functional magnetic resonance imaging

Patients had two sessions of imaging: one session consisted of resting fMRI plus structural brain imaging including DTI, and a second session consisted of a task fMRI.

2.3.2.1.1 Resting functional magnetic resonance imaging

Resting fMRI was acquired for 8 minutes when patients were instructed to close their eyes and relax. Following this, all patients performed a task in the scanner.

Imaging pre-processing

Data pre-processing was completed using FSL (Oxford Centre for Functional Magnetic Resonance Imaging of the Brain Software Library www.fmrib.ox.ac.uk). Image preprocessing comprised, motion correction (using MCFLIRT), spatial smoothing (using a Gaussian kernel of FWHM 6mm), and temporal bandpass filtering (high-pass temporal filtering). As a final preprocessing step, each individual's time series was normalized by registration to the Montreal Neurological Institute (MNI) template, with 4mm resolution.

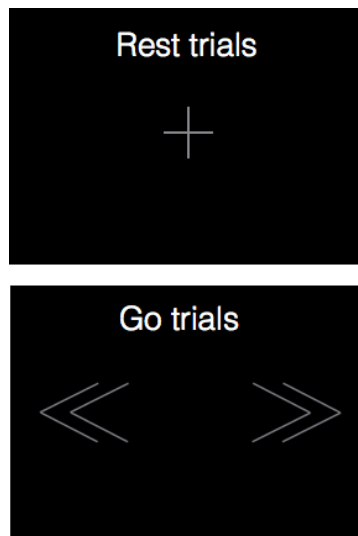
2.3.2.1.2 Task functional MRI imaging

A simple choice reaction time (CRT) task was selected to assess sustained attention in HIV-infected participants. The paradigm was developed in collaboration with Robert Leech at the C3NL laboratory at the Hammersmith campus, Imperial College London.

The paradigm was programmed using Matlab psychophysics toolbox using the Psychtoolbox-3 available here www.psychtoolbox.org. Stimuli were presented through an IFI-SA system *in vivo*. Responses were recorded through a fiber optic response box (Nordicneurolab), interfaced with the stimulus presentation PC running Matlab. In the task, participants were presented with an initial fixation cross for 350 ms followed by a response cue as an arrow pointing either to the left or the right lasting 1400 ms (Figure 2.2).

Participants were instructed to press the button box with the index finger accordingly, as quick and as accurately as possible. Presentation was blocked, with five repeated blocks of 10 response trials, 10 rest trials, and four response trials at the start of the experiment. The total duration of the scan was 5.06 minutes. The inter-stimulus interval was 1750 ms.

Figure 2.2: Example of rest and go trials stimulus in the choice reaction time task.



[Legend Figure 2.2: Patients were instructed to click a button box when the arrow was present on the screen; rest trials: initial fixation cross before task was on. Go trials: stimulus presented to patients when task was on (arrows pointing either left or right)]

Imaging pre-processing

The first four volumes were discarded to allow for T1 equilibration effects. Imaging pre-processing was performed using FSL (FMRIB software library) (<http://www.fmrib.ox.ac.uk/fsl>). The following pre-processing steps were conducted: motion correction using MCFLIRT, high-pass temporal filtering and spatial smoothing using a Gaussian kernel of 5mm full-width-half-maximum (FWHM). Removal of brain structures from the imaging volumes was carried out using the BET tool from the FSL software. Linear imaging registration tool (FLIRT) (216) was used to register the fMRI volumes into the individual participant's structural scan and standard MNI152 space images. T1 weighted were also acquired for each participant (TR: 2300ms, TE: 2.98ms, voxel size 1x1x1 mm).

2.3.2.2 Diffusion tensor imaging

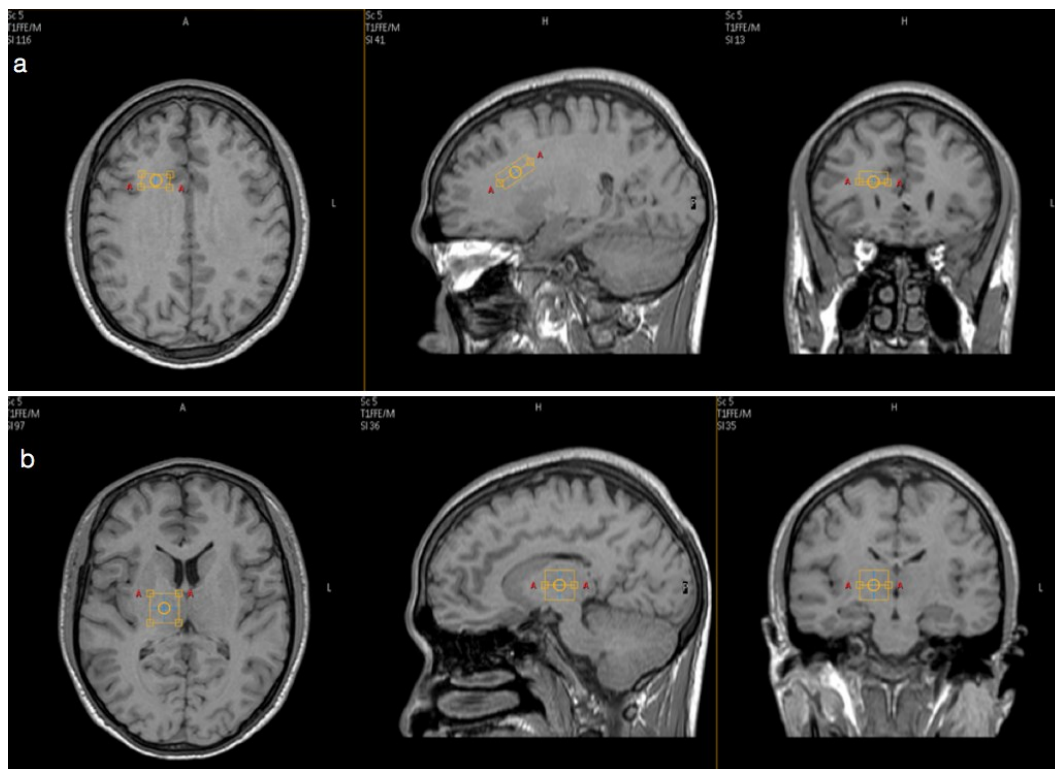
Diffusion-weighted volumes with gradients applied, resulting in a total of 68 directions. The following parameters were used: 52 contiguous slices, slice thickness=2.0 mm, field of view (FOV) 224 mm³, voxel size=1.8x1.8x2.0 mm, b value=1000s/mm², imaging runs with diffusion weighting (b=1 s/mm²).

2.3.2.3 Magnetic resonance imaging spectroscopy (^1H -MRS)

MRS data acquisition:

^1H -MRS data acquisition was performed on a Siemens MAGNETOM[®] Verio 3.0 tesla magnetic resonance MR scanner (Siemens, Germany) as described above (section 2.1.1). MRS data were acquired by a single voxel examination in 2-voxel locations: right frontal white matter, and the right basal ganglia (Figure 2.3).

Figure 2.3 MRS voxel locations selected for the study



[Legend Figure 2.3 Voxel locations selected for the study in yellow (a) Frontal white matter (voxel size (AP) 18mm x (RL) 18mm x (HF) 10mm. (b) Basal ganglia (voxel size (AP) 18mm x (RL) 18mm x (HF) 18mm if no good try (AP) 10mm x (RL) 18mm x (HF) 18mm]

These anatomical voxels were selected because previous imaging studies have described cerebral metabolite abnormality patterns in such voxels in HIV-infected individuals (110, 153). A double-spin-echo sequence was performed in point-resolved spectroscopy with the following settings: TE: 30 ms, TR of 3000 ms, a spectral width of 2500 Hz, 2048 data points and 128 data acquisitions. Spectra from

the MR imaging were post-processed using the manufacturer's software for automated water signal suppression and water shimming. Each examination lasted 20 min.

2.4 Neurocognitive testing

For the PET study only, neurocognitive testing was performed in HIV-infected individuals using a commercially available cognitive test battery CogState (CogState Ltd, Melbourne, Australia). CogState is a computerised cognitive test battery designed to measure cognitive performance. This tool has been validated for the detection of cognitive impairment in HIV-infected subjects when compared with formal neurocognitive assessment (107, 217)

The battery consisted of eleven individual tasks in the form of card-games or shapes that were presented in a computer screen to minimise language and cultural differences (Table 2.1). During testing, each participant is seated in a quiet room where the instructions were given verbally by the test supervisor (J.V). A practice session was conducted during the screening visit, followed by the baseline assessment conducted during the scanning visit.

2.5 Blood and CSF biomarkers

2.5.1 Lumbar puncture

Lumbar punctures were performed by J.V within 1 month of the scanning visit as a day case procedure at St Mary's Hospital, Imperial College London. Lumbar punctures were performed using aseptic technique under local anaesthesia (2-10mL 2% lignocaine) to obtain CSF. CSF was collected in universal containers.

2.5.2 Blood samples and CSF processing

Blood samples were collected in EDTA bottles during the screening visit and the LP visit. CSF samples were collected in universal containers. 3mL of CSF were sent to the Clinical Biochemistry and Microbiology Laboratory at St Mary's Hospital, London for the analysis of CSF protein, glucose, gram stain, and microscopy. An additional 2mL of CSF sample was sent to the Virology Laboratory at St Mary's Hospital, London for the analysis of CSF HIV RNA. CSF HIV RNA was quantified using the real-time PCR (Qjagen probe PCR). The limit of detection was 10 copies/mL (218).

Blood and remaining CSF (5mL) samples were centrifuged after 10 minutes (1500g, 3000 rpm) in a 4°C refrigerated centrifuge. Plasma and CSF samples were then transferred to storage tubes and store at -80 C°.

2.5.3 Analysis of plasma and CSF biomarkers

2.5.3.1 Chemokines

The MSD human chemokine 9-Plex-and 7-Plex cytokine assay (ultrasensitive kit K15001C, Meso Scale Discovery) for human chemokines (eotaxin, MIP-1 β , TARC, IP-10, IL-8, MCP-1, MDC, MCP-4) was used for the analysis of plasma and/or CSF. The arrays were pre-incubated with 25 μ L/well of assay diluent for 30 minutes, with shaking at room temperature. Following the pre-incubation, 25 μ L of sample or calibrator in duplicate was added into the corresponding wells. Thereafter the plate was incubated for 2 hours, with vigorous shaking (600rpm). The array was then washed three times with phosphate buffered saline containing 0.005% of Tween 20, and then 25 μ L of detection antibody reagent was added into each well. The plates were incubated in room temperature for 2 hours and the array wash three times. The plates were analyzed immediately after adding 2x reading buffer. Chemokine concentrations in blood and CSF samples were determined with MSD Discovery Workbench® software, using the software's curving fit model. The lower limit of detection in both plasma and CSF chemokines/cytokines (pg/mL) were: eotaxin (23), MIP-1 β (7.5), TARC (15), IP-10 (12), IL-8 (0.6), MCP-1 (8), MDC (334), MCP-4 (8.4).

2.5.3.2 Soluble CD14

A commercially available kit (R&D Systems: Quantikine, Human sCD14 Immunoassay) was used to measure sCD14 in plasma samples stored at -80 degrees. All reagents and samples were brought to room temperature. Plasma samples were diluted 200X into calibrator diluent. CSF samples were diluted 40x. Lyophilised soluble CD14 standard was reconstituted with 5mL calibrator diluent, giving a stock solution of 16,000 pg/mL. The stock solution was gently agitated for 15 minutes prior to use. The resulting standard concentrations were as follows: 16,000 pg/mL, 8,000 pg/mL, 4,000 pg/mL, 2,000 pg/mL, 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 0 pg/mL. 100ul of assay diluent was added to each of the microplate wells, followed by 100 μ L of either standard, diluted sample or control per well in duplicate. A spiked control of known sCD14 concentration was run on every plate. The

microplate was covered with an adhesive plate sealer and incubated at room temperature for 3 hours then aspirated and washed 4X before 200 μ L of sCD14 conjugate was added to each well. The microplate was covered with a plate sealer and incubated at room temperature for 1 hour followed by a further wash step. 200 μ L of substrate solution was added to each well and the plate was incubated in the dark at room temperature for 30 minutes. The reaction was stopped with 50 μ L of stop solution and read at 450nm with a background reading at 540nm. The standard curve was calculated in GraphPad Prism using a 4PL fit and any sample duplicates with CVs exceeding 15% were repeated. This assay was performed in collaboration with Dr Adriano Boasso from Chelsea and Westminster Hospital.

2.5.3.3 CD163

A commercially available kit (R&D Systems: Quantikine, Human CD163 Immunoassay) was used to measure CD163 in CSF samples stored at -80 degrees. All reagents and samples were brought to room temperature. CSF samples were used neat. Lyophilised soluble CD163 standard was reconstituted with 1mL of distilled water, giving a stock solution of 200ng/mL. The stock solution was gently agitated for 15 minutes prior to using to produce a dilution series. 100 μ L of assay diluent was added to each of the microplate wells, followed by 50 μ L of either standard, sample or control per well in duplicate. A spiked control of known CD163 concentration was run on every plate. The microplate was covered with an adhesive plate sealer and incubated at room temperature for 2 hours then aspirated and washed 4X before 200 μ L of CD163 conjugate was added to each well. The microplate was covered with a plate sealer and incubated at room temperature for 1 hour followed by a further wash step. 200 μ L of substrate solution was added to each well and the plate was incubated in the dark at room temperature for 30 minutes. The reaction was stopped with 50 μ L of stop solution and read at 450nm with a background reading at 540nm. The standard curve was calculated in Excel using a lin-lin fit and any sample duplicates with CVs exceeding 15% were repeated. This assay was performed in collaboration with Dr Adriano Boasso from Chelsea and Westminster Hospital

2.5.3.4 16s Ribosomal DNA

Bacterial DNA was measured in plasma and CSF samples stored at -80 using quantitative polymerase chain reaction (qPCR). DNA was extracted from 200 μ L

plasma or CSF using an EZI Advanced robot and kit (Qiagen DNA EZI, DNA EZI mini kit v2.0). PCR was carried out with Platinum Syber Green Supermix UDG (Invitrogen) with 16s primers (Eurofons MWG Operon (forward (5'-3' – TGG AGC ATG TGG TTT AAT TCG A) reverse (5'- 3' – TGC GGG ACT TAA CCC AAC A))) at a final concentration of 0.05µM. Molecular grade water controls and spiked plasma controls were run on each plate. A standard curve was made from purified *E.coli* DNA and ranged from 2×10^7 to 2×10^1 copies per reaction. Quantitative real time PCR was performed in an Applied Biosystems ABI PRISM® 7000 using the following conditions; activation of Taq DNA polymerase for 10 minutes at 95°C, 40 cycles of denaturing for 40 seconds at 94°C, annealing for 45 seconds at 60°C, and extension for 45 seconds at 72°C. A PCR run was accepted if the slope of the standard fell between -3.6 and -3.3, representing 90-100% efficiency, and the curve fit was above 0.985. Samples were run in triplicate and repeated if the standard deviation of the quantification cycle (Cq) was over 0.300. This assay was also performed in collaboration with Dr Adriano Boasso from Chelsea and Westminster Hospital

2.6 Statistical analysis

A detailed description of the specific statistical methods employed in this thesis is presented in each results chapter. The statistical packages used in this thesis were: SPSS (Version 21.0) and GraphPad Prism (Version 6.0)

2.7 Ethical approval

Ethical and Trust approval were obtained via the UK national research ethics service to conduct the following studies presented in this thesis:

- The neuroinflammation study (chapters 3) approval granted by NRES (reference number 12/LO/1570)
- Permission to administer [^{11}C] PBR28 was granted by the administration of radioactive substances advisory committee (ARSAC) of the UK (reference number 630/3764/29163)
- The SIV chemokine study (chapter 6) obtained ethical approval for housing and research of cynomolgus macaques from the Home office

Table 2.1 Tasks and cognitive domain assessed using CogState

Task performed	Unit of measurement	Cognitive function tested	Interpretation of score
Speed and visual attention			
Detection (DET)	Log ₁₀ milliseconds	Speed of processing	Lower score=better performance
Identification (IDN)	Log ₁₀ milliseconds	Attention	Lower score=better performance
Executive function			
Groton maze learning test (GMLT)	Total errors	Executive function	Lower score=better performance
Continuous paired learning (CPAL)	Total errors	Paired associated learning and memory	Lower score=better performance
Accuracy and visual learning			
One card memory (OCL)	Arcsine proportion correct	Visual learning/memory	Higher score=better performance
One back memory (ONB)	Arcsine proportion correct	Working memory	Higher score=better performance
Memory and verbal learning			
International shopping list task (ISL)	Number of correct responses	Verbal learning/memory	Higher score=better performance
International shopping list- recall (ISLR)	Number of correct responses	Verbal learning/memory	Higher score=better performance

Chapter 3

**Neuroinflammation in HIV-infected individuals
stable on cART: a [^{11}C] PBR28 PET CT study**

Chapter 3: Neuroinflammation in HIV-infected individuals stable on cART: a [^{11}C] PBR28 PET CT study

3.1 Introduction	68
3.2 Methods.....	69
3.2.1 Subject selection.....	69
3.2.1 Study procedures.....	69
3.2.2 Neurocognitive testing	69
3.2.3 Brain PET scanning with [^{11}C] PBR28.....	70
3.2.3.1 Imaging analysis of [^{11}C] PBR28 CT PET results	70
3.2.3.2 Kinetic analysis.....	70
3.2.3.3 V_T normalisation.....	71
3.2.4 Statistical analysis	71
3.3 Results	71
3.3.1 Neurocognitive testing results	72
3.3.2 [^{11}C] PBR28 volume of distribution (V_T) in HIV-infected subjects compared to HIV-negative controls	72
3.3.3 Associations of [^{11}C] PBR28 V_T and clinical parameters.....	80
3.4 Discussion	88

3.1 Introduction

The exact role of neuroinflammation in treated HIV-infected individuals remains controversial, as inflammatory brain changes associated with microglial activation have been reported in both effectively treated and untreated HIV-infected individuals (40). Despite effective control of HIV RNA in plasma and the CSF with cART, there is evidence that the initial inflammatory process following HIV-infection of the brain is likely to persist. Studying neuroinflammation in cognitively asymptomatic HIV-infected patients may shed light on whether microglial activation is an earlier contributor to the pathogenesis of HIV-associated NCI

The identification of TSPO a mitochondrial transmembrane protein, highly expressed in activated microglia and astrocytes, has allowed the *in vivo* quantification of brain inflammation using PET. TSPO response to injury seems to be directly associated with the degree of damage. There is a significant increase in TSPO density with neuronal cell loss (219, 220), and demyelination (221, 222). What the relative contributions of activated microglia and activated astrocytes to the overall TSPO imaging signal is not known. However, Banati et al. demonstrated that microglial cells are the main source of increase TSPO expression when the BBB is intact (223)

The inflammatory response to brain injury can be studied *in vivo* using TSPO ligands such as [^{11}C] PBR28. [^{11}C] PBR28 is a second-generation TSPO ligand with greater sensitivity and significantly better signal-to-noise ratio compared with first generation ligands (190). Although there is a signal of greater microglial activation in HIV-infected subjects with severe NCI compared to controls (188), evidence for microglial activation in treated HIV-infected subjects with milder NCI or without evidence of neurological disease is not conclusive (189, 195, 214).

The detection of neuroinflammation in treated HIV-infected individuals could provide a unique opportunity to further explore the pathogenic mechanisms of HIV-associated brain disease and to establish whether microglial activation has a deleterious effect on brain function. This chapter will address the first two hypotheses of this thesis; first, that microglial activation will be present in neuroasymptomatic

HIV-infected individuals stable on cART and secondly that microglial activation will be associated with abnormalities in brain function parameters.

3.2 Methods

3.2.1 Subject selection

Male patients attending St Mary's Hospital, Imperial College London NHS Healthcare were eligible. HIV-negative control data were obtained from [^{11}C] PBR28 PET CT studies conducted at Imanova.

HIV-infected subjects were required to be HIV-1 antibody positive for at least 2 years, proficient in the English language, age between 20 to 50 years of age, receiving stable cART with a plasma HIV RNA of <50 copies/mL at screening. Although, patients with any nadir CD4 T-cell counts were eligible, I enrolled a similar number of participants with high (>300 cells/uL) and low (<300 cells/uL) nadir CD4 T-cell counts, in order to determine difference in TSPO binding associated with this HIV-clinical parameter. Exclusion criteria included any active, or past history of neurological or psychiatric disease, current use of anti-inflammatories or benzodiazepines, current use of recreational drugs or alcohol abuse, and low affinity binder affinity to TSPO on genotype testing.

3.2.1 Study procedures

All participants provided informed consent. During the screening visit, demographic information was collected and included, age, current CD4 and CD8 T-cell count, nadir CD4 T-cell count, time since HIV diagnosis, time since starting cART, and pre-treatment HIV RNA. Pre-treatment HIV RNA level was defined as the highest HIV RNA value before initiating cART.

3.2.2 Neurocognitive testing

Neurocognitive testing was performed only in HIV-infected subjects using the CogState battery as described in section 2.4. All subjects had neurocognitive testing before any imaging was performed. All subjects completed neurocognitive testing at screening (practice run) and on the scanning day (study neurocognitive testing).

Analysis of neurocognitive tests scores

Scores from each task were obtained and as a measure of overall cognitive performance, composite scores were calculated from combining all tasks in the battery. To do this each score at the study cognitive examination was standardised (Z-scores) against age-matched normative population data (n=879) provided by the manufacturer. Cognitive impairment was defined as the presence of either a cognitive task score below one standard deviation (SD) in at least two cognitive tasks.

3.2.3 Brain PET scanning with [¹¹C] PBR28

PET CT scanning and T1-weighted cerebral MR imaging were performed as described in section 2.3.1.

3.2.3.1 Imaging analysis of [¹¹C] PBR28 CT PET results

Dynamic PET data were corrected for motion via frame-to-frame image registration and aligned with the individual's structural T1 MRI image using SPM5 (Wellcome Trust Center for Neuroimaging, <http://www.fil.ion.ucl.ac.uk/spm>) with a mutual information cost function.

The CIC neuroanatomical atlas (224) was nonlinearly deformed into the individual's space, via T1 MRI data mapping, to obtain a personalised anatomical parcellation of regions of interest. Attention focused on regions of moderate and high binding based: brain stem, thalamus, hippocampus, global cortical regions, cerebellum and basal ganglia. Each region of interest was then applied to the dynamic PET data to derive regional time-activity curves (TACs).

3.2.3.2 Kinetic analysis

Two-tissue compartment model, utilising the metabolite corrected plasma input function, was applied to the dynamic PET data using a fixed blood volume correction of 5%. For each ROI examined, the total volume of distribution (V_T) was estimated from the rate constants as described previously by Gunn et al. (225). The Logan graphical method (226) employing a plasma input, 5% fixed blood volume, and a linear start time at 35 min was also used to estimate the absolute V_T of each region of interest and further applied at the voxel level to produce a parametric V_T map for each subject. The main outcome measure is the total V_T for each defined brain region and a parametric V_T map for each subject. The V_T for a radioligand is defined as the

ratio of concentration in tissue to that in plasma, and it is an alternative to the binding potential (density of the neuroreceptor) in the absence of a region where the neuroreceptor is not present (reference region).

3.2.3.3 V_T normalisation

Besides V_T , the distribution volume ratio (DVR) in each region of interest was also estimated by normalizing the V_T to cortical grey matter in order to evaluate local regional differences. Normalisation can control for unknown physiological factors (unrelated to inflammation) that may affect radioligand brain uptake in TSPO PET imaging studies (227). The grey matter was selected as an area of control for normalisation because its relative uniform pattern of high baseline binding in healthy subjects. The main outcome measured is a normalized $V_{T(DVR)}$ for each defined brain region. Model fitting and parameter estimation was performed using software implemented in Matlab R2008b (The MathWorks Inc., Natick, MA, USA).

3.2.4 Statistical analysis

Differences between patients and controls were studied both on region of interest level using ANOVA with genetic effect as a fixed factor using SPSS (SPSS, Version 21, IBM, USA), and on a parametric level using FSL randomize whilst accounting for affinity status. Partial correlation was performed to investigate the relationship between TSPO binding and clinical parameters. Each HIV-infected individual enrolled in the study was evaluated by examining the statistical parametric maps and V_T from all brain regions in order to identify any associations between increase PBR28 binding and HIV-clinical and cognitive parameters.

3.3 Results

Patient demographics and clinical parameters for HIV-infected subjects are presented in Table 3.1. 12 male, HIV-infected subjects (8 HABs and 4 MABs), median (range) ages 43.5 (26-49) years were recruited and completed all study procedures. 10 Male, healthy, HIV-negative controls (6 HABs and 4 MABs), median age 44 years (28-52), matched by age and gender were included in the analysis. Median (range) nadir CD4 T-cell count (cells/uL) was 196 (70-350) and the median (range) time elapsed since HIV diagnosis (years) was 15 (2-19). HIV-clinical parameters for each HIV-infected individual are presented in Table 3.2

3.3.1 Neurocognitive testing results

Results from neurocognitive testing for the HIV-infected group are presented in Table 3.3, Table 3.4 and Table 3.5. No evidence of NCI in HIV-infected subjects was observed when compared against age-matched normative data.

HIV-infected patients performed well across the range of cognitive task of varying complexity. However in one of the tasks, continuous pair associated learning (CPAL), which evaluates visual and learning memory, HIV-infected subjects had significantly more errors compared with controls ($P=0.04$). In the CPAL task subjects must learn to remember the location of pictures presented on a screen that is hidden beneath different locations on the screen. For the CPAL task the lower the number of errors the better the performance.

3.3.2 [^{11}C] PBR28 volume of distribution (V_T) in HIV-infected subjects compared to HIV-negative controls

[^{11}C] PBR28 Total V_T results

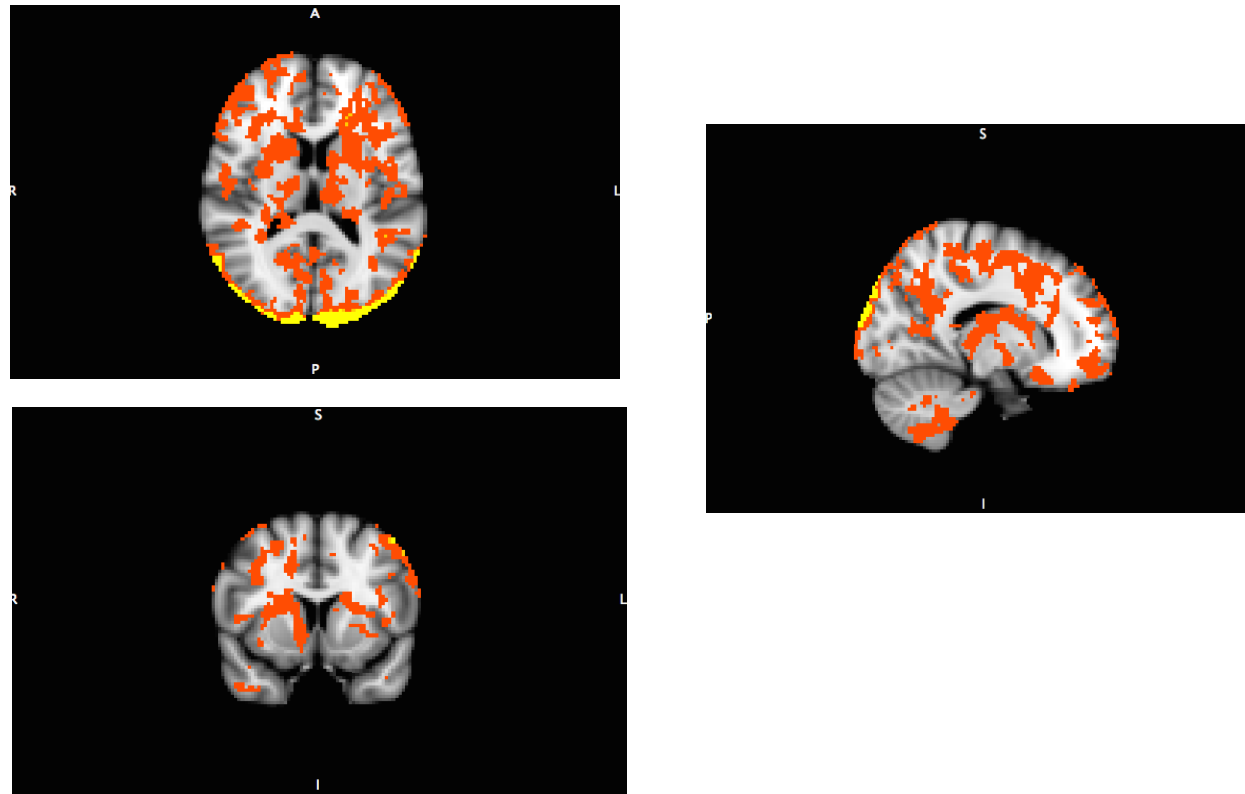
An increase in mean total [^{11}C] PBR28 V_T in HIV-infected subjects compared to HIV-negative controls in several brain regions was observed (Figure 3.1).

When only HABs were considered, the region of interest analysis showed a significant increase in total V_T in the left occipital lobe ($P=0.032$), and a trend towards increase total V_T in the left basal ganglia ($P=0.095$), striatum ($P=0.081$), left caudate ($P=0.057$), globus pallidus ($P=0.091$) and medulla ($P=0.089$) in HIV-infected subjects compared to controls, Figure 3.2

[^{11}C] PBR28 V_T from selected brain regions for each HIV-infected participant (high and mixed affinity binders) enrolled in the study is presented in Figure 3.3 and 3.4.

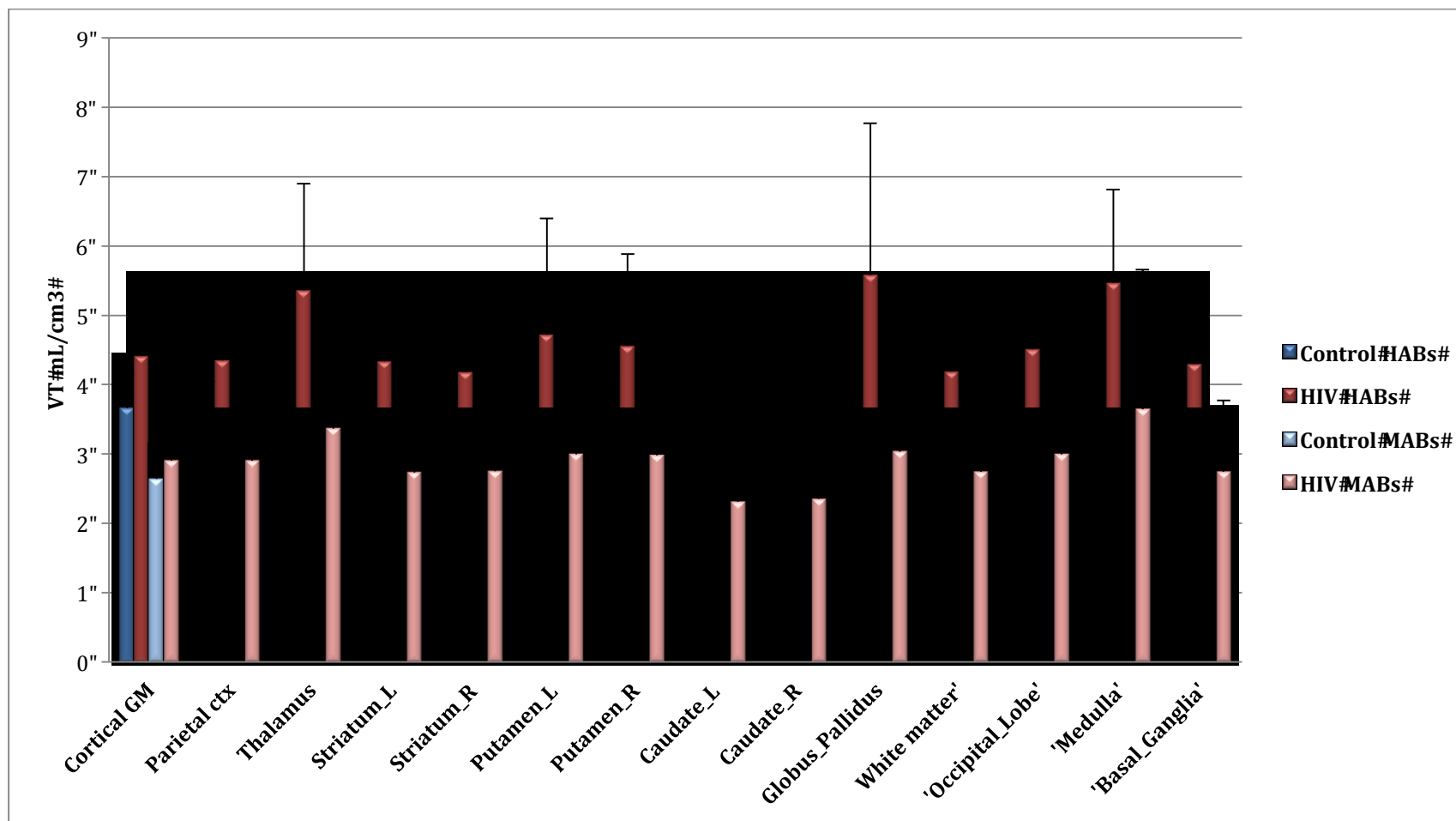
HIV-infected individuals (subjects: 03, 06, 10, 11 and 13) had an increase in [^{11}C] PBR28 V_T binding in most brain regions examined. In contrast, subjects 05, 07 and 12 had brain regions with increase PBR28 binding and reduce PBR28 binding compared to controls. The parametric [^{11}C] PBR28 V_T maps in Figure 3.5 show that subject 11 and subject 06 are the HIV-infected participants with the greatest levels of PBR28 binding when compared individually with other HIV-infected individuals and the HIV-negative controls; although these 2 individuals are neurologically asymptomatic with no evidence of cognitive impairment.

Figure 3.1 Parametric map for HIV-infected patients vs HIV-negative controls.



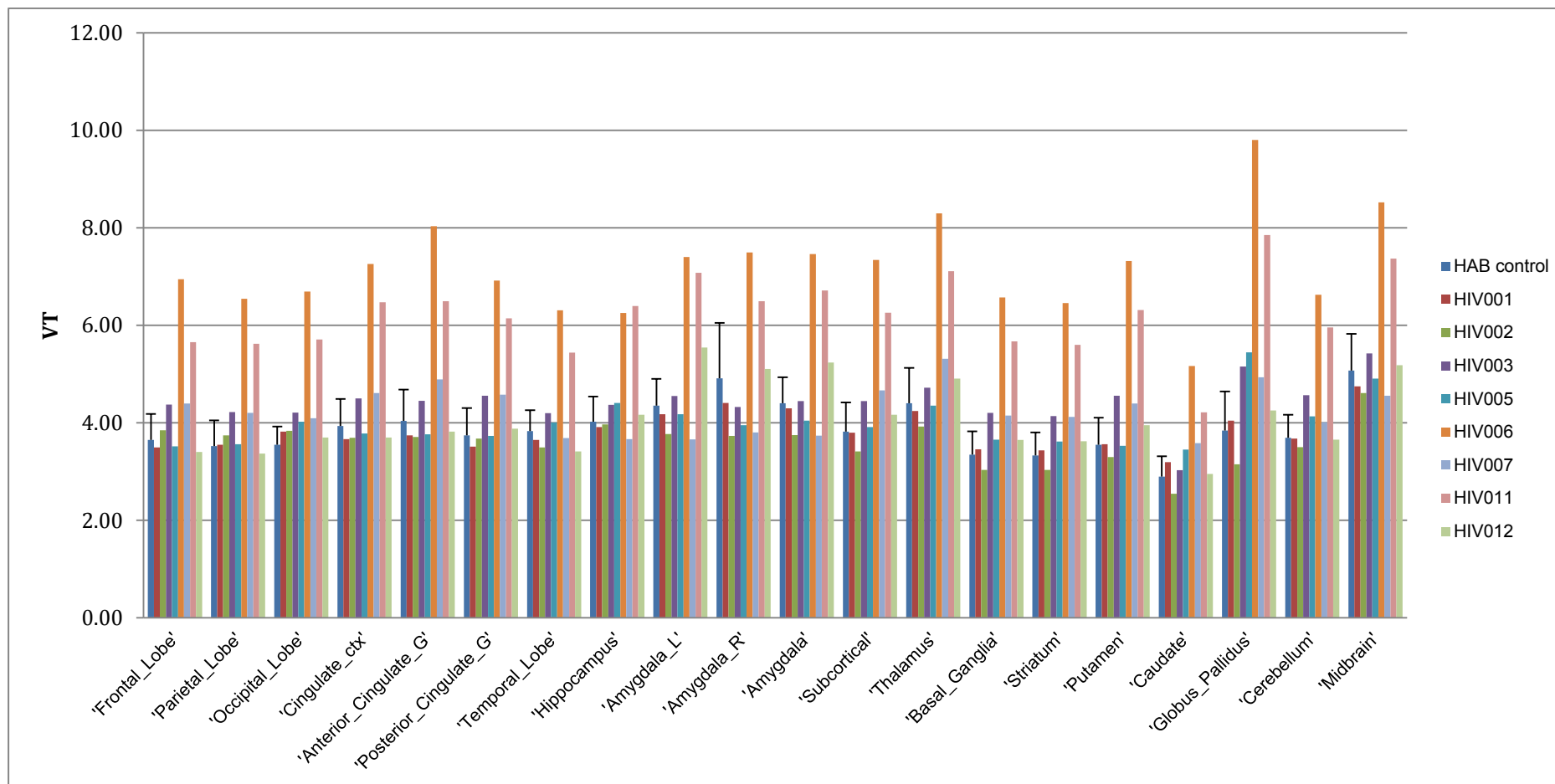
[Legend Figure 3.1: These images represent the parametric analysis showing significant increase in $[^{11}\text{C}]$ PBR28 total V_T in HIV-infected patients compared to controls, ($P=0.001$). The parametric analysis includes both affinity groups high mixed affinity and mixed affinity binders after correction for affinity phenotype.]

Figure 3.2 [^{11}C] PBR28 total V_T in selected brain regions investigated for HIV-infected cases and controls.



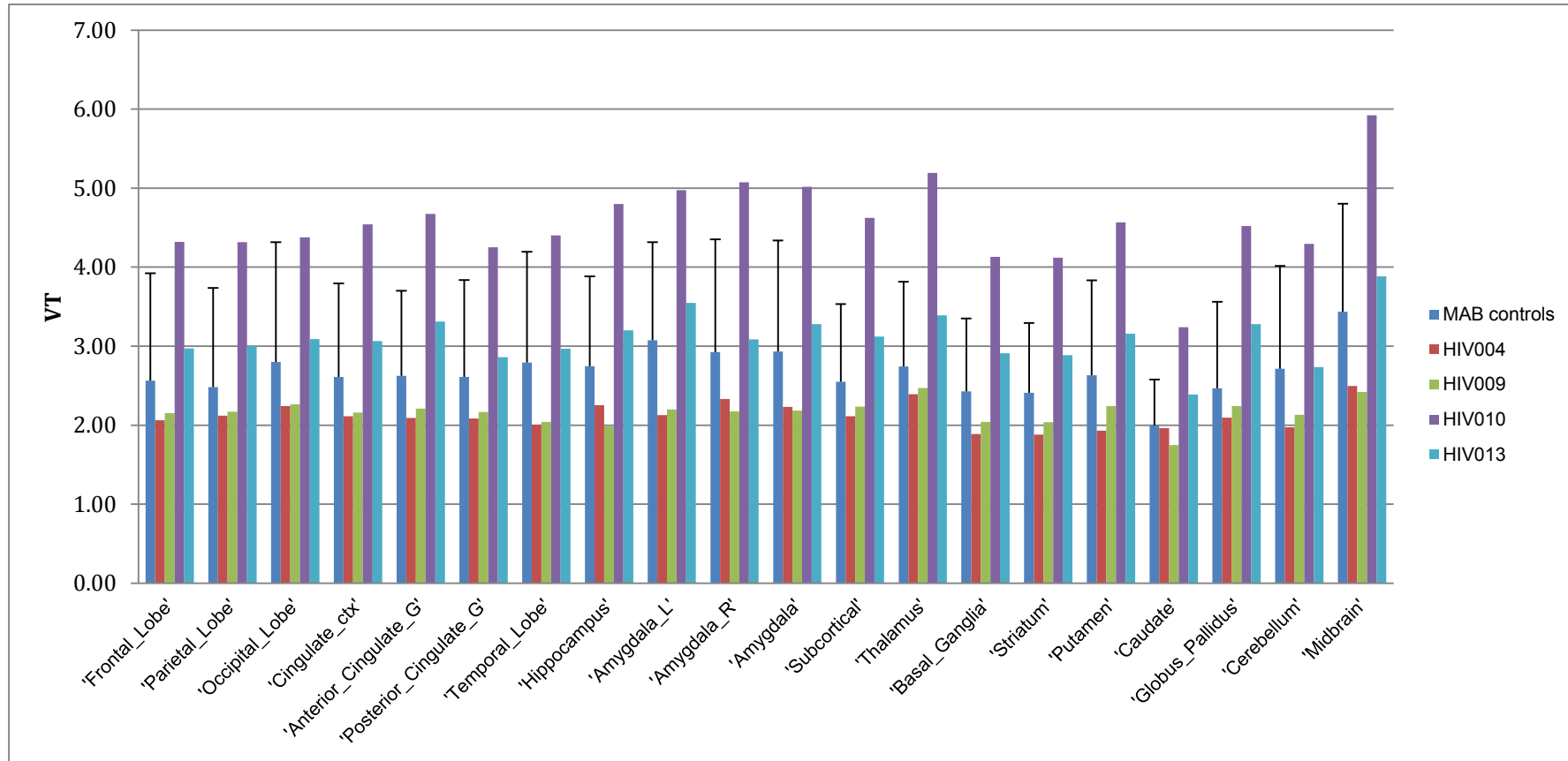
[Legend Figure 3.2: HAB= high affinity binders, MAB: mixed affinity binders, GM=grey matter, ctx= Cortex, L= left, R= right]

Figure 3.3 [^{11}C] PBR28 total V_T in selected brain regions of each HIV-infected participant (high affinity binders) enrolled in the study.



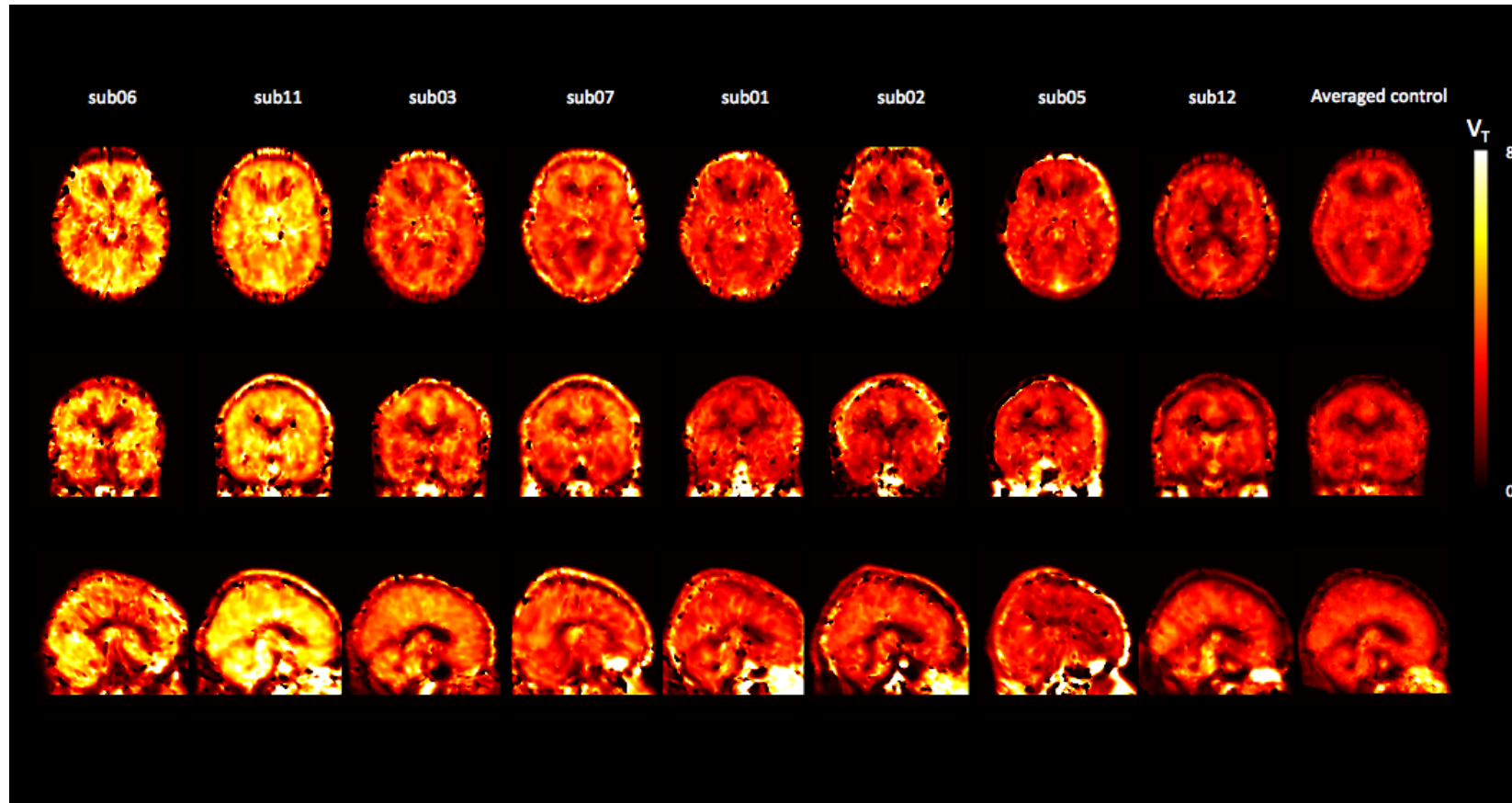
[Legend Figure 3.3: Bars represented [^{11}C] PBR28 total V_T from selected brain regions for each PBR28 high affinity binder, HIV-infected participant enrolled in the study. Average [^{11}C] PBR28 total V_T for controls is presented in dark blue with a black limit line; HAB= high affinity binders, ctx= cortex]

Figure 3.4 [^{11}C] PBR28 total V_T in selected brain regions of each HIV-infected participant (mixed affinity binders) enrolled in the study



[Legend Figure 3.4: Bars represented [^{11}C] PBR28 total V_T from selected brain regions for each PBR28 mixed affinity binder, HIV-infected participant enrolled in the study. Average [^{11}C] PBR28 total V_T for controls is presented in dark blue with a black limit line; MAB= high affinity binders, ctx= cortex]

Figure 3.5 Comparison between parametric V_T maps of HIV-infected patients and healthy controls.

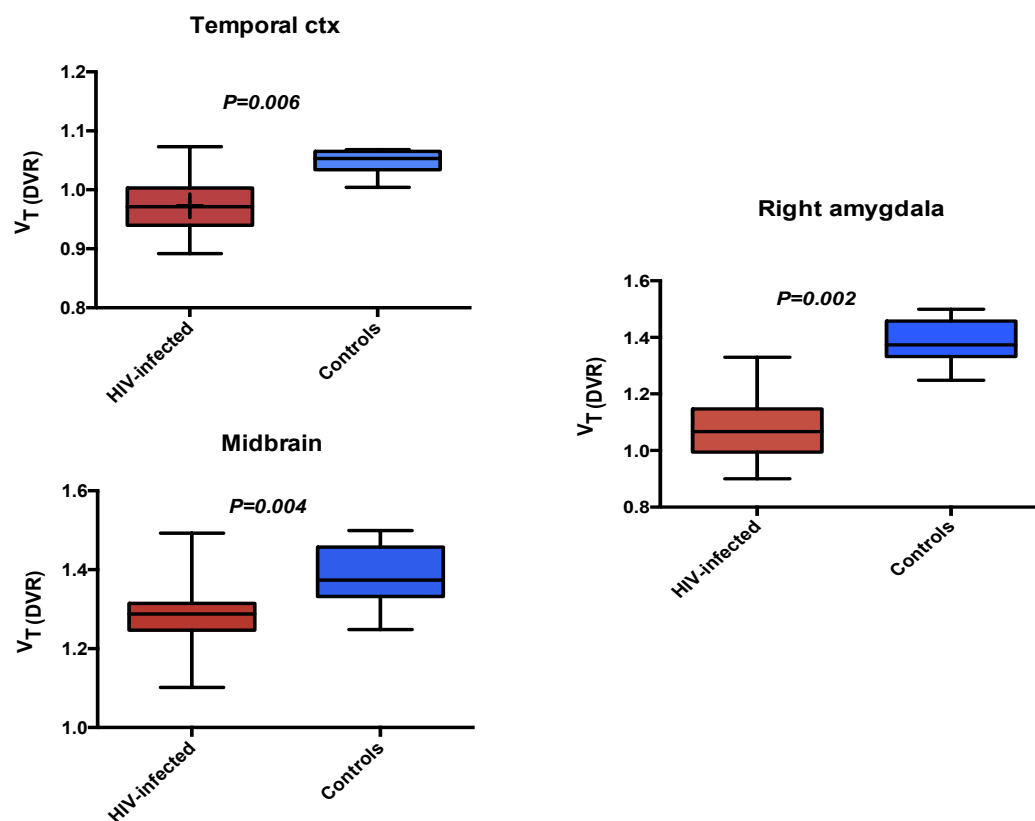


[Legend Figure 3.5: Images represent parametric maps of [^{11}C] PBR28 total V_T of individual HIV-infected subjects with high affinity to PBR28 compared to the average HIV-negative controls with high affinity for PBR28]

$[^{11}\text{C}]$ PBR28 normalized $V_{T(DVR)}$ results

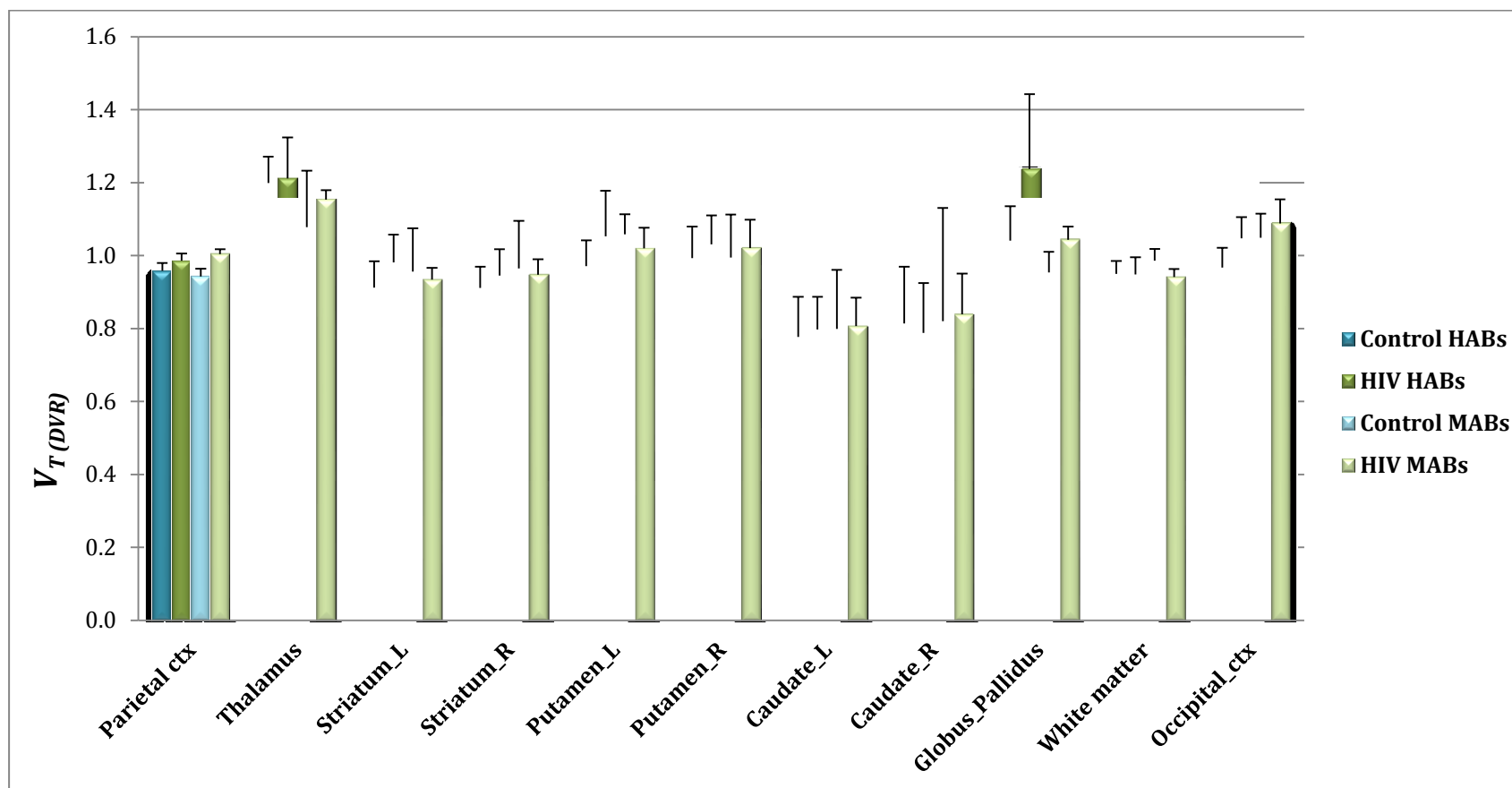
HIV-infected subjects (high affinity and mixed affinity genotype) had significant regional increase in $V_{T(DVR)}$ in the parietal lobe ($P=0.001$), occipital lobe ($P=0.046$) and globus pallidus ($P=0.035$) (Figure 3.7). A significant decrease in $V_{T(DVR)}$ in the midbrain ($P=0.05$) and the temporal lobe ($P=0.036$) were observed in HIV-infected patients compared to controls (Figure 3.6). When only HABs were considered, aside from the brain regions described, the left striatum showed an increase in $V_{T(DVR)}$, and the right amygdala a decrease in $V_{T(DVR)}$ in HIV-infected cases compared to controls, see Table 3.4

Figure 3.6 Lower $[^{11}\text{C}]$ PBR28 $V_{T(DVR)}$ in three brain regions in HIV-infected cases compared to controls.



[Legend Figure 3.6: A reduction in $[^{11}\text{C}]$ PBR28 binding in the temporal cortex, midbrain and right amygdala were demonstrated in HIV-infected individuals compared to controls. This data suggest differences in expression of TSPO in different brain regions of HIV-infected individuals on cART]

Figure 3.7 [^{11}C]PBR28 $V_{T(\text{DVR})}$ in selected brain regions investigated for all HIV-infected cases and controls.



[Legend Figure 3.7: Bars represent the [^{11}C]PBR28 $V_{T(\text{DVR})}$ for all HIV-infected participants vs HIV-negative controls; HAB= high affinity binders, MAB: mixed affinity binders, GM= grey matter, ctx= cortex, L= left, R= right]

3.3.3 Associations of [^{11}C] PBR28 V_T and clinical parameters

[^{11}C] PBR28 V_T and cognitive performance

After adjusting for TSPO affinity genotype and education, I looked for associations between [^{11}C] PBR28 $V_{T(\text{DVR})}$ and performance on neurocognitive tests. HIV-infected patients showed significant negative associations between greater [^{11}C] PBR28 $V_{T(\text{DVR})}$ and poorer cognitive global performance, see Table 3.7 and Table 3.8. The strongest correlations were between [^{11}C] PBR28 $V_{T(\text{DVR})}$ and cognitive tasks evaluating memory and verbal learning. Also, when high affinity binders were only considered, the correlations were still observed between [^{11}C] PBR28 $V_{T(\text{DVR})}$ and global cognitive performance, and composite memory and verbal learning Z-scores. Interestingly, the partial correlation analysis for the composite speed Z-scores, demonstrated a positive correlation between [^{11}C] PBR28 $V_{T(\text{DVR})}$ in several brain regions and performance in speed processing. However, these associations were not present when looking at the individual speed processing tasks (DET and IDN). The individual evaluation of HIV-infected participants demonstrated that subjects with greater [^{11}C] PBR28 $V_{T(\text{DVR})}$ binding (subjects: 06 and 11) had more errors when performing the continuous pair associated learning (CPAL) compared to other HIV-infected-individuals and HIV negative controls, see Table 3.4. The CPAL cognitive task evaluates verbal and visual memory.

[^{11}C] PBR28 V_T and HIV-clinical parameters

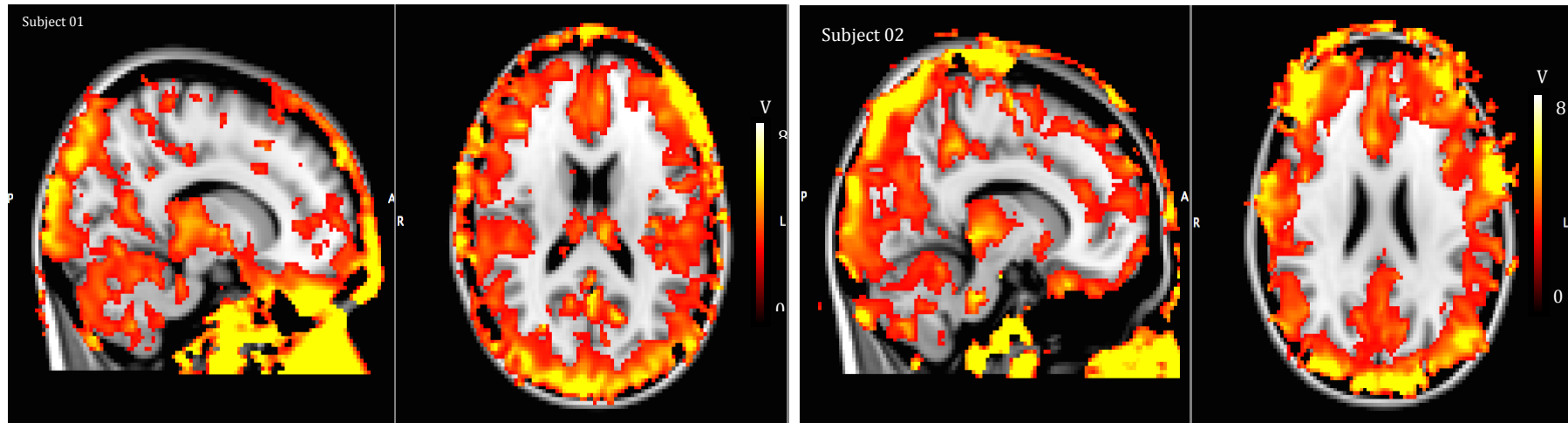
After adjusting for TSPO affinity genotype, I investigated associations between [^{11}C] PBR28 $V_{T(\text{DVR})}$ and HIV clinical parameters using correlation analysis. In addition, I examined the clinical parameters of each HIV-individual enrolled in the study to identify clinical characteristics associated with high PBR28 signal, see Figure 3.8

In the group analysis several correlations were identified. First, greater [^{11}C] PBR28 $V_{T(\text{DVR})}$ was correlated with lower CD4/CD8 ratio in several brain regions, see Table 3.9. Second, pre-treatment HIV RNA was associated with greater [^{11}C] PBR28 $V_{T(\text{DVR})}$ in the occipital lobe and parietal lobe. Interestingly, lower nadir CD4 count was

not significantly associated with [^{11}C] PBR28 $V_{T(\text{DVR})}$ in any brain region evaluated. Finally, associations between any other HIV clinical parameters and [^{11}C] PBR28 $V_{T(\text{DVR})}$ including, type of cART and years since HIV-infection or years since starting cART were not observed ($P \geq 0.1$).

The individual evaluation of HIV-infected participants showed that HIV-infected individuals with greater [^{11}C] PBR28 $V_{T(\text{DVR})}$ binding (subjects: 06,11,10,13) had higher pre-treatment HIV-RNA compared to HIV-infected individuals with lower [^{11}C] PBR28 V_T binding. Associations between other HIV clinical parameters and [^{11}C] PBR28 V_T were not identified. Parametric [^{11}C] PBR28 V_T maps, clinical and cognitive data for each HIV-infected individual enrolled in the study are presented in Figure 3.8, and Table 3.2

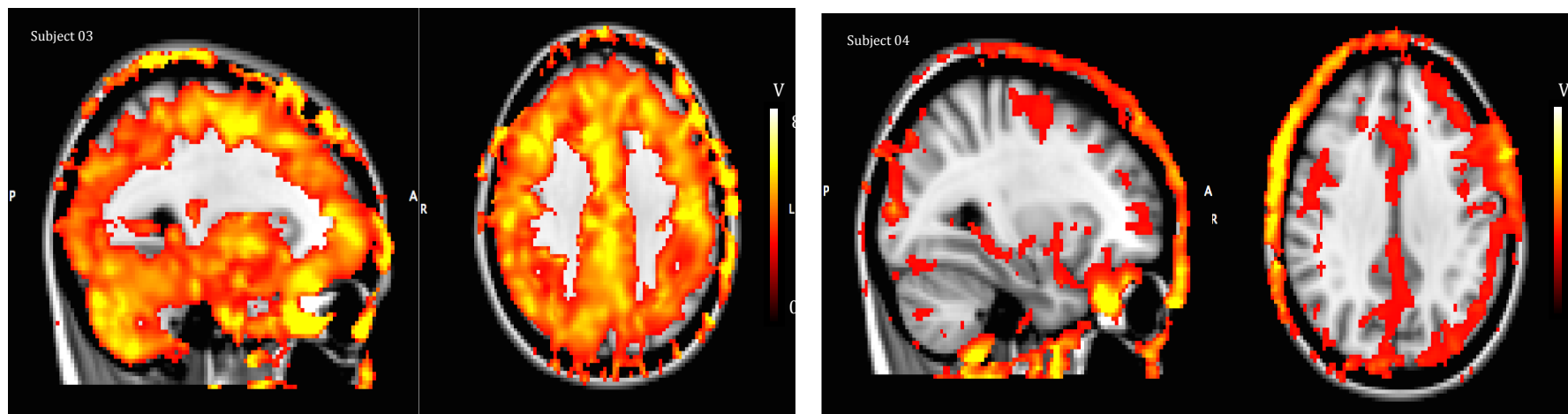
Figure 3.8 PBR28 statistical parametric maps, clinical and cognitive parameters for each HIV-infected participant enrolled in the study



Study case	PBR28 affinity	Age (years)	Current CD4 (cells/mL)	CD4/CD8 ratio	Nadir CD4 (cells/mL)	Pre-treatment HIV RNA (copies/mL)	Years since cART	DET	IDN	CPAL	GMLT	OCL	ONB	ISLT
01	High	39	900	0.70	350	n/a	15	2.434	2.63	106	44	1.074	1.322	30
02	High	45	700	0.82	110	69388	13	2.588	2.754	9	51	1.009	1.393	24

[Legend Figure 3.8: Parametric maps superimposed over a T1 MRI image from the MNI152 atlas, showing areas of increase PBR28 binding; for DET, IDN, CPAL and GMLT: lower scores=better performance; for OCL, ONB and ISLT: higher score=better performance; yellow indicates areas with greater binding]

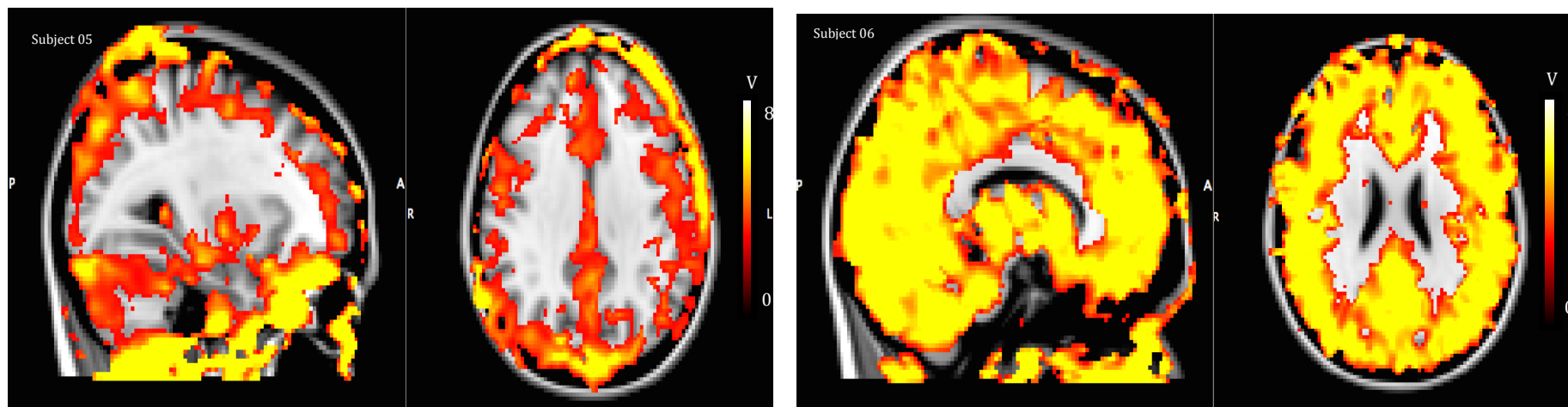
Figure 3.8 (Continued)



Study case	PBR28 affinity	Age (years)	Current CD4 (cells/mL)	CD4/CD8 ratio	Nadir CD4 (cells/mL)	Pre-treatment HIV RNA (copies/mL)	Years since cART	DET	IDN	CPAL	GMLT	OCL	ONB	ISLT
03	High	43	350	0.74	150	151	3	2.404	2.667	78	47	1.034	1.393	28
04	Mixed	39	730	0.75	220	57998	16	2.498	2.714	73	51	1.0	1.322	27

[DET, IDN, CPAL and GMLT: lower scores=better performance; for OCL, ONB and ISLT: higher score=better performance]

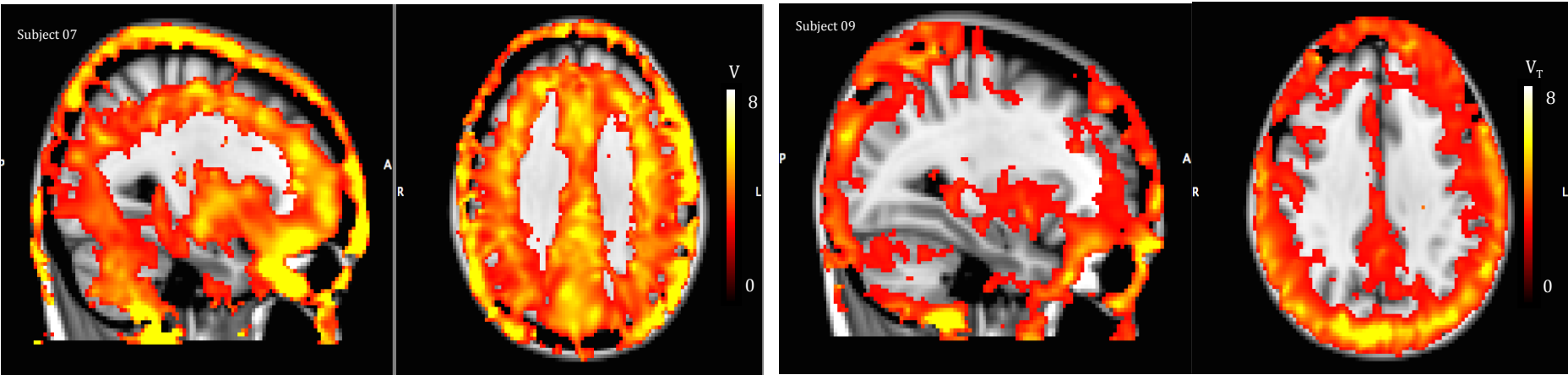
Figure 3.8 (Continued)



Study case	PBR28 affinity	Age (years)	Current CD4 (cells/mL)	CD4/CD8 ratio	Nadir CD4 (cells/mL)	Pre-treatment HIV RNA (copies/mL)	Years since cART	DET	IDN	CPAL	GMLT	OCL	ONB	ISLT
05	High	49	500	0.36	228	261970	3	2.64	2.814	2	76	1.13	1.226	27
06	High	49	720	0.72	250	1624782	19	2.429	2.675	233	40	1.101	1.322	28

[DET, IDN, CPAL and GMLT: lower scores=better performance; for OCL, ONB and ISLT: higher score=better performance]

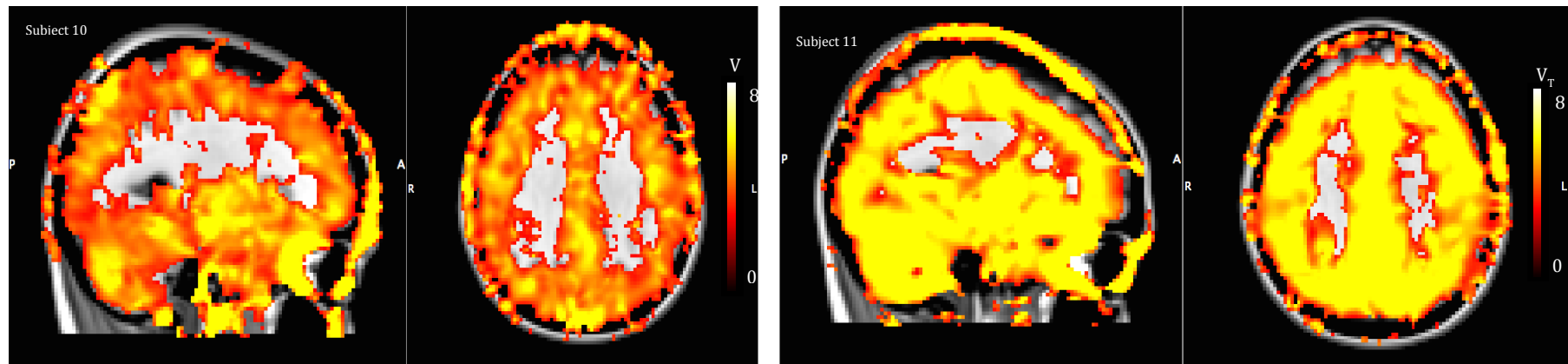
Figure 3.8 (Continued)



Study case	PBR28 affinity	Age (years)	Current CD4 (cells/mL)	CD4/CD8 ratio	Nadir CD4 (cells/mL)	Pre-treatment HIV RNA (copies/mL)	Years since cART	DET	IDN	CPAL	GMLT	OCL	ONB	ISLT
07	High	43	1240	0.72	345	35000	2	2.523	2.742	44	64	1.021	1.322	29
09	Mixed	46	150	0.70	70	38529	3	2.426	2.703	168	58	1.034	1.269	31

[DET, IDN, CPAL and GMLT: lower scores=better performance; for OCL, ONB and ISLT: higher score=better performance]

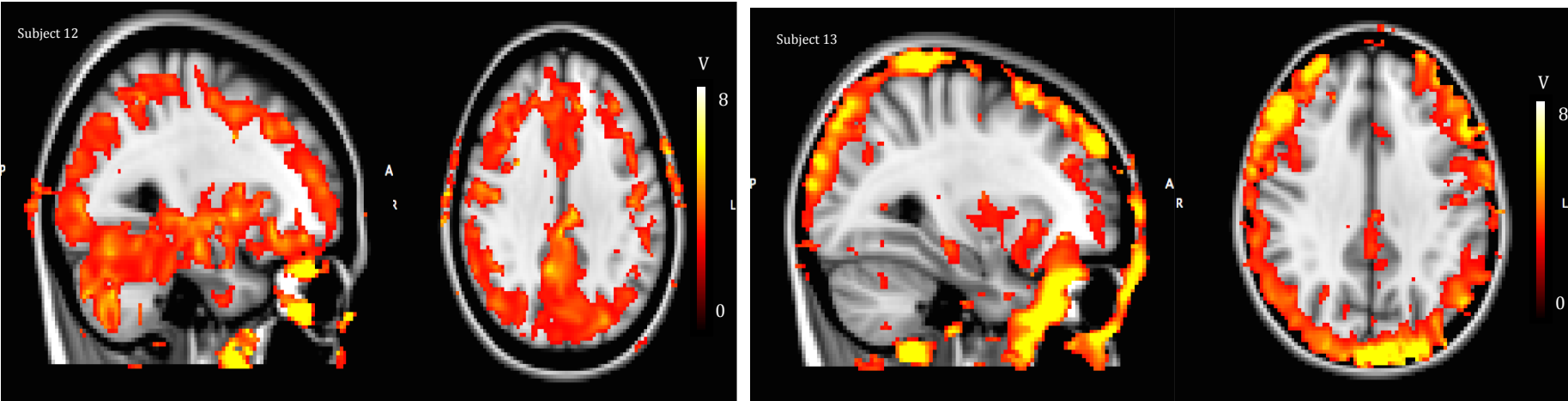
Figure 3.8 (Continued)



Study case	PBR28 affinity	Age (years)	Current CD4 (cells/mL)	CD4/CD8 ratio	Nadir CD4 (cells/mL)	Pre-treatment HIV RNA (copies/mL)	Years since cART	DET	IDN	CPAL	GMLT	OCL	ONB	ISLT
10	Mixed	40	600	0.50	130	452457	16	2.407	2.578	48	61	1.009	1.393	28
11	High	44	440	0.67	160	187890	4	2.448	2.756	268	44	1.06	1.189	25

[DET, IDN, CPAL and GMLT: lower scores=better performance; for OCL, ONB and ISLT: higher score=better performance]

Figure 3.8 (Continued)



Study case	PBR28 affinity	Age (years)	Current CD4 (cells/mL)	CD4/CD8 ratio	Nadir CD4 (cells/mL)	Pre-treatment HIV RNA (copies/mL)	Years since cART	DET	IDN	CPAL	GMLT	OCL	ONB	ISLT
12	High	49	160	0.15	100	N/A	6	2.401	2.732	5	132	0.987	1.571	23
13	Mixed	49	690	0.40	240	500000	3	2.516	2.724	1.28	42	1.101	1.571	27

[DET, IDN, CPAL and GMLT: lower scores=better performance; for OCL, ONB and ISLT: higher score=better performance]

3.4 Discussion

In this study using [^{11}C] PBR28 as an *in vivo* biomarker of TSPO expression, I investigated the presence of microglial activation in individuals with chronic HIV-infection, stable on effective cART, without cognitive or neurological symptoms. When compared to HIV-negative control subjects, matched for age and gender, I found evidence of microglial activation in HIV-infected subjects.

Unlike, previous reports on TSPO expression in neuroasymptomatic HIV-infected individuals; I observed increases in microglial activation, in several cortical and subcortical structures. The strongest signal for neuroinflammation is coming from subcortical brain regions, particularly basal ganglia structures (globus pallidus, caudate and striatum). These provide a link between *in vivo* microglial activation and evidence from neuropathological and MRS imaging studies, showing involvement of basal ganglia structures in HIV-infected individuals with and without HIV-associated brain disease (12, 228).

Although in HIV-infected subjects TSPO expression was increased in most brain regions, I identified three brain structures (temporal lobe, midbrain and the right amygdala) with reduced TSPO expression in HIV-infected subjects compared to controls. To my knowledge, this is the first time reduced TSPO expression has been identified in the brains of HIV-infected subjects compared to HIV-negative controls. Brain regions with presence of both reduced and increased microglial activation could indicate that microglial activation is exhibiting different responses to HIV infection of the brain. *In vitro* studies of microglia have demonstrated that microglia from specific brain regions express different cell surface proteins (81), suggesting that in response to brain injury, microglia might express distinctive activation patterns in different brain regions.

One important question is whether microglial activation (which could be of a more M1-phenotype) is detrimental or not to cognitive function and potentially a risk factor for the development of NCI. I found evidence that increase microglial activation was associated with poorer global cognitive function, mainly because of poorer performance in tasks evaluating verbal learning and memory. Subcortical-frontal networks have been implicated with poorer performance in verbal learning and

memory (229). Performance of patients with abnormalities in this network, such poor attention, problem-solving difficulties and general problems with retention and recall, have been reported in HIV-infected subjects (30, 31, 230). Although, HIV-infected individuals had no evidence of NCI, the association between microglial activation in subcortical brain regions and poorer cognitive performance is interesting, further longitudinal data are essential to assess whether neuroinflammation can eventually lead to cognitive impairment.

Finally, I found a strong relationship between microglial activation in several brain regions and two HIV clinical parameters: low CD4/CD8 ratio and high pre-treatment HIV RNA. Regarding CD4/CD8 ratio, in virally suppressed HIV-infected subjects lower CD4/CD8 ratio has shown to independently predict immune activation (231) and non-AIDS comorbidities such as cardiovascular disease, bone disease, and NCI (232, 233). The association, between lower CD4/CD8 ratio greater [^{11}C] PBR28 $V_{T(\text{DVR})}$ in several brain regions could indicate a direct relationship between an imaging marker of inflammation and an immunological marker of immune activation, consistent with abnormally persistent microglial activation. Individual analysis of HIV-infected individuals revealed some interesting associations. HIV-infected subjects with greater pre-treatment HIV viral load were associated with greater PBR28 binding in most brain regions. Although, subjects with greater levels of neuroinflammation were neurologically asymptomatic and had normal cognitive function by the study definition, they had more errors in a cognitive task evaluating visual and verbal memory (continuous paired associated learning task). One subject in particular, not only had more errors on the cognitive task described before, but had reduced brain activation on fMRI when performing a task designed to evaluate sustained attention, the choice reaction time task. These findings suggest a possible detrimental effect of neuroinflammation on brain function.

Despite studying individuals with a range of both high and low nadir CD4 T cell-counts, I did not find associations between greater [^{11}C] PBR28 total V_T or $V_{T(\text{DVR})}$ and nadir CD4 T-cell counts in any of the brain regions studied. Similarly when comparing HIV-infected individuals with low and high nadir CD4 T-cell counts, no differences in [^{11}C] PBR28 total V_T or $V_{T(\text{DVR})}$ were observed. The lack of association with low nadir CD4 T cell-count could be due to the small sample size.

Pre-treatment HIV RNA has been proposed as a surrogate measure of an individual's HIV-disease activity and a predictor of non-HIV associated diseases (234, 235). In this study I found that increase $[^{11}\text{C}] \text{PBR28 } V_{T(\text{DVR})}$ in the occipital and parietal lobes, caudate and striatum were associated with greater pre-treatment HIV RNA. These regions are the same regions that showed an increase in microglial activation compared to HIV-negative controls. HIV enters the CNS early during infection, after entry, activated macrophages and microglia act as both the major targets for HIV-infection and a source of neurotoxins that eventually disrupts the blood brain barrier facilitating, brain infection (61). In this context higher plasma HIV RNA could cause more active virus to enter the brain, leading to significantly more microglial activation and early brain injury. The consequence of this process is likely to be persistent microglial activation despite effective cART.

Limitations to this chapter include the small number of subjects recruited, restricting the power to detect other differences and associations. However, the sample size is similar to other TSPO PET studies. Also, the control group was only matched by age and sex, but not by other factors such as ethnicity and sexual orientation. Further limitations include the lack of cognitive data from the controls used for the $[^{11}\text{C}] \text{PBR28}$ study and the absence of longitudinal follow-up PET data to identify any other changes in $[^{11}\text{C}] \text{PBR28 } V_T$. Finally, the control data provided by CogState to perform the cognitive analysis are only matched by age.

Another important aspect is the potential effect of antiretroviral therapy on TSPO function. It is recognised mitochondrial toxicity can be induced by nucleoside reverse transcriptase inhibitors (NRTIs) (236). Nucleoside analogues inhibit HIV replication because of their affinity for the viral enzyme reverse transcriptase (viral DNA polymerase); However, NRTIs can also bind to other human DNA-polymerases like mitochondrial DNA polymerase gamma. By inhibiting mitochondrial DNA polymerase gamma, NRTIs can lead to depletion of mitochondrial DNA resulting in dysfunction of oxidase phosphorylation processes (237). Importantly, it appears that mitochondrial toxicity occurs only until a specific threshold of dysfunction has been reached (238). The affinity for the mitochondrial DNA polymerase determines the propensity of a NRTI drug to induce toxicity (239). TSPO has been implicated with several important

mitochondrial processes including regulation of cellular respiration, energy production (185, 240), regulation of reactive oxygen species, apoptosis, and uptake of cholesterol to the mitochondria (241). It is possible that NTRIs such as tenofovir or abacavir affect TSPO expression that could affect the quantification of brain TSPO binding. Most patients in this study were on cART that contained a NRTI medication. The presence of increase TSPO signal in these patients could therefore have been influenced positively (increasing the expression of TSPO) or negatively (decreasing the expression of TSPO) by NRTI containing regimens.

In summary, this study shows that neuroasymptomatic HIV-infected individuals on stable cART have an increase in TSPO expression, mostly in the basal ganglia and subcortical brain regions usually affected by HIV-brain disease. In these same regions [^{11}C] PBR28 was associated with poorer cognitive function and alterations of clinical markers of HIV-disease activity (CD4/CD8 ratio and pre-treatment HIV RNA). This study addresses the first two hypotheses of my thesis, providing evidence that microglial activation is present in neuroasymptomatic individuals with treated chronic HIV-infection and that microglial activation may be detrimental to cognitive function.

Longitudinal studies to detect disease progression from asymptomatic neuroinflammation to HIV-NCI using *in vivo* measurement of TSPO expression would be crucial to define the role of microglial activation in the pathogenesis of HIV brain disease.

Table 3.1 HIV-infected subjects demographics and clinical parameters

Demographic / clinical parameter	Median (range) unless otherwise stated
Number, n	12
Male gender, n (%)	12 (100)
Age, mean years (SD)	42 (6.4)
Ethnicity, n (%)	
White	10 (83.3)
Black	2 (16.7)
Education years, mean (SD)	12 (2.5)
Current CD4/CD8 ratio,	0.7 (0.2-0.9)
Current CD4 cell count (cells/uL)	645 (350-1240)
Nadir CD4 cell count (cells/uL)	190 (70-350)
Baseline plasma HIV RNA level<50 copies/mL, (%)	12 (100)
Pre-treatment HIV RNA copy years (Log 10copies/mL)	5.0 (2.1- 6.2)
Time-elapse since HIV diagnosis (years)	15 (2-19)
Time-elapse since starting cART (years)	4 (2-16)
Antiretroviral therapy, n (%)	12 (100)
PI based, n(%)	4 (33)
DRV/r	1
DRV/r TDF FTC	2
LOP/r TDF FTC	1
NNRTI based, n(%)	8 (77)
NVP TDF FTC	4
NVP ABC 3TC	1
EFV TDF FTC	2
RLP TDF FTC	1

[Legend Table 3.1: TDF=tenofovir, DRV=darunavir, r=ritonavir, LOP=lopinavir, NP=Nevirapine, EFV=efavirenz, RLP=rilpivirine, ABC=abacavir, 3TC= lamivudine, FTC= emtricitabine]

Table 3.2 Clinical parameters for each HIV-infected participant enrolled in the study

Study case	PBR28 affinity	Age (years)	Current CD4 (cells/mL)	CD4/CD8 ratio	Nadir CD4 (cells/mL)	Pre-treatment HIV RNA (copies/mL)	Years since cART
01	High	39	900	0.70	350	Not available	15
02	High	45	700	0.82	110	69388	13
03	High	43	350	0.74	150	151	3
05	High	49	500	0.36	228	261970	3
06	High	49	720	0.72	250	1624782	19
07	High	43	1240	0.72	345	35000	2
11	High	44	440	0.67	160	187890	4
12	High	49	160	0.15	100	Not available	6
04	Mixed	39	730	0.75	220	57998	16
09	Mixed	46	150	0.70	70	38529	3
10	Mixed	40	600	0.50	130	452457	16
13	Mixed	49	690	0.40	240	500000	3

Table 3.3 Neurocognitive testing results for HIV-infected subjects vs HIV-negative controls

Task performed	Unit of measurement	Cognitive function tested	Interpretation of score	HIV-infected Mean (SD)	Normative data* Mean (SD)	Significance P=value
Speed and visual attention						
Detection (DET)	Log ₁₀ milliseconds	Speed of processing	Lower score=better performance	2.49(0.08)	2.49(0.09)	0.867
Identification (IDN)	Log ₁₀ milliseconds	Attention	Lower score=better performance	2.71(0.64)	2.70(0.07)	0.912
Executive function						
Groton maze learning test (GMLT)	Total errors	Executive function	Lower score=better performance	52.2(13.02)	55.3(22.42)	0.391
Continuous paired learning (CPAL)	Total errors	Paired associated learning and memory	Lower score=better performance	73.1(81.3)	8.78 (10.23)	0.004
Accuracy and visual learning						
One card memory (OCL)	Arcsine proportion correct	Visual learning/memory	Higher score=better performance	1.06(0.044)	1.01(0.12)	0.591
One back memory (ONB)	Arcsine proportion correct	Working memory	Higher score=better performance	1.32(0.077)	1.34(0.19)	0.392
Memory and verbal learning						
International shopping list task (ISL)	Number of correct responses	Verbal learning/memory	Higher score=better performance	27.2(2.1)	22.79(5.4)	0.812
International shopping list- recall (ISLR)	Number of correct responses	Verbal learning/memory	Higher score=better performance	10(1.7)	8.29(3.16)	0.563

[Legend Table 3.3: * Independent t-test analysis between HIV and normative data from HIV-negative individuals provided by CogState (n=873), P<0.05 considered significant]

Table 3.4. Neurocognitive testing results for each HIV-infected participant enrolled in the study

Study case	DET Lower score=better performance	IDN Lower score=better performance	CPAL Lower score=better performance	GMLT Lower score=better performance	OCL Higher score=better performance	ONB Higher score=better performance	ISLT Higher score=better performance
01	2.434	2.63	106	44	1.074	1.322	30
02	2.588	2.754	9	51	1.009	1.393	24
03	2.404	2.667	78	47	1.034	1.393	28
05	2.64	2.814	2	76	1.13	1.226	27
06	2.429	2.675	233	40	1.101	1.322	28
07	2.523	2.742	44	64	1.021	1.322	29
11	2.448	2.756	268	44	1.06	1.189	25
12	2.401	2.732	5	132	0.987	1.571	23
04	2.498	2.714	73	51	1.009	1.322	27
09	2.426	2.703	168	58	1.034	1.269	31
10	2.407	2.578	48	61	1.009	1.393	28
13	2.516	2.724	1.28	42	1.101	1.571	27

[Legend Table 3.4: Results presented here are the scores from cognitive tasks evaluated in the study, details of each score and domains evaluated are presented in Table 3.3]

Table 3.5 Composite (Z-scores) neurocognitive group results for HIV-infected individuals

Composite	Interpretation of score	HIV-infected cases Mean (SD)
Speed: (DET+IDN)	Lower score=better performance	0.03(0.09)
Accuracy and visual learning: (OCL+ONB)	Higher score=better performance	0.2(0.5)
Memory and verbal learning: (ISL+ISLR)	Higher score=better performance	0.001(0.8)
Executive function: (CPAL+GMLT)	Lower score=better performance	0.2 (0.5)
Global composite score: (DET+IDN+OCL+ONB+ISL+ISLR+CPAL+GMLT)	Higher score=better performance	-0.006(0.3)

[Legend Table 3.5: Composite mean scores for the normative data equal to zero (standardized Z-scores), SD not available from CogState, therefore it is not possible to obtain Z-scores to perform statistical comparisons with HIV-infected cases]

Table 3.6 Regional [^{11}C] PBR28 $V_{T(\text{DVR})}$ in HIV-infected subjects vs HIV negative controls

Brain region	HIV HABs		Control HABs		HIV MABs		Control MABs		F	Mean square	Significance (<i>P</i> -value)
	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
Frontal_Lobe	0.99	0.02	1.01	0.04	0.97	0.02	0.99	0.01	1.88	0.002	0.187
Parietal_Lobe	0.96	0.02	0.99	0.02	0.94	0.02	1.00	0.01	27.99	0.010	0.001
Occipital_Lobe	0.97	0.03	1.03	0.04	1.05	0.04	1.04	0.03	1.75	0.002	0.203
Cingulate_ctx	1.07	0.02	1.06	0.05	1.01	0.06	1.02	0.01	0.00	0.000	0.983
Insular_ctx	1.08	0.04	1.08	0.05	1.03	0.13	1.06	0.08	0.35	0.002	0.562
Temporal_Lobe	1.05	0.02	0.97	0.05	1.06	0.02	0.98	0.02	22.02	0.033	0.001
Hippocampus	1.10	0.07	1.06	0.11	1.07	0.11	1.04	0.08	0.63	0.006	0.439
Amygdala	1.20	0.07	1.13	0.18	1.12	0.16	1.08	0.05	0.79	0.015	0.386
Subcortical	1.04	0.06	1.07	0.08	1.00	0.12	1.04	0.02	0.93	0.006	0.348
Thalamus	1.20	0.07	1.21	0.11	1.08	0.15	1.15	0.03	0.96	0.010	0.341
Basal_Ganglia	0.91	0.05	0.97	0.07	0.96	0.11	0.94	0.03	0.36	0.002	0.557
Striatum	0.91	0.05	0.96	0.07	0.96	0.12	0.94	0.03	0.23	0.001	0.636
Putamen	0.97	0.03	1.04	0.09	1.02	0.08	1.02	0.06	1.34	0.006	0.263
Caudate	0.80	0.14	0.80	0.09	0.82	0.23	0.82	0.08	0.00	0.000	0.960
Globus_Pallidus	1.04	0.09	1.24	0.20	0.95	0.06	1.04	0.04	5.22	0.102	0.035
Cerebellum	1.01	0.03	1.02	0.05	1.03	0.03	0.96	0.04	3.67	0.006	0.071
Brain_Stem	1.27	0.13	1.28	0.15	1.19	0.07	1.18	0.11	0.00	0.000	0.971
Midbrain	1.38	0.09	1.29	0.11	1.35	0.14	1.24	0.10	4.78	0.055	0.042
Pons	1.29	0.19	1.30	0.17	1.12	0.10	1.17	0.12	0.14	0.004	0.715
Medulla	1.12	0.24	1.25	0.20	1.18	0.09	1.20	0.20	0.71	0.029	0.409
White matter	0.95	0.03	0.95	0.05	0.99	0.03	0.94	0.02	1.80	0.003	0.196

[Legend Table 3.6: Factorial ANOVA analysis of comparing PBR28 binding between HIV-infected cases vs controls using genotype as a fixed factor]

Table 3.7 Correlation coefficients (r) between [¹¹C] PBR28 V_{T(DVR)} and neurocognitive testing, adjusted for TSPO affinity status and education years

Region	DET	IDN	CPAL	GMLT	OCL	ONB	ISLT	ISLR
Occipital lobe	-0.590*	-0.526**	-0.115	-0.031	0.536**	-0.178	0.186	-0.547**
Hippocampus	-0.141	-0.185	0.123	-0.571**	-0.017	0.208	-0.659*	-0.657*
Amygdala_R	0.472	0.160	0.005	-0.660*	-0.201	0.461	-0.567**	-0.752*
Thalamus_L	0.593*	0.122	0.243	-0.676*	-0.357	0.282	-0.041	-0.539**
Striatum	0.572**	0.312	-0.119	-0.311	0.152	0.268	0.176	-0.570**
Putamen	0.783*	0.430	-0.323	-0.20	-0.30	0.332	0.076	-0.612*
Globus pallidus	0.107	-0.146	-0.447	-0.005	0.640*	-0.311	0.175	-0.146
Midbrain	0.290	0.218	0.022	-0.534**	-0.23	0.521	-0.554**	-0.648*
Medulla	0.191	0.053	0.350	-0.639*	-0.219	0.392	-0.589*	-0.685*
White matter	0.477	-0.030	0.134	-0.573**	-0.381	0.142	-0.493	-0.635**
Subcortical	0.517	0.088	0.040	-0.565**	-0.094	0.206	-0.029	-0.591*

[Legend Table 3.7: Results of correlation analyses between PBR28 binding and neurocognitive testing results adjusted for TSPO status.

**P<0.05, **P<0.1; DET: detection, IDN: identification, CPAL: Continuous paired learning, GMLT: Groton maze learning test, OCL: one card memory, ONB: one back memory, ISLT: international shopping list task, ISLTR: international shopping list task-recall; 88 correlation coefficients were calculated (11 brain regions multiplied by 8 cognitive tasks)]*

Table 3.8 Correlation coefficients (r) between [¹¹C] PBR28 V_{T(DVR)} and composite neurocognitive Z-scores adjusted for TSPO affinity status and education years

Region	Composite Speed	Composite accuracy and visual learning	Composite memory and verbal learning	Composite executive function	Global cognitive score
Occipital lobe	0.072	-0.602*	0.145	0.301	0.390
Hippocampus	-0.771*	-0.175	-0.624*	-0.156	-0.624*
Amygdala_R	-0.750*	0.403	-0.636*	0.210	-0.635*
Thalamus	-0.609*	0.250	-0.650*	-0.023	-0.651*
Parietal lobe	0.269	-0.506	0.090	-0.534*	0.244
Putamen_L	0.075	0.658*	0.130	0.247	-0.135
Midbrain	-0.689*	0.275	-0.518	0.408	-0.528**
Medulla	-0.749*	0.133	-0.672*	0.139	-0.672*
White matter	-0.665*	0.248	-0.681*	-0.200	-0.681*

*[Legend Table 3.8: Results of correlation analyses between PBR28 binding and neurocognitive testing Z-scores results adjusted for TSPO status *P<0.05, ** P<0.1; 55 correlation coefficients were calculated (11 brain regions multiplied by 5 cognitive composite domains)]*

Table 3.9 Correlation coefficients (r) between [¹¹C] PBR28 V_{T(DVR)} and HIV clinical parameters adjusted for TSPO affinity status

Region	Current CD4 T-cell count	Nadir CD4 T-cell count	CD4/CD8 ratio	Pre-treatment HIV RNA
Occipital lobe	-0.181	-0.408	0.175	0.684*
Hippocampus	-0.533**	-0.572**	-0.596*	-0.003
Parietal lobe	-0.146	0.483	0.100	0.662*
Temporal lobe	-0.177	0.500	-0.553**	0.284
Amygdala_L	-0.599*	-0.529**	-0.759*	0.259
Thalamus_L	0.285	-0.24	-0.525*	-0.214
Caudate_R	0.059	0.507	-0.308	0.600**
Basal ganglia	0.230	0.315	-0.555**	-0.080
Striatum	-0.137	0.221	0.333	0.604**
Putamen	-0.084	0.453	0.562	0.040
Globus pallidus	-0.243	0.315	0.059	0.115
Midbrain	0.040	-0.068	-0.732*	-0.062
Medulla	-0.633*	0.089	-0.740*	0.263

[Legend Table 3.9: Results of correlation analyses between PBR28 binding and clinical characteristics of HIV infected individuals adjusted for TSPO status. * $P < 0.05$, ** $P < 0.1$; 48 correlation coefficients were calculated (12 brain regions multiplied by 4 HIV clinical parameters)]

Chapter 4

Cross-sectional analysis of the relationship between microglial activation and functional, structural and metabolic cerebral MRI imaging parameters in HIV-infected individuals on cART

Chapter 4: Cross-sectional analysis of the relationship between microglial activation and functional, structural and metabolic MRI imaging parameters in HIV-infected individuals

4.1 Introduction	104
4.2 Methods.....	104
4.2.1 Functional magnetic resonance imaging	104
4.2.1.1 Resting magnetic resonance imaging	104
4.2.1.2 Task functional MRI.....	105
4.2.2 Diffusion tensor imaging	106
4.2.3 Cerebral proton spectroscopy ([¹ H]-MRS).....	106
4.2.4 Statistical analysis	108
4.3 Results	108
4.3.1 Functional MRI.....	108
4.3.1.1 Resting functional MRI	108
4.3.1.2 Task functional MRI.....	109
4.3.2 Diffusion tensor imaging	113
4.3.3 Cerebral proton spectroscopy	115
4.7 Discussion	115

4.1 Introduction

Neuroimaging techniques that allow the evaluation of brain function, brain metabolism and white matter integrity can improve our understanding of cognitive deficits in HIV-infected individuals with HIV-associated brain disease.

Several advanced MRI techniques have been developed that can noninvasively evaluate brain function, chemistry and structure. fMRI allows the mapping of activated brain regions when subjects are either at rest or performing a particular cognitive task. DTI evaluates white matter integrity *in vivo*, while MRS detects changes in cerebral chemical concentrations. All these techniques have been used independently to study HIV-associated brain disease in both treated and untreated HIV-infected individuals (Chapter 1, section 1.5). However, the correlations between microglial activation measured by TSPO PET CT imaging and MRI biomarkers of function, chemistry, and structure have not been evaluated to date.

To study the second hypothesis of this thesis, this study aimed to investigate whether microglial activation measured by [^{11}C] PBR28 PET CT is associated with abnormalities of structural, metabolic and functional imaging parameters on MRI in neuroasymptomatic HIV-infected individuals on effective cART.

4.2 Methods

12 HIV-infected subjects that participated in the [^{11}C] PBR28 PET CT study presented in Chapter 3, underwent brain MRI scans at Imanova. All subjects completed one MRI imaging assessment including: [^1H]-MRS, fMRI and DTI. Data acquisition parameters for the MRI methods employed in this study are described in detail in section 2.3.2. All subjects completed the MRI scans before the PET CT was performed

4.2.1 Functional magnetic resonance imaging

4.2.1.1 Resting magnetic resonance imaging

Analysis were carried out using Tensorial Independent Component Analysis (ICA) (242) as implemented in MELODIC (Multivariate Exploratory Linear Decomposition into Independent Components) Version 3.13, part of FSL (FMRIB's Software Library,

www.fmrib.ox.ac.uk/fsl). The following data pre-processing was applied to the input data: masking of non-brain voxels; voxel-wise de-meaning of the data and normalisation of the voxel-wise variance.

Temporal concatenation ICA was performed across all functional datasets from each subject using automatic dimensionality estimation. Pre-processed data were whitened and projected into a 15-dimensional subspace using Principal Component Analysis. The whitened observations were decomposed into sets of vectors which describe signal variation across the temporal domain (time-courses), the session/subject domain and across the maps by optimising for non-Gaussian spatial source distributions using a fixed-point iteration technique (243). Estimated component maps were divided by the standard deviation of the residual noise and thresholded by fitting a mixture model to the histogram of intensity values $P < 0.05$ (244). The default mode network was identified by visual inspection and compared to previously published data (169). Individual Z-values of the independent component representing the default mode network were tested for correlations with [^{11}C] PBR28 total V_T and $V_{T(DVR)}$ values from brain regions that demonstrated increase and decrease [^{11}C] PBR28 $V_{T(DVR)}$ binding in the PET study presented in Chapter 3. Partial correlation analysis was performed adjusting for TSPO affinity status.

4.2.1.2 Task functional MRI

Two different analyses were performed: First, I employed a model-based fMRI data analysis using the FEAT (FMRI Expert Analysis Tool), which is part of the FSL software. FEAT is based on a general linear modeling (GLM) otherwise known as multiple regression analysis. Higher-level analyses such as mean group effect, and the effect of clinical and other imaging covariates were conducted using FLAME (FMRIB'S Local Analysis of Mixed Effects) (242). Final statistical images were thresholded (Gaussian random field-based cluster) using clusters determined by $Z \geq 2.3$ and a cluster significance threshold of $P < 0.005$ corrected for multiple comparisons. This resulted in two statistical maps of voxels, one map of voxels significantly activated by the choice reaction time task and a separate map of voxels showing relative deactivation on the same task.

For the second analysis, I used FSL tool (Featquery) to extract the time series within selected ROI having pre-processed and performed the group analysis of the

raw fMRI data from each participant. The region of interest selected for this analysis where regions with significant increase and decrease in $[^{11}\text{C}]$ PBR28 V_T in HIV-infected subjects. Using the extracted time series, linear correlation analysis was performed between the time series values in each ROI and clinical parameters and other quantifiable imaging measurements.

4.2.2 Diffusion tensor imaging

Diffusion-weighted images were registered to the $b=0$ image by affine transformations to minimise distortion due to motion and eddy currents, and then brain-extracted using the Brain Extraction Tool (BET), part of the FSL image processing toolbox (216, 245). DTI parameter maps were generated using FMRIBs Diffusion Toolbox (FDT) in FSL for FA (fractional anisotropy) and MD (mean diffusivity). The FA and MD output images were used as input for tract-base spatial statistics, a voxelwise approach for analysis of FA and MD data (246). A mean FA and MD image were created and thinned to create a mean FA and MD skeleton that represents the centers of all tracts common to the group. Each subject's aligned FA data was then projected onto this skeleton. Next the individual subject was registered and projected onto the group mean FA and MD skeleton. Mean FA and MD in major white matter tracts for the HIV-infected subjects were calculated by projecting each individual skeleton through masks for the major white matter tracts contained within the JHU white-matter tractography atlas, alongside additional cortico-thalamic and trans-collosal tracts defined by tractography (247, 248).

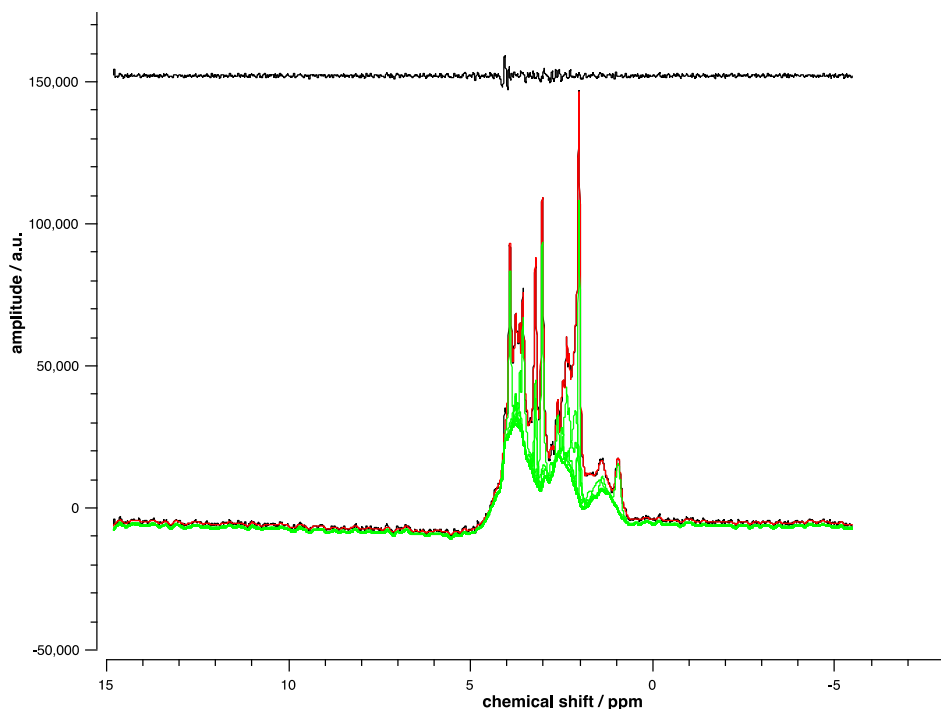
FA and MD data from the HIV-infected group were compared with data from 10 aged-matched, HIV-negative controls available from Imanova. HIV-negative controls employed the same MRI scanner, and imaging acquisition data used in my study. Independent t -test was used to compare both study groups. $P < 0.05$ were considered significant at a 95% confidence interval.

4.2.3 Cerebral proton spectroscopy ($[^1\text{H}]$ -MRS)

Details of data acquisition are described in section 2.3.2. All spectra were analyzed and quantified by a single observer (J.V.). The data were processed and fitted with TARQUIN (version 4.1.1) software (249, 250). The fitting was performed with the standard metabolite library provided by TARQUIN, which consisted on 16 different metabolites and nine lipids and macromolecular components (Figure 4.1). A

simulated negative singlet at 3.94 parts per million (ppm) (-CrCh₂) was included to account for attenuation of the CH₂ peak of Cr.

Figure 4.1. An example fit plot of a HIV-infected individual frontal white matter spectra, using TARQUIN.



[Legend Figure 4.1. Tarquin performs the fitting of cerebral metabolite peaks, allowing the calculation of the cerebral metabolites. Tope line is the residual obtained after the fitting process [ppm=part per million; a.u= arbitrary units]

The quantitation of metabolites was performed using the spectrum acquired without water suppression as reference. Two quality control criteria were taken into account in order to include the samples in the analysis: (1) A quality fit (Q) lower or equal to 2, where Q is supplied by TARQUIN and defined as the standard deviation of the frequency domain residual between 0.2 and 4.0 ppm divided by the standard deviation of the spectral noise (249, 251); and (2) a signal-to-noise ratio (SNR) of the spectra greater than 5. The following metabolites were investigated: NAA, Cr, Cho and ml. All metabolites were expressed as ratios with respect to cerebral Cr.

4.2.4 Statistical analysis

Associations between MRI imaging parameters and [^{11}C] PBR28 V_T and HIV-related clinical parameters were performed using partial correlation analysis adjusted for TSPO affinity status. Multiple comparisons were corrected by bootstrapping (252). P values below 0.05 were considered statistically significant.

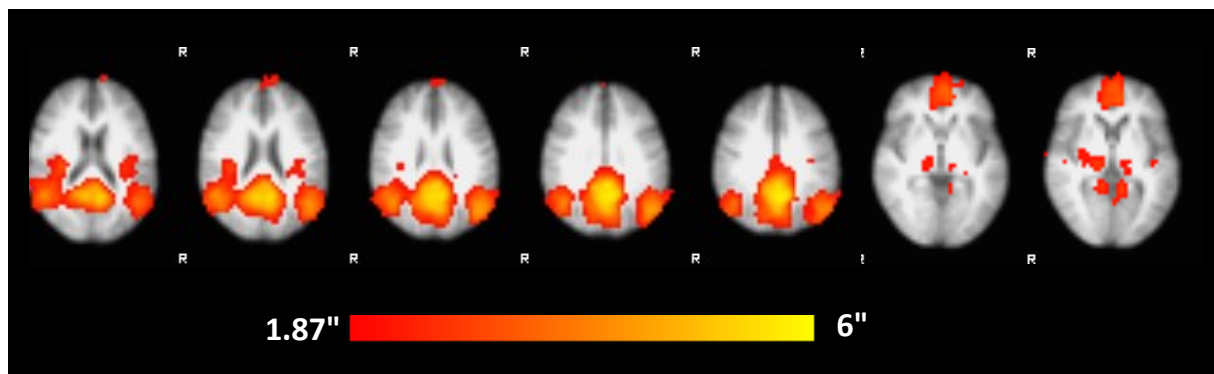
4.3 Results

4.3.1 Functional MRI

4.3.1.1 Resting functional MRI

After decomposition of the functional data by means of ICA 15 independent components were obtained. The DMN was identified by visual inspection as the network comprising the anterior cingulate cortex, posterior cingulate cortex, the precuneus and lateral parietal inferior gyri (Figure 4.2).

Figure 4.2 Spatial map of the default mode network (DMN) of HIV-infected individuals.



[Legend Figure 4.2: Hot colours represent brain activation in the brain areas associated with the default mode network: posterior cingulate cortex, retrosplenial cortex, ventromedial prefrontal cortex, medial temporal lobe and ventral precuneus. The DMN are active when the individual is in a wakeful rest. In this study participants were instructed to close their eyes during scanning]

Associations between BOLD signal in the default mode network and [^{11}C] PBR28 $V_{T(DVR)}$

No significant correlations between [^{11}C] PBR28 $V_{T(DVR)}$ in any brain region (regions and BOLD signal in the default mode network were observed (Table 4.1). No significant associations between PBR28 binding and the default mode network were identified in the resting fMRI data of each HIV-infected participant enrolled in the study

4.3.1.2 Task functional MRI

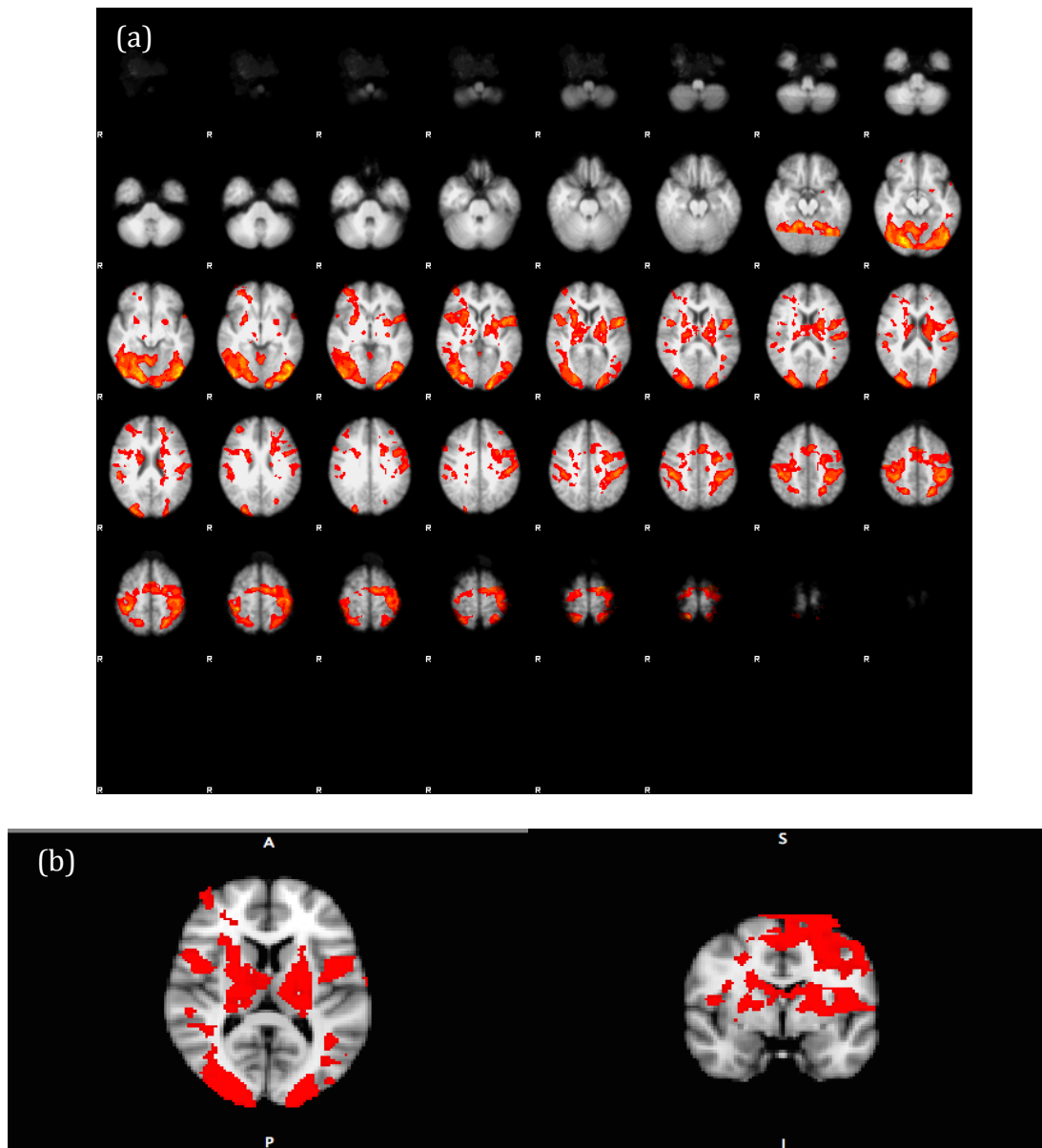
Brain activation in HIV-infected individuals following a choice reaction time task

A pattern of activation within bilateral motor and sensory regions, including superior parietal, frontal, occipital regions and the thalamus were observed in the whole brain event-related analysis (task-on). This pattern of activation is similar to the one reported in HIV-negative individuals performing an almost identical CRT task (253). In HIV-infected individuals task-related activation was also present in the basal ganglia (Figure 4.3). As expected, task-related deactivation (task-off), showed deactivation within sensory-motor areas.

Brain activation on each HIV-infected participant enrolled in the study following a choice reaction time task

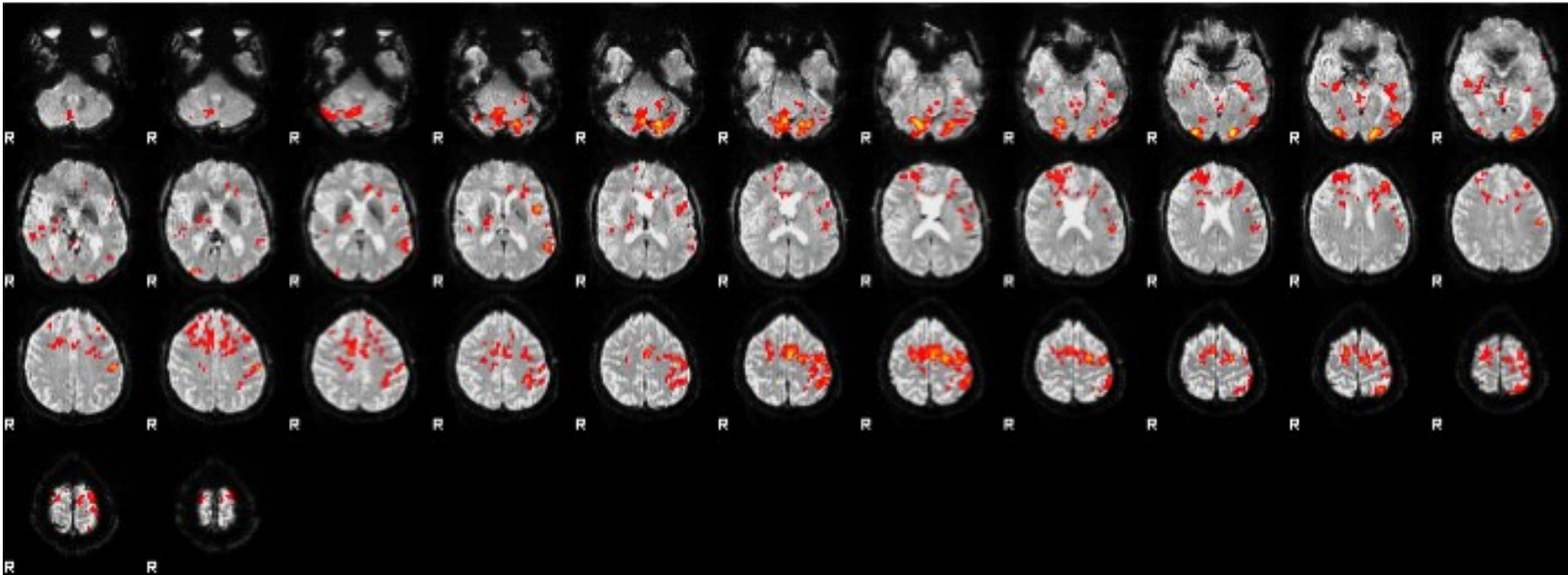
When looking at the pattern of brain activation of each HIV-infected subject, there were no differences among subjects with motor and sensory regions being activated during the task. However, subject 06 showed a clear pattern of reduced brain activation during the choice reaction task (Figure 4.4). This HIV-infected participant is had the greatest degree of PBR28 binding in the PET study presented in Chapter 3

Figure 4.3 Statistical maps of task-induced brain activation in HIV-infected individuals



[Legend Figure 4.3: Statistical maps of task-induced brain activation in HIV-infected individuals. Task induced brain activation for HIV-infected individuals (a) voxels showing significant brain activation during the choice reaction task versus 'rest'; (b) Brain activation observed in thalamus and the basal ganglia (MNI coordinates Z=40). A standard Z-statistic threshold of 2.3 was used combined with a corrected cluster significance threshold probability of $P < 0.005$. Results are shown superimposed on sagittal and axial slices of the MNI 152 T1 1-mm brain template

Figure 4.4. Statistical maps of task-induced brain activation of study case 06



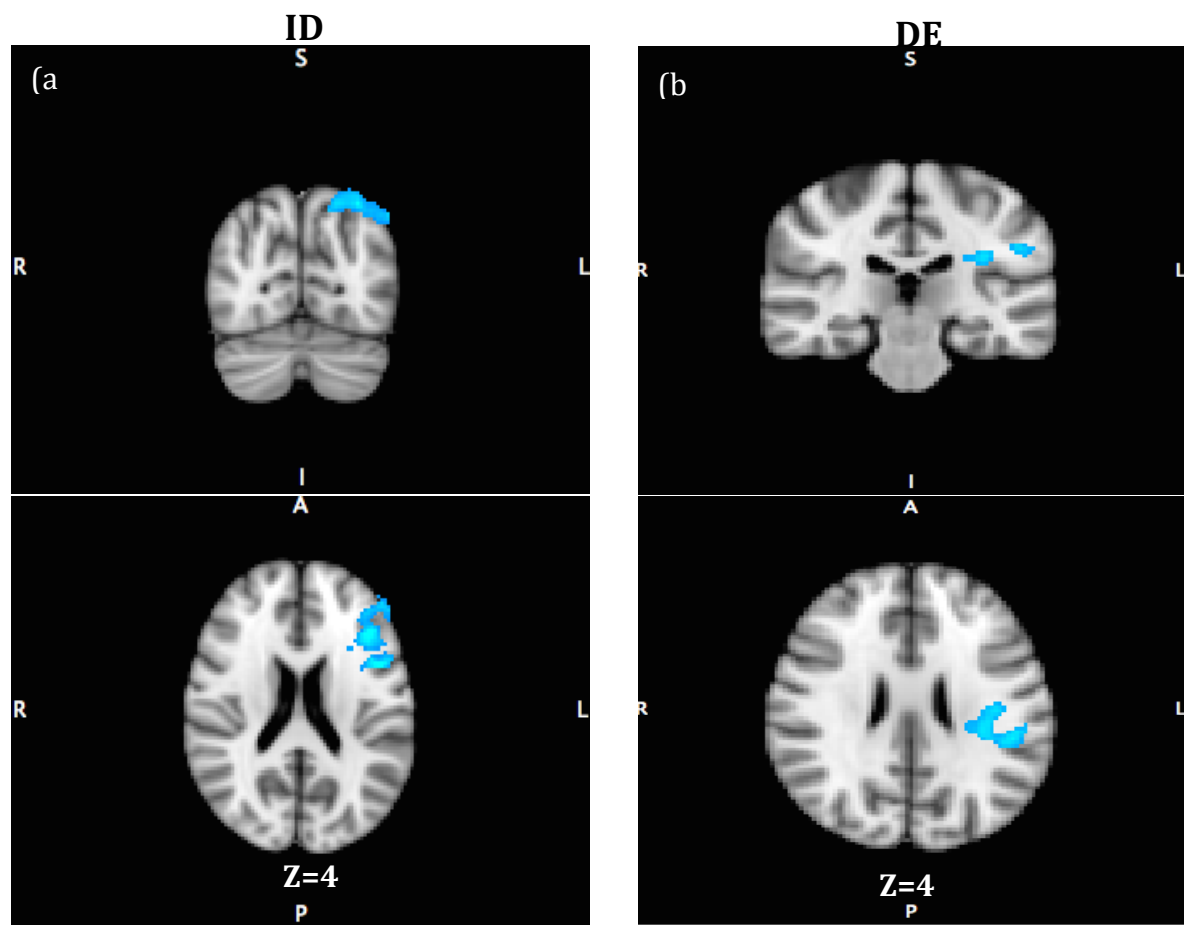
Study case	PBR28 affinity	Age (years)	Current CD4 (cells/mL)	CD4/CD8 ratio	Nadir CD4 (cells/mL)	Pre-treatment HIV RNA (copies/mL)	Years since cART
06	High	49	720	0.72	250	1624782	19

[Legend Figure 4.4:(a)Task induced brain activation for subject 06 (a) voxels showing reduced brain activation during the choice reaction task versus rest];(b). HIV-clinical parameters for subject 06. This subject had the highest degree of PBR28 binding on CT PET imaging]

Associations between task-related brain activation and cognitive function

A significant correlation between greater activation in the left frontal and left parietal lobes, and cognitive performance in tasks evaluating speed of processing information (detection and identification) was observed in the voxelwise analysis (Figure 4.5). No significant associations between task-related activation and cognitive function were identified in the individual analysis of HIV-infected participants.

Figure 4.5 Statistical maps of correlations between task-related brain activation and tasks evaluating speed of processing information.



[Legend Figure 4.5: (a) Voxels showing significant correlation between brain activation in the left frontal and parietal lobes and performance on IDN task. (b) voxels showing significant correlation between brain activation in the left parietal lobe and performance on DET task. MNI coordinates for brain slices are shown. IDN=identification, DET=detection. Statistic images were thresholded using clusters determined by $Z > 2.3$ and a (corrected) cluster significance threshold of $P = 0.05$]

Associations between [^{11}C] PBR28 $V_{T(\text{DVR})}$ and task-related brain activation

Associations between [^{11}C] PBR28 $V_{T(\text{DVR})}$ and task-related brain activation overall were not observed in the group voxelwise analysis. However, the ROI analysis showed a significant correlation between increase [^{11}C] PBR28 $V_{T(\text{DVR})}$ in the globus pallidus, hippocampus, caudate and reduced task-related brain activation in basal ganglia structures, see Table 4.2. No significant associations between task-related activation and PBR28 binding were identified in the individual analysis of HIV-infected participants.

Associations between task-related brain activation and HIV clinical parameters

No significant associations between task-related brain activation and HIV-clinical parameters including nadir CD4 count, current CD4 count, CD4/CD8 ratio, pre-treatment HIV RNA, years since HIV diagnosis and years since cART were observed in both the group voxelwise and ROI analysis ($P > 0.1$, for all observations). No significant associations between task-related activation and HIV-clinical parameters were identified in the individual analysis of HIV-infected participants.

4.3.2 Diffusion tensor imaging

FA and MD values for HIV-infected subjects and HIV-negative controls are presented in Table 4.3 and Table 4.4

A trend towards reduced FA in the HIV-infected group compared with HIV-negative controls was observed across all the evaluated white matter tracts after correcting for multiple comparisons, with significant reduced FA in the splenium of the corpus callosum ($P=0.04$) and the right superior temporal fasciculus ($P=0.05$). A trend towards increase MD was also observed in several white matter tracts in HIV-infected individuals when compared to HIV-negative controls (Table 4.4).

Associations between DTI parameters and [^{11}C] PBR28 $V_{T(\text{DVR})}$ for HIV-infected patients:

I investigated associations between brain regions that in the PET analysis showed significantly greater [^{11}C] PBR28 $V_{T(\text{DVR})}$ when compared to controls (basal ganglia,

globus pallidus, amygdala, midbrain, and occipital, parietal, and temporal lobes) and DTI parameters.

A significant correlation between increase $[^{11}\text{C}]$ PBR28 $V_{T(\text{DVR})}$ in the amygdala and reduced FA in the cingulum cingulate ($r=-0.525$, $P=0.001$) and inferior longitudinal fasciculus ($r=-0.537$, $P=0.001$) white matter tracks was observed. When investigating associations between the brain regions of interest described above and MD, significant positive correlations between increase $[^{11}\text{C}]$ PBR28 $V_{T(\text{DVR})}$ in these regions and greater MD across several white matter tracks were observed (Table 4.5). No significant associations between DTI parameters and PBR28 binding were identified in the individual analysis of HIV-infected participants.

Associations between DTI parameters and clinical parameters for HIV-infected patients

Among clinical parameters for HIV-infected patients only CD4/CD8 ratio had significant correlations with DTI parameters. An increase in FA in several white matter tracts correlated with an increase in the CD4/CD8 ratio; left corticospinal ($r=0.670$, $P=0.048$), left superior longitudinal fasciculus ($r=0.703$, $P=0.035$), left uncinate fasciculus ($r=0.661$, $P=0.052$) and body of the corpus callosum ($r=0.822$, $P=0.007$). No other associations between HIV-clinical parameters and DTI parameters were observed in the group and individual analysis of HIV-infected participants enrolled in the study

Associations between DTI parameters cognitive performance for HIV-infected patients

A significant correlation between increase MD in white matter tracts and a cognitive task evaluating executive function/spatial problem learning, was observed.

Reduced scores in the groton mask learning test (GMLT) (indicating poorer performance) was significantly associated with increase MD in the body of the corpus callosum ($r=-0.532$, $P=0.092$), left uncinate fasciculus ($r=-0.578$, $P=0.063$), left superior longitudinal fasciculus ($r=-0.692$, $P=0.018$), left superior longitudinal temporal fasciculus ($r=-0.714$, $P=0.014$) and left inferior fronto-occipital tract ($r=-$

0.619, $P=0.042$). No significant associations between DTI parameters and cognitive function were identified in the individual analysis of HIV-infected participants

4.3.3 Cerebral proton spectroscopy

Results of baseline CMRs in the FWM and RBG for HIV-infected subjects are shown in Table 4.6.

Associations between [^{11}C] PBR28 $V_{T(DVR)}$ and cerebral metabolite ratios in HIV-infected patients

A significant correlation between greater [^{11}C] PBR28 $V_{T(DVR)}$ in several brain regions and reduced ml/Cr and Cho/Cr in the RBG were observed, see Table 4.7. Interestingly, reduced NAA/Cr in the RBG correlated with greater [^{11}C] PBR28 $V_{T(DVR)}$ in the putamen. There were no significant correlations between concentrations of any cerebral metabolites in the FWM and [^{11}C] PBR28 $V_{T(DVR)}$; ($P=>0.1$), see Table 4.8

4.7 Discussion

In this study I investigated the relationship between microglial activation as measured by [^{11}C] PBR28 and cerebral MRI biomarkers. This is the first study to describe the effects of microglial activation *in vivo* on brain activation, brain network connectivity, white matter structure, and cerebral metabolism in HIV-infected individuals stable on cART.

I have made several interesting observations. First, I studied the effects of microglial activation on brain function using fMRI. Initially I investigated the effects of microglial activation on resting fMRI brain networks affected in HIV-infected individuals such as the default mode network (175). In this study, I found no associations between microglial activation and disruption of resting brain connectivity networks. However, in HIV-infected patients performing a cognitive task that evaluates sustained attention, a significant association between reduced brain activation in the basal ganglia and increased microglial activation in the globus pallidus and the caudate was observed. Traditionally, the basal ganglia has been associated with motor control (254). Although, basal ganglia structures play a critical role on motor execution and motor learning recent data suggest that they are also involved in

memory, attention, and learning that is not directly tied to motor functioning (255, 256). For instance, the caudate has been associated with instrumental learning where it is involved with integration of information about performance and cognitive control demands (257).

Interestingly, a positive correlation between [^{11}C] PBR28 $V_{T(\text{DVR})}$ in the hippocampus and brain activation in the basal ganglia was observed. These results suggest that some brain regions are more susceptible to the effects of inflammation than others. This finding strengthens the observation from the PET study, that brain regions in HIV-infected individuals showed different degrees of microglial activation when compared to HIV-negative controls.

I also examined the relationship between task-related brain activation and cognitive function using a voxelwise analysis. I observed an association between increase brain activation and performance on cognitive tests evaluating speed of processing information. Although all the HIV-infected individuals in this study were cognitively intact, a greater use of their brain capacity to maintain the task performance is suggested. These findings are consistent with those of other task-related fMRI studies and in HIV-infected individuals (163, 166)

The second interesting observation was the relationship between microglial activation and white matter injury. First, I observed a trend towards a decrease in FA and increased MD in HIV-infected individuals compared to HIV-negative controls. White matter integrity was affected in temporal, parietal, occipital white matter regions including the corpus callosum. These findings are consistent with previous reports demonstrating white matter injury in HIV-infected individuals despite effective cART (180). Most, notably in brain regions with an increase in microglial activation I observed a significant correlation between greater [^{11}C] PBR28 V_T and increased MD in most white matter regions examined.

The observed deleterious effect of microglial activation on white matter is particularly noteworthy, as it suggests a direct effect of inflammation on white matter integrity in the brain of HIV-infected individuals on effective cART. The microstructural changes responsible for the abnormal diffusivity and anisotropy in HIV-infected patients are

unclear. Inflammation has been demonstrated to increase tissue water affecting water diffusivity in animal models of brain inflammation (258) and disturbances in water diffusivity are known to reduce anisotropy and to increase mean diffusivity (259).

I also explored the effects of abnormal white matter integrity on cognitive function and found a correlation between poorer performance on task evaluating executive function and increase MD in frontal, temporal and occipital white matter regions, including the corpus callosum. These results indicate that microglial activation affects white matter integrity, which could potentially lead to cognitive decline.

Finally, I studied the relationship between microglial activation and cerebral metabolites as measured by cerebral proton spectroscopy. Surprisingly I found an increase in microglial activation to be associated with reduce cerebral metabolite markers of glial activation in the RBG. These results were unexpected as ml and Cho are cerebral metabolites known to be associated with neuroinflammation. A explanation for these results could be that the area investigated was not affected by microglial activation, especially because the voxel in the RBG was placed in the right thalamus, an area that did not show evidence of microglial activation compared to HIV-negative controls in the PET study. Alternatively, the degree of inflammation might not be sufficient to increase the concentrations of ml.

The study described in this chapter has several limitations, and therefore data must be interpreted with caution. First, the small sample size limits the power to detect relationships. Second, I did not have control data from HIV-negative individuals for the fMRI and cerebral proton spectroscopy analyses; therefore, interpretation of the data is limited. However, as a proof of principle this study demonstrates the potential effect of microglial activation on cerebral function, structure and metabolism of HIV-infected individuals on cART.

Table 4.1 Correlation coefficients (r) between [^{11}C] PBR28 $V_{T(\text{DVR})}$ and BOLD signal in the default mode network adjusted for TSPO affinity status

Region	Correlation coefficients (r)
Occipital lobe	0.264
Temporal lobe	0.259
Parietal lobe	0.213
Frontal lobe	0.255
Anterior cingulate	0.246
Posterior cingulate	0.264
Basal ganglia	0.243
Globus pallidus	0.212
Midbrain	0.259
Medulla	0.221

[Legend Table 4.1: Correlation results between PBR28 binding in anatomical brain regions related to the default mode network and BOLD fMRI from the default mode network. $P > 0.1$ for all regions investigated]

Table 4.2 Correlation coefficients (r) between [^{11}C] PBR28 $V_{T(DVR)}$ and task-related brain activation adjusted for TSPO affinity status

Brain region	Subcortical	Thalamus	Striatum	Globus pallidus	Putamen	Caudate	Cingulate	Basal ganglia
Globus pallidus $V_{T(DVR)}$	-0.667**	-0.712*	-0.721*	-0.546**	-0.613*	-0.800*	-0.662**	-0.650**
Caudate $V_{T(DVR)}$	-0.125	-0.184	-0.233	-0.157	-0.131	-0.606*	-0.395	-0.342
Hippocampus $V_{T(DVR)}$	0.480	0.526	0.429	0.431	0.429	0.644*	0.545**	0.404

*[Legend Table 4.2: Results of correlation analyses between PBR28 binding in selected brain regions and task-related brain activation results obtained using the FEAT tool from the FSL software; * $P < 0.05$, ** $P < 0.1$; 88 correlation coefficients were calculated ($[^{11}\text{C}]$ PBR28_(DVR) values from 11 brain regions multiplied by values from task related activation from 8 brain regions)]*

Table 4.3 Fractional anisotropy (FA) results from independent t-test between HIV-infected subjects and HIV-negative controls

White matter tract	HIV-infected		HIV-negative controls		Mean difference	P= value	CI (95%)
	Mean	SD	Mean	SD			
Anterior thalamic left	0.49	0.02	0.50	0.02	-0.01	0.13	-0.03 - 0.00
Cingulum cingulate left	0.53	0.02	0.55	0.03	-0.02	0.18	-0.04 - 0.01
Cingulum hippocampus left	0.47	0.01	0.46	0.04	0.01	0.62	-0.02 - 0.03
Corticospinal left	0.57	0.02	0.58	0.02	-0.01	0.29	-0.02 - 0.01
Inferior fronto-occipital left	0.48	0.02	0.50	0.03	-0.02	0.07	-0.04 - 0.00
Inferior longitudinal fasciculus left	0.47	0.02	0.49	0.03	-0.02	0.06	-0.04 - 0.00
Superior longitudinal fasciculus left	0.48	0.02	0.49	0.03	-0.02	0.11	-0.04 - 0.00
Superior fasciculus temporal left	0.49	0.02	0.50	0.03	-0.02	0.12	-0.04 - 0.01
Uscinate fasciculus left	0.45	0.02	0.46	0.03	-0.01	0.23	-0.04 - 0.01
Corpus callosum body	0.66	0.03	0.68	0.03	-0.02	0.17	-0.04 - 0.01
Corpus callosum genu	0.60	0.02	0.61	0.03	0.00	0.64	-0.03 - 0.02
Corpus callosum splenium	0.70	0.02	0.72	0.02	-0.02	0.04	-0.03 - 0.00
Forceps major	0.57	0.05	0.57	0.02	0.00	0.88	-0.04 - 0.03
Forceps minor	0.53	0.02	0.54	0.03	-0.01	0.32	-0.03 - 0.01
Anterior thalamic right	0.49	0.02	0.46	0.03	-0.02	0.10	-0.04 - 0.00
Cingulum cingulate right	0.52	0.02	0.50	0.03	-0.01	0.31	-0.04 - 0.01
Cingulum hippocampus right	0.51	0.02	0.48	0.03	-0.01	0.20	-0.04 - 0.01
Corticospinal right	0.57	0.02	0.49	0.02	-0.01	0.29	-0.03 - 0.01
Inferior fronto-occipital right	0.49	0.02	0.51	0.03	-0.02	0.14	-0.04 - 0.01
Inferior longitudinal fasciculus right	0.47	0.02	0.57	0.02	-0.01	0.49	-0.03 - 0.01
Superior longitudinal fasciculus right	0.47	0.02	0.52	0.02	0.00	0.82	-0.02 - 0.02
Superior fasciculus temporal right	0.48	0.02	0.55	0.03	-0.03	0.05	-0.05 - 0.00
Uscinate fasciculus right	0.49	0.02	0.50	0.02	-0.01	0.33	-0.03 - 0.01

[Legend Table 4.3 and 4.4: FA= fractional anisotropy; MD= mean diffusivity; SD=standard deviation; CI: coefficient interval]

Table 4.4 Mean Diffusivity (MD) results from independent t-test between HIV-infected subjects and HIV-negative controls

White matter tract	HIV-infected		HIV-negative controls		Mean difference	P= value	CI (95%)	
	Mean (10 ³)	SD	Mean (10 ³)	SD				
Anterior thalamic left	1.374	0.001	1.306	0.001	-0.0001	0.19	-0.0002	0.00
Cingulum cingulate left	1.158	0.001	1.138	0.001	-0.0001	0.68	-0.0002	0.00
Cingulum hippocampus left	1.913	0.001	1.668	0.002	0.0000	0.00	-0.0001	0.0001
Corticospinal left	1.223	0.001	1.098	0.001	0.0000	0.00	-0.0001	0.0001
Inferior fronto-occipital left	1.176	0.001	1.148	0.001	-0.0002	0.52	-0.0004	-0.0001
Inferior longitudinal fasciculus left	1.247	0.001	1.188	0.001	-0.0002	0.20	-0.0004	-0.0001
Superior longitudinal fasciculus left	1.089	0.001	1.067	0.001	-0.0001	0.62	-0.0002	0.00
Superior fasciculus temporal left	1.072	0.001	1.043	0.001	-0.0001	0.47	-0.0002	0.00
Uscinate fasciculus left	1.080	0.001	1.090	0.001	0.00	0.76	-0.0001	0.0001
Corpus callosum body	1.763	0.001	1.604	0.002	0.00	0.02	-0.0001	0.0001
Corpus callosum genu	1.407	0.002	1.392	0.002	-0.0001	0.85	-0.0002	0.00
Cospus callosum splenium	1.875	0.001	1.683	0.001	-0.0001	0.01	-0.0002	0.00
Forceps major	1.393	0.001	1.292	0.001	0.00	0.02	-0.0001	0.0001
Forceps minor	1.214	0.002	1.221	0.001	0.00	0.90	-0.0001	0.0001
Anterior thalamic right	1.393	0.001	1.287	0.001	0.00	0.04	-0.0001	0.0001
Cingulum cingulate right	1.057	0.001	1.036	0.001	0.00	0.48	-0.0001	0.0001
Cingulum hippocampus right	1.752	0.001	1.509	0.001	0.00	0.01	-0.0001	0.0001
Corticospinal right	1.236	0.001	1.104	0.001	0.00	0.01	-0.0001	0.0001
Inferior fronto-occipital right	1.185	0.001	1.132	0.001	-0.0002	0.14	-0.0003	0.00
Inferior longitudinal fasciculus right	1.294	0.001	1.221	0.001	-0.0002	0.07	-0.0003	0.00
Superior longitudinal fasciculus right	1.088	0.001	1.051	0.001	0.00	0.40	-0.0002	0.0002
Superior fasciculus temporal right	1.035	0.001	9.945	0.001	0.00	0.30	-0.0002	0.0002
Uscinate fasciculus right	1.123	0.001	1.086	0.001	-0.0002	0.33	-0.0003	-0.0001

[Legend Table 4.4: *P<0.05, ** P<0.1; 22 correlation coefficients were calculated per brain region ([¹¹C] PBR28_(DVR) values from one brain region multiplied by parameters from 22 MD white matter tracts) 11 brain regions were investigated)]

Table 4.5 Correlation coefficients (r) between [¹¹C] PBR28 V_{T(DVR)} and mean diffusivity parameters from white matter tracts, adjusted for TSPO affinity status

Brain regions with increase [¹¹ C] PBR28 V _{T(DVR)}	Anterior thalamic left	Cingulum hippocampus left	Corticospinal left	Superior L_fasciculus left	Superior L_fasciculus temporal left	Uscinate fasciculus left	Corpus callosum body	Cospos callosum splenium	Anterior thalamic right	Cingulum cingulate right	Cingulum hippocampus right	Corticospinal right	Inferior fronto-occipital right
Basal ganglia	0.606*	0.528**	0.741*	0.662*	0.630*	0.578**	0.687*	0.614*	0.639*	0.736*	0.762*	0.790*	0.706*
Amygdala	0.572**	0.375	0.581**	0.606*	0.577**	0.612*	0.615*	0.489	0.575**	0.640*	0.603*	0.574**	0.549**
Globus pallidus	0.671*	0.600*	0.777*	0.716*	0.695*	0.637*	0.700*	0.658*	0.711	0.807*	0.803*	0.834*	0.773*
Midbrain	0.563**	0.430	0.642*	0.573**	0.536**	0.577*	0.664*	0.526**	0.595*	0.620*	0.646*	0.666*	0.594*
Temporal lobe	0.573**	0.486	0.706*	0.565**	0.522**	0.565**	0.608*	0.553**	0.621*	0.630*	0.704*	0.630*	0.654*
Occipital lobe	0.575**	0.516	0.715*	0.612*	0.575*	0.572**	0.686*	0.539**	0.607*	0.661*	0.696*	0.750*	0.657*

[Legend Table 4.5: Results of correlation analyses between PBR28 binding and mean diffusivity in selected white matter tracts. *P<0.05, **

P<0.1; 22 correlation coefficients were calculated per brain region ([¹¹C] PBR28_(DVR) values from one brain region multiplied by parameters from 22 MD white matter tracts) 11 brain regions were investigated)]

Table 4.6 Results of cerebral [¹H]-MRS in HIV-infected individuals

Cerebral metabolite ratios	HIV-infected cases Mean (SD)
Frontal white matter	
NAA/Cr	1.76 (0.31)
Cho/Cr	0.73 (0.32)
ml/Cr	0.85 (0.34)
Right basal ganglia	
NAA/Cr	1.73 (0.32)
Cho/Cr	0.11 (0.08)
ml/Cr	0.84 (0.5)

[Legend Table 4.6: Mean results of cerebral metabolites for the frontal white matter and right basal ganglia of HIV-infected individuals. SD=standard deviation, NAA= N-acetylaspartate, Cho=Choline, ml=myoinositol, Cr=Creatine]

Table 4.7 Correlation coefficients (r) between [^{11}C] PBR28 $V_{T(DVR)}$ and [^1H]-MRS in the right basal ganglia adjusted for TSPO affinity status

Brain region	Right basal ganglia		
	NAA/Cr	ml/Cr	Cho/Cr
Basal ganglia	-0.504	-0.804*	-0.773*
Subcortical	-0.401	-0.691*	-0.757*
Thalamus	-0.254	-0.630*	-0.656
Striatum	-0.585**	-0.795*	-0.747*
Putamen	-0.694*	-0.768*	-0.798*
Caudate	-0.144	-0.442	-0.630**
Globus pallidus	-0.671	-0.769*	-0.797*
Anterior cingulate	-0.595**	-0.474	-0.520
Hippocampus	0.687**	0.059	-0.208

*[Legend Table 4.7: Results of correlation analysis between PBR28 binding in corresponding brain regions and right basal ganglia cerebral metabolites. * $P < 0.05$, ** $P < 0.1$, NAA= N-acetylaspartate, Cho=Choline, ml=myoinositol, Cr=Creatine; 33 correlation coefficients were calculated ([^{11}C] PBR28_(DVR) values from 11 brain regions multiplied by values from 3 CMRs in the RBG)]*

Table 4.8 Correlation coefficients (r) between [^{11}C] PBR28 $V_{T(DVR)}$ and [^1H]-MRS in the frontal white matter adjusted for TSPO affinity status

Brain region	Frontal White Matter		
	NAA/Cr	ml/Cr	Cho/Cr
Basal ganglia	-0.488	-0.159	0.273
Subcortical	-0.438	-0.251	0.281
Thalamus	-0.566	-0.254	0.255
Striatum	-0.505	-0.193	0.261
Putamen	-0.532	-0.250	0.294
Caudate	-0.039	0.171	-0.172
Globus pallidus	-0.468	-0.117	0.462
Anterior cingulate	-0.583	-0.382	0.350
Hippocampus	-0.246	-0.022	0.101

*[Legend Table 4.8: Results of correlation analysis between PBR28 binding in corresponding brain regions and frontal white matter cerebral metabolites. * $P < 0.05$, ** $P < 0.1$, NAA= N-acetylaspartate, Cho=Choline, MI=myoinositol, Cr=Creatine; 33 correlation coefficients were calculated ($[^{11}\text{C}]$ PBR28 $_{(DVR)}$ values from 11 brain regions multiplied by values from 3 CMRs in the FWM)*

Chapter 5

**Cross-sectional analysis of the relationship
between microglial activation and cerebral
inflammatory biomarkers in HIV-infected
individuals on cART**

Chapter 5: Cross-sectional analysis of the relationship between microglial activation and blood and CSF inflammatory biomarkers in HIV-infected individuals on cART

5.1 Introduction	129
5.2 Methods.....	131
5.2.1 Statistical analysis	131
5.3 Results	131
5.3.1 Soluble CD14.....	131
5.3.2 Soluble CSF CD163.....	131
5.3.3 Ribosomal 16s.....	132
5.3.4 Plasma and CSF chemokines.....	132
5.3.5 Assessment of plasma and CSF biomarkers of individual HIV-infected participants enrolled in the study.....	134
5.4 Discussion	134

5.1 Introduction

Chronic immune activation is the hallmark of progressive HIV-disease, and it is likely to play a pivotal role in the pathogenesis of HIV-associated NCI (117). Plasma and CSF biomarkers of monocyte activation and microbial translocation can provide important information about the relationships between chronic inflammation, gut disease and NCI in HIV-infected individuals on cART. Although, there are no specific immune activation biomarkers of HIV-associated NCI in treated HIV-infected individuals, several candidates linked to monocyte activation and microbial translocation have been proposed as biomarkers for the assessment of milder and severe forms of HIV-associated NCI.

Plasma soluble sCD163, a product of the hemoglobin scavenger receptor CD163 that is expressed exclusively by monocytes and macrophages of the M2 phenotype (260, 261) has been suggested as novel marker of monocyte activation in HIV-disease. sCD163 is produced by the shedding of CD163 following monocyte activation. Increase levels of sCD163 in plasma have been found to correlate with HIV RNA prior to and after cART in both individuals with early and chronic HIV-infection (262). Sustained elevation of plasma sCD13 compared with HIV-negative controls has been associated with NCI in virologically suppressed HIV-infected individuals (262), and higher plasma sCD163 was specifically associated with impairments in the domains of executive function and learning, two of the most common and consistent areas of cognitive impairment in the era of cART (263).

Soluble CD14 (sCD14) is another monocyte activation marker that is produced by the shedding CD14, a glycoprotein expressed on the surface of monocytes and macrophages. Monocyte activation leads to increase release of sCD14 from monocytes, particularly after stimulation of circulation products of microbial translocation such as bacterial lipopolysaccharide (LPS) (264). Elevated levels of sCD14 correlate with HIV-related chronic immune activation and disease progression (265, 266). sCD14 has been associated with NCI in HIV-infected individuals with advanced disease and detectable HIV RNA on cART (267).

Microbial translocation is characterised by the translocation of microbial products derived from the gastrointestinal tract of HIV-infected individuals to the bloodstream. Circulating microbial products have been found to contribute to HIV-related systemic immune activation (117). Concentrations of plasma LPS, a biomarker of microbial translocation has been associated with HIV-infection, immune activation and HAD (116, 117). The relationship between LPS-induced systemic inflammation and microglial activation has been evaluated in an animal model where baboons were scanned with [^{11}C] PBR28 PET CT before and after intravenous administration of LPS. LPS administration increased [^{11}C] PBR28 binding, and this effect was mediated by an increase in inflammatory chemokines such as IL-6 and IL-1 β (268).

LPS is only present on a proportion of enteric bacterial microbiota (gram-negative bacteria) (117) and for this reason quantification of bacterial DNA encoding the ribosomal 16S rRNA gene has been proposed as a more sensitive biomarker of microbial translocation than LPS (269). Plasma levels of bacterial 16S rDNA in individuals have been found to be significantly higher in HIV-infected individuals, and they correlated with plasma LPS levels (270). In HIV-infected individuals, higher levels of plasma ribosomal 16S rDNA are also associated with increased T-cell activation (270).

Brain chemokines and their receptors play an important role in many physiological and inflammatory processes and altered expression of α and β chemokines have been documented in HIV-infected individuals with HIV-associated NCI (summary Table 1.2, Chapter 1).

Studies of biomarkers for HIV-associated brain disease have focused on investigating relationships between immune activation markers and cognitive testing parameters. Few studies have examined the associations between immune activation biomarkers and cerebral imaging parameters of structural metabolic or functional disease (271, 272).

To investigate the second hypothesis of this thesis, this study aimed to examine the relationship between *in vivo* microglial activation parameters as measured by [^{11}C]

PBR28 PET CT and biomarkers of monocyte and microbial translocation in CSF and blood of neuroasymptomatic HIV-infected individuals on effective cART.

5.2 Methods

Blood and CSF biomarker analyses were performed as described in section 2.5.

5.2.1 Statistical analysis

Associations between plasma/CSF biomarker concentrations and [^{11}C] PBR28 V_T and HIV-related clinical parameters were performed using partial correlation analysis adjusted for TSPO affinity status. Multiple comparisons were corrected by bootstrapping (252). P values below 0.05 were considered statistically significant.

Each HIV-infected participant was examined separately to examine the clinical characteristics and biomarker profile of HIV-infected individuals with greater PBR28 binding.

5.3 Results

10 HIV-infected individuals agreed to have a lumbar puncture. All HIV-infected participants consented for blood sampling (n=12). Descriptive statistics for the plasma and CSF biomarkers evaluated are presented in Table 5.1.

Biomarker concentrations for each HIV-infected case enrolled in the study are presented in Table 5.2

5.3.1 Soluble CD14

No association between plasma or CSF sCD14 levels and [^{11}C] PBR28 $V_{T(DVR)}$ was observed in any brain region. Similarly no associations between plasma or CSF sCD14 levels and any HIV-related clinical parameters were found ($P > 1$).

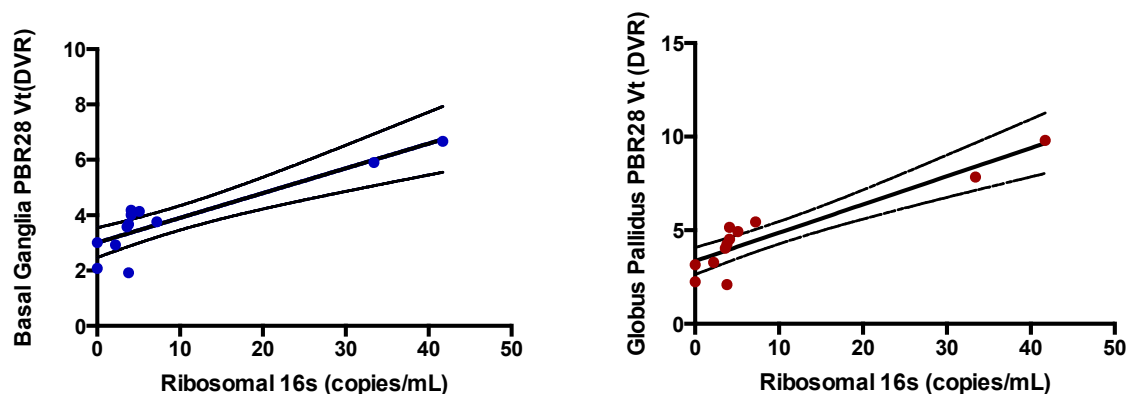
5.3.2 Soluble CSF CD163

No association between CSF sCD163 levels and [^{11}C] PBR28 $V_{T(DVR)}$ was observed in any brain region. No associations between sCD163 in the CSF and other imaging biomarkers or HIV-related clinical parameters were observed ($P > 1$)

5.3.3 Ribosomal 16s

A significant association between greater concentration of ribosomal 16s in plasma and increase $[^{11}\text{C}]$ PBR28 $V_{T(\text{DVR})}$ was observed in brain regions with significant microglial activation including the basal ganglia and the globus pallidus, Figure 5.2 and Table 5.2. Plasma ribosomal 16s was also correlated with an increase in ml/Cr ($r=0.682$; $P=0.01$) in the RBG and greater mean diffusivity in the forceps major ($r=0.532$; $P=0.07$), right inferior longitudinal fasciculus ($r=0.601$; $P=0.03$) and right inferior fronto-occipital fasciculus ($r=0.509$; $P=0.09$) on DTI, see Figure 5.3

Figure 5.2. Scatter plot of correlation between basal ganglia and globus pallidus $[^{11}\text{C}]$ PBR28 $V_{T(\text{DVR})}$ and concentration of plasma ribosomal 16S DNA in HIV-infected individuals stable on cART



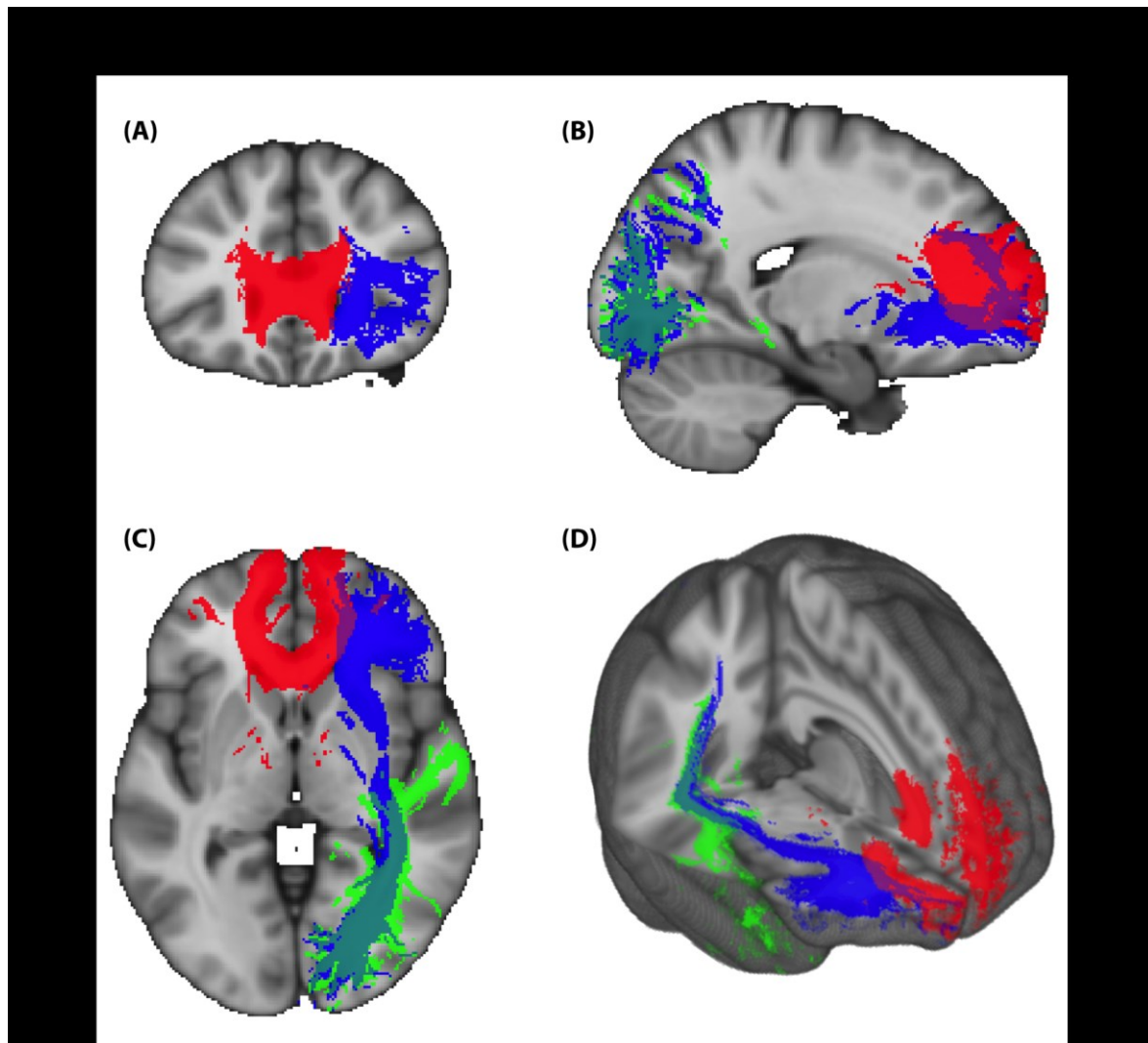
[Legend Figure 5.2. Scatter plot shows the correlation between concentration of ribosomal 16s DNA and PBR28 binding in two brain regions of HIV-infected subjects]

No correlations between ribosomal 16s in CSF and $[^{11}\text{C}]$ PBR28 $V_{T(\text{DVR})}$ in any brain region were observed. Similarly, no associations between ribosomal 16s in CSF and other imaging biomarkers and HIV-related clinical parameter were observed ($P \geq 1$).

5.3.4 Plasma and CSF chemokines

Three (Eotaxin-3, MIP- β , IL-8) out of nine chemokines measured in plasma and CSF were within the detection range of the assay and therefore available for correlation analyses.

Figure 5.3 DTI map showing the MD of brain regions that correlated positively with plasma ribosomal 16s



[Legend Figure 5.3. This figure represents the mean diffusivity (MD) map of three brain regions that correlated positively with plasma concentrations of ribosomal 16s; red corresponds to the forceps major, green is the right inferior longitudinal fasciculus and blue is the right inferior fronto-occipital fasciculus: a. coronal view, b. sagittal view, c. axial view, d. three dimensional view]

Concentrations of IL-8, eotaxin-3, and MIP- β in both the CSF and plasma are presented in Table 5.1. No significant correlations between levels of individual chemokines in plasma and CSF were identified. Significant associations between increase concentrations of eotaxin-3, MIP- β , IL-8 in the CSF, and [^{11}C] PBR28 $V_{\text{T(DVR)}}$ in brain regions with increased microglial activation in the PET study were observed

(Table 5.2). Increase concentrations of IL-8 and MIP- β in the CSF were also associated with greater levels of ml/ Cr in the RBG (IL-8; $r=0.610$; $P=0.06$), (MIP- β ; $r=0.639$; $P=0.04$)

Interestingly, there was a significant positive correlation between concentrations of plasma ribosomal 16s I, a marker of microbial translocation and concentrations of the proinflammatory chemokine IL-8 in the CSF ($r=0.599$; $P=0.024$, $CI=0.152$ to 0.956) No associations between plasma or CSF chemokines and other imaging biomarkers and HIV-related clinical parameters were observed ($P=>1$).

5.3.5 Assessment of plasma and CSF biomarkers of individual HIV-infected participants enrolled in the study

When evaluating plasma and CSF biomarkers data from each HIV-infected individual involved in the study, subjects 06 and 11 had increase concentrations of CSF IL-8 and plasma ribosomal 16s compared to other HIV-infected participants. Subject 06 also had the highest concentration of eotaxin-3, MIP- β and sCD163 in the CSF. Interestingly, subjects 06 and 11 had the greatest levels of PBR28 binding in the PET study compared to other HIV-infected participants.

5.4 Discussion

This study attempted to investigate the association between plasma and CSF biomarkers of inflammation and microbial activation using [^{11}C] PBR28 CT PET. A significant correlation between increase plasma ribosomal 16s, a marker of microbial translocation and greater microglial activation in most brain regions was observed suggesting an effect of gut microbial translocation on cerebral immune activation. This finding became more significant after observing a positive correlation between plasma ribosomal 16s and MD in several white matter tracts on DTI.

Hannestad et al. observed an increase in levels of TSPO detected with [^{11}C] PBR28 PET after intravenous administration of *E.coli* lipopolysaccharide (LPS), a marker of microbial translocation using a nonhuman primate brain model. The study demonstrated that systemic inflammation is associated with microglial activation in the brain of nonhuman primates. The increase in microglial activation after LPS correlated with increase inflammatory cytokine levels, suggesting that microglial activation was mediated by LPS-induced inflammatory cytokines such as IL-6 and IL-

1 β (268). In the study presented in this chapter, I found a significant correlation between plasma ribosomal 16s and levels of the inflammatory chemokine IL-8, which are in agreement with the findings by Hannestad et al. where microglial activation was induced by bacterial byproducts through mediation of inflammatory chemokines.

This evidence suggests that translocation of bacterial byproducts in the systemic circulation through the damaged gastrointestinal barrier may contribute to immune activation in chronic HIV-infection, and high levels of microbial translocation markers such as LPS have been associated with HAD independently of plasma viral load and CD4 counts (116). Interestingly, the association between plasma levels of ribosomal 16s and increase MD in white matter tracts further suggests a potential effect of inflammation on white matter integrity.

Although, I did not observe any correlation between sCD14 and CSF sCD163 and microglial activation, increased concentrations of IL-8, eotaxin-3 and MIP- β in the CSF were significantly associated with brain regions where microglial activation was identified.

This study supports my second hypothesis, that microglial activation is associated with biomarkers of inflammation. Furthermore, this pilot study has shown an association between systemic inflammation, microglial activation, and markers of injury. This suggests a model whereby microglial activation is driven by systemic inflammation, which in HIV-infected individuals is maintained by the translocation of microbial products from an impaired gut-blood barrier.

The model is consistent with immune activation mediated injury via non-specific activation (273) rather than HIV infection in the privileged CNS compartment. Perry et al. described the molecular and cellular components that mediate the communication between peripheral inflammation and the brain, all which lead to the production of cytokines/chemokines and inflammatory mediators, which are associated with neuronal injury (74). Systemic inflammatory markers could provide indices to guide risk-assessment and treatment of HIV-associated brain disease

Table 5.1 Descriptive statistics of plasma and CSF biomarkers for HIV-infected individuals

Biomarker (units)	CSF Mean (SD)	Plasma Mean (SD)
sCD14 (ng/mL)	120 (0.03)	1480(0.29)
sCD163 (ng/mL)	34.1 (12)	N/A
Ribosomal 16s (copies/mL)	4.7(2.8)	10.9(14.2)
IL-8 (ng/mL)	0.028 (4.6)	0.0031 (1.3)
Eotaxin-3 (ng/mL)	0.1295 (11.7)	0.112 (51)
MIP- β (ng/mL)	14.622 (18)	57.621(26)

[Legend Table 5.1: Mean results for biomarkers measured in both plasma and CSF in HIV-infected individuals]

Table 5.2 Correlation coefficients (r) between [¹¹C] PBR28 V_{T(DVR)} and plasma /CSF biomarkers adjusted for TSPO affinity status

Biomarker	Basal ganglia	Globus pallidus	Temporal lobe	Parietal lobe	Occipital lobe	Caudate	Striatum	Medulla	Midbrain
Ribosomal 16s (plasma)	0.881*	0.928*	0.818*	0.832*	0.861*	0.877*	0.871*	0.645*	0.811*
IL-8 (CSF)	0.727*	0.801*	0.766*	0.722*	0.761*	0.847*	0.692*	0.734*	0.736*
MIP-B (CSF)	0.688*	0.696*	0.749*	0.728*	0.731	0.682**	0.444	0.653*	0.730**
Eotaxin (CSF)	0.668**	0.622**	0.603**	0.650**	0.641**	0.499**	0.586**	0.661**	0.691**

*[Legend Table 5.2: Results of correlations between PBR28 binding and biomarkers in the CSF and plasma in HIV-infected individuals. *P<0.05, ** P<0.1; 22 correlation coefficients calculated per biomarker with the exception of sCD163 (each biomarker in plasma and CSF multiplied by [¹¹C] PBR28 V_{T(DVR)} values from 11 brain regions)]*

Table 5.3 Plasma and CSF biomarkers results for each HIV-infected participant enrolled in the study

Study case	CD4/CD8 ratio	Pre-treatment HIV RNA (copies/mL)	sCD14 CSF (ng/mL)	sCD14 blood (ng/mL)	sCD163 CSF (ng/mL)	Ribosomal 16s blood (copies/mL)	IL-8-CSF (ng/mL)	Eotaxin-3-CSF (ng/mL)	MIP- β -CSF (ng/mL)
01	0.70	n/a	0.1	1.27	26.78	3.6	0.023	0.148	11.03
02	0.82	69388	0.13	1.29	34.32	0	0.034	0.142	12.52
03	0.74	151	0.06	1.5	18.24	4.1	0.025	0.124	15.51
04	0.75	57998	0.12	1.44	44.02	3.8	0.026	0.032	8.89
05	0.36	261970	0.11	2.3	30.48	7.2	0.057	0.174	13.01
06	0.72	1624782	0.18	1.26	59.71	41.7	0.065	0.180	23.53
07	0.72	35000	0.09	1.64	27.34	5.1	0.034	0.165	9.71
09	0.70	38529	0.09	1.23	37.99	0	0.029	0.026	15.81
10	0.50	422457	n/a	1.45	n/a	4.1	0.03	n/a	n/a
11	0.67	187890	0.08	1.34	22.14	33.4	0.053	0.237	12.93
12	0.15	n/a	n/a	1.45	n/a	3.8	n/a	n/a	n/a
13	0.40	500000	0.06	1.65	40.13	2.2	0.07	0.039	19.69

[Legend Table 5.3: Subjects with higher PBR28 binding such as subjects 06 and 11 had increased concentration of IL-8-CSF and blood ribosomal 16s]

Chapter 6

Cerebral inflammatory chemokines in cynomolgus macaques infected with SIVmac251

Chapter 6: Cerebral inflammatory chemokines in cynomolgus macaques infected with SIVmac251

6.1 Introduction	142
6.2 Methods.....	143
6.2.1 Statistical analysis	144
6.3 Results	144
6.3.1 Plasma chemokine changes following SIVmac251 infection	145
6.3.2 CSF chemokine changes between SIV-infected and naïve macaques.....	145
6.3.3 Correlation between plasma chemokines and CSF chemokines during acute and chronic SIV-infection.....	145
6.3.4 Correlation between plasma SIV-RNA viral load and plasma/CSF chemokines during acute and chronic SIV-infection.....	145
6.4 Discussion	148

6.1 Introduction

SIV-infection of non-human primates can be used as models of systemic and neurological manifestations of HIV-disease in humans (274). The most frequently utilised models to investigate brain pathology associated with HIV-infection in humans induce rapid development of disease and neuropathology through infection of pig tail macaques (*Macaca nemestrina*) with either a SIV-infection in combination with CD8 T-cell depletion or co-inoculation of the immunosuppressive SIVdeltaB670 with the neurovirulent SIVmac17E-Fr (45, 275, 276). However, since the advent of cART, the rapid development of severe neurological disease is not representative of the current HIV-associated neurological phenotype characterized by mild cognitive impairment in HIV-infected individuals on effective cART. Therefore, a model displaying a more gradual disease development may be more suitable to study the pathogenesis of HIV-associated brain disease in the modern antiretroviral era.

Unlike the rhesus (*Macaca mulatta*) and pig tale macaque models of neuropathology using single strain SIV-infection of cynomolgus macaques (*Macaca fascicularis*), without immunosuppression, results in a more slowly developing neuropathology. A recent study by Clarke et al. demonstrated that non-accelerated infections of cynomolgus macaques with SIV-strains (including non-neurotropic strains) can induce pathological changes in the brain despite limited peripheral viral replication or evidence of disease progression (50). In addition, the viral replication kinetics of this model are characterised by a low peripheral viral set point (when SIV RNA levels reach a plateau after the acute phase) following the initial replicative burst (maximum SIV RNA levels measured within the first 2 months after inoculation) (276) with low CSF viral loads (50, 277). The observed neuropathology in cynomolgus macaques infected with both neurotropic and non-neurotropic SIV-strains is that of (277) absence of multinucleated giant cell formation, similar to the neuropathology observed in HIV-brain disease in HIV-infected patients on cART.

Very little is known about blood and CSF chemokine responses in cynomolgus macaques infected with SIV. Microglial activation is associated with the release of inflammatory chemokines and other immune mediators. Chemokines and chemokine

receptors play an important role in orchestrating the immune responses following brain infection with HIV. The longitudinal evaluation of chemokines in blood and CSF in this model of SIV-infection could provide important information about the immune responses associated with microglial activation following early HIV-infection of the brain.

The aim of this study was to test the second hypothesis of this thesis, that microglial activation is associated with disturbances in biomarkers of inflammation. To test this hypothesis, I examined the concentrations of inflammatory chemokines in CSF and blood from a cynomolgus model of SIV-infection

6.2 Methods

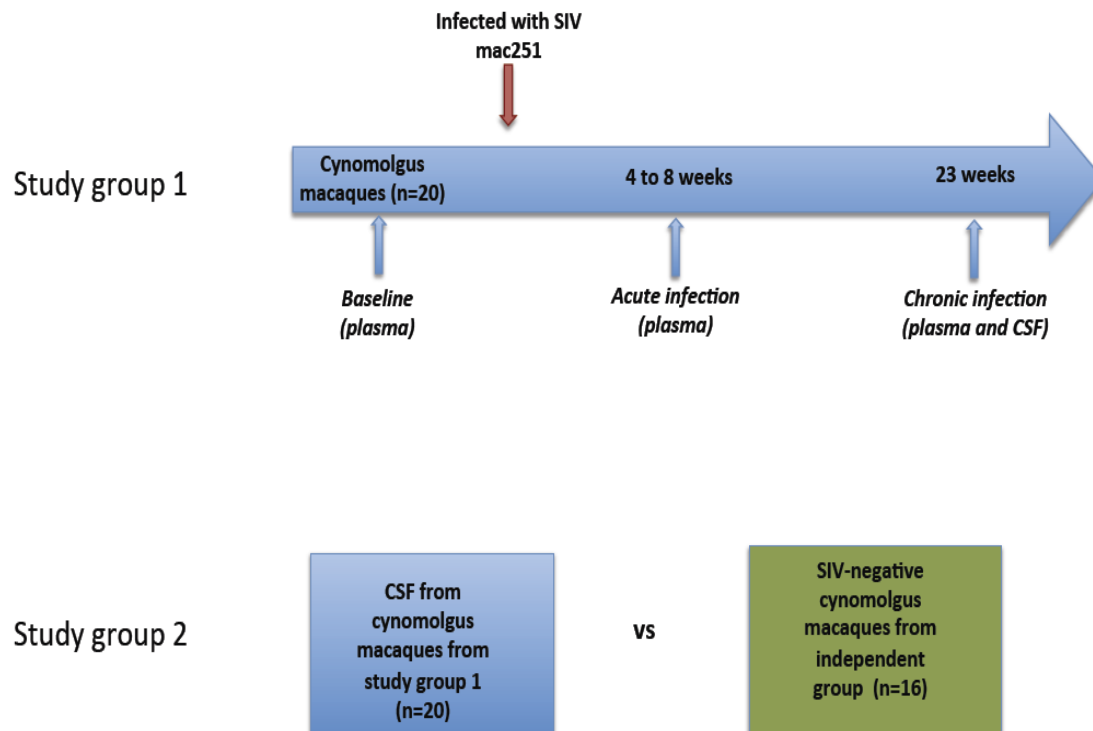
This study consisted of two different study groups. Study group 1: plasma samples at baseline (before inoculation of SIVmac251), during acute infection (between weeks 4 and 8 post detectable infection) and at termination (chronic infection, 23 weeks post-infection) were examined. Study group 2: CSF samples from the study group 1 were collected 23 weeks after infection (at termination, during necropsy) and compared with samples of CSF from a different group of SIV-naïve animals (n=16), see Figure 6.1.

Infection of animals was undertaken at the National Institute of Biological Standards and Control (Hertfordshire, UK) by Dr Deborah Fergusson. Following a SIV-gag based intra dermal immunisation protocol, where 20 animals received a single atraumatic low dose intra-rectal inoculation of SIVmac251 ($150_{\text{TCD}_{50}}$) on a weekly basis for up to 10 weeks. Blood samples were monitored weekly for presence of virus via quantitative real-time reverse transcriptase PCR, as described previously (276) and following a positive result the animal removed from the inoculation schedule. SIV replication was detected in brain tissue by *in situ* hybridisation in all animals challenged with SIVmac251.

I performed the chemokine analysis at Imperial College London, St Mary's Campus. Chemokines (IL-8, eotaxin, IP 10, MCP-1, MCP-4, and MDC) were analysed using human ELISA ultrasensitive Multiplex platform (MSD Meso Scale, USA) as described in section 2.5.3. IL-8, eotaxin, IP-10, MCP-1 and MDC were selected because they

have been associated with regulation of HIV-disease and cognitive impairment in HIV-infected individuals (45, 278-281)

Figure 6.1 Study design



6.2.1 Statistical analysis

Wilcoxon matched pairs rank test was used to determine differences in chemokine concentrations in plasma at baseline, acute and chronic stages of SIV-infection. To determine differences between CSF chemokines in SIV-infected and uninfected macaques a Mann Whitney *U*-test was employed. Associations between plasma and CSF concentrations and relationship to SIV-RNA levels were evaluated using Pearson's correlation analysis. P values below 0.05 were considered statistically significant.

6.3 Results

All vaccinated animals became infected with SIVmac251 during the 10-week challenge period. All measured chemokines were within the detectable range in the

plasma and CSF specimens at all-time points (Table 6.1). Median plasma SIV-RNA (copies per mL) during acute infection was 1.87×10^5 (range: 3.25×10^2 to 4.25×10^7) and at termination was 6.2×10^3 , (range: 0 to 3.04×10^5). CSF SIV-RNA measurements were not available for analysis.

6.3.1 Plasma chemokine changes following SIVmac251 infection

Significant changes in plasma concentrations of IL-8, MCP-1, MCP-4 and eotaxin were observed in cynomolgus macaques following SIV infection (Figure 6.2). Greater concentrations of eotaxin and MCP-4 and lower levels of MCP-1 and IL-8 were seen at termination compared with baseline. No changes in plasma concentrations of IP-10 or MDC were observed between baseline and acute or chronic stages of SIV-infection (Table 6.2)

6.3.2 CSF chemokine changes between SIV-infected and naïve macaques

There were significant differences in concentrations of all chemokines between SIV-infected and naïve macaques. Greater concentrations of IL-8 and lower concentrations of eotaxin, MCP-4, MDC, MCP-1 and IP-10 were observed in SIV-infected animals compared to controls (Figure 6.3).

6.3.3 Correlation between plasma chemokines and CSF chemokines during acute and chronic SIV-infection

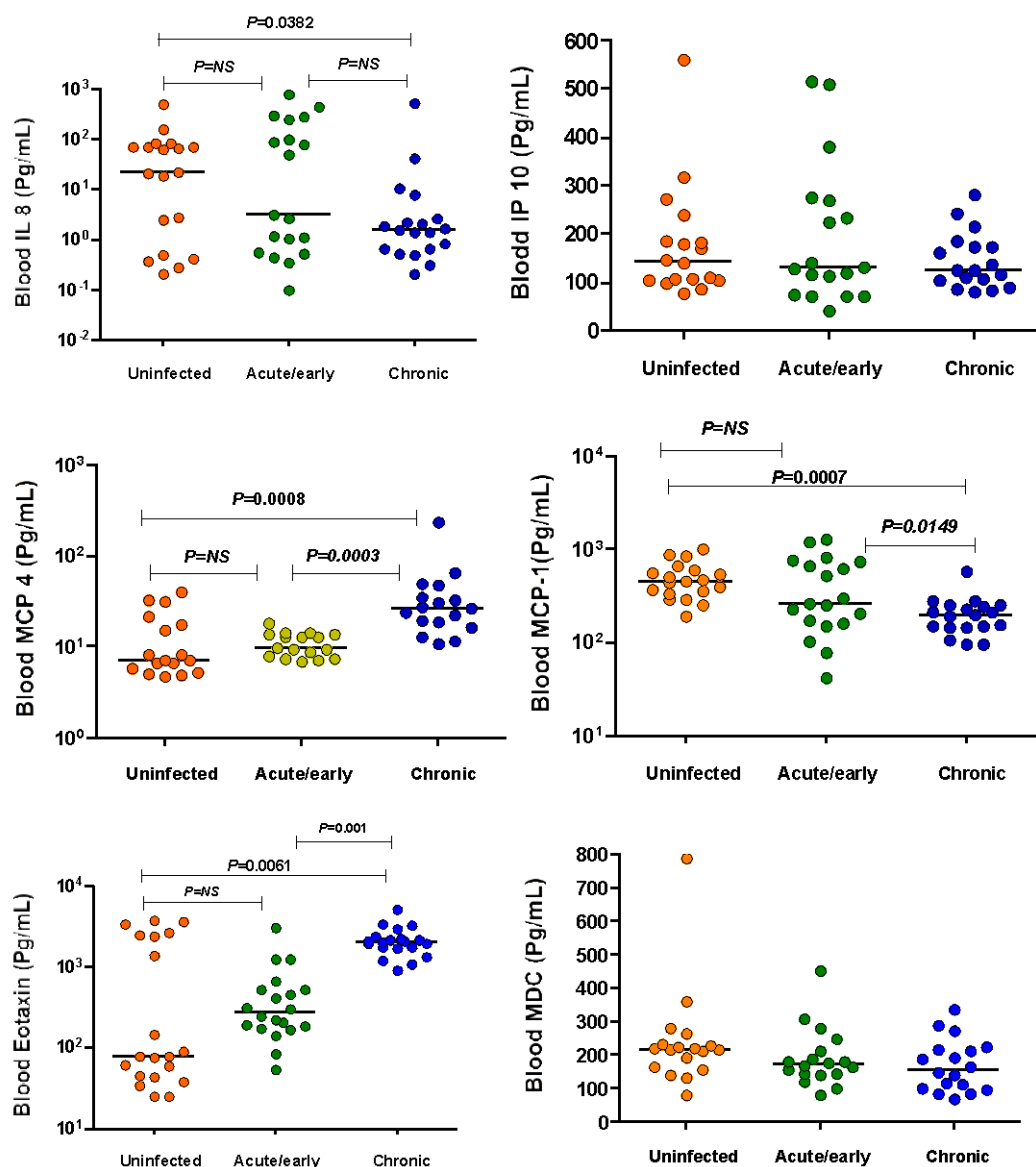
Greater concentrations of plasma eotaxin and IP-10 at termination were associated with high concentrations in the CSF (eotaxin, $r=0.492$, $P=0.033$); (IP-10: $r=0.568$, $P=0.009$). No other associations between plasma and blood chemokine concentrations were observed ($P>1$)

6.3.4 Correlation between plasma SIV-RNA viral load and plasma/CSF chemokines during acute and chronic SIV-infection

During acute SIV-infection, there was a significant correlation between plasma SIV-RNA viral load and plasma concentration of IP-10, IL-8 and MCP-1 with higher SIV-RNA viral load associated with higher concentration of IP -10 ($r=0.492$, $P=0.033$), IL-8 ($r=0.551$, $P=0.012$) and MCP-1 ($r=0.404$, $P=0.077$). During chronic SIV infection there was a significant positive correlation between plasma SIV-RNA viral load and plasma concentration of IP-10 ($r=0.590$, $P=0.006$) at termination. Finally, no

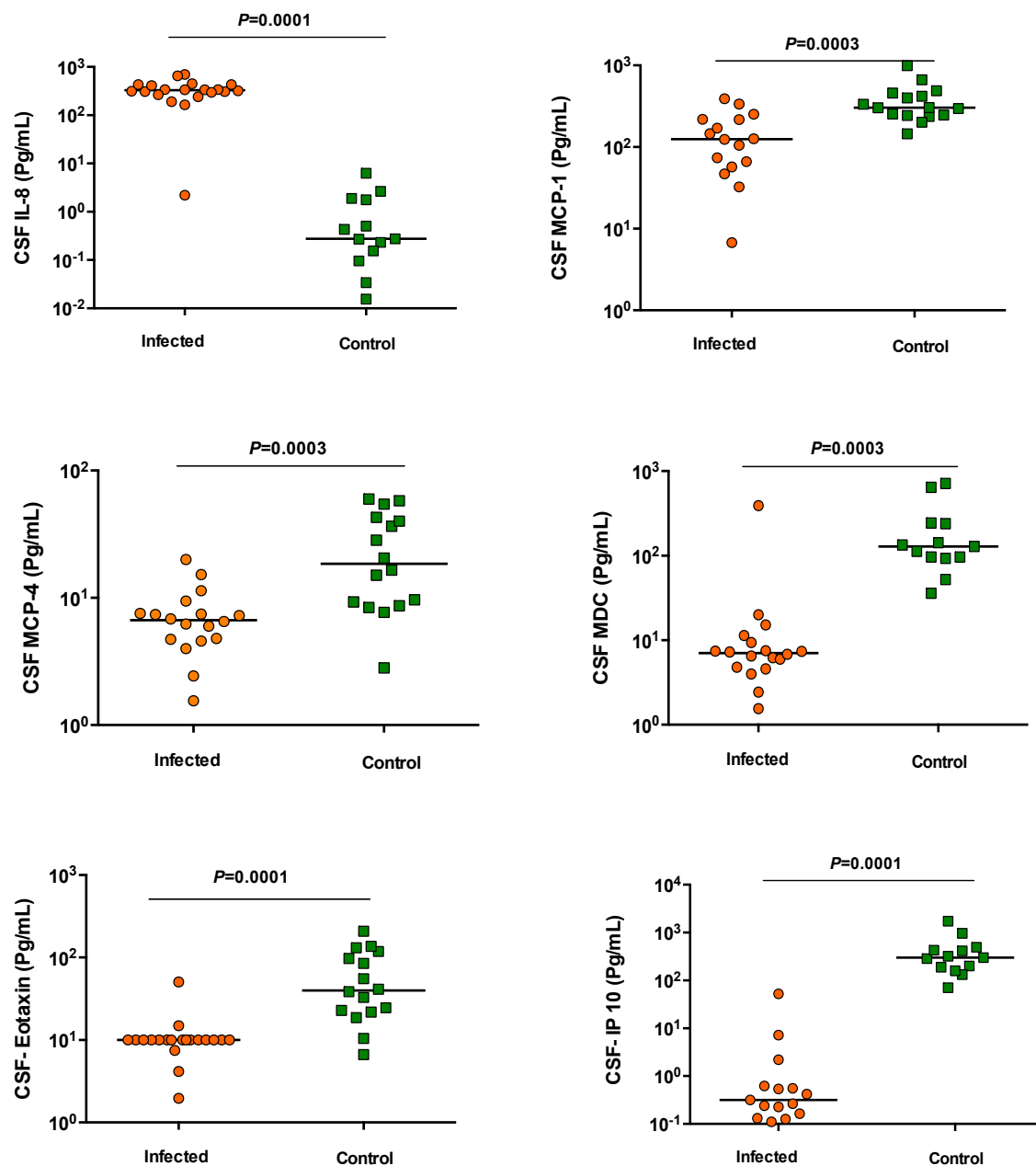
associations between plasma SIV RNA viral load at termination and other CSF chemokines were observed.

Figure 6.2 Plasma chemokines in Cynomolgus macaques infected with SIVmac251.



[Legend Figure 6.2: Plasma levels of chemokines were determined before and after infection (acute and chronic/termination). P value was calculated by the Wilcoxon matched-pairs test

Figure 6.3 CSF chemokine concentrations in *Cynomolgus* macaques.



[Legend Figure 6.3: CSF levels of chemokines were determined in infected and uninfected individuals. P values were calculated by the Mann Whitney U-test]

6.4 Discussion

In order to investigate the second hypothesis of this thesis, this cross-sectional study explored changes to plasma and CSF inflammatory chemokines associated with microglial activation in cynomolgus macaques infected with SIVmac251 during different stages of SIV-infection. This is the first study investigating changes in biomarkers of microglial activation in the SIV/cynomolgus macaque model of CNS infection. Overall, lower levels of inflammatory chemokines in CSF and plasma (eotaxin, IP-10, MCP-1, MCP-10 and MDC) were observed in SIV-infected cynomolgus macaques compared with uninfected controls.

There are several possible explanations for these results. First, as explained in the introduction section of this chapter, this CNS model is characterised by mild CNS disease (171), thus, it is possible that although these animals were systemically infected with SIV, and SIV replication was detected in brain tissue by *in situ* hybridisation in all animals challenged with the SIVmac251, microglial activation was not present. However, microglial activation in the brains of cynomolgus macaques infected with SIVmac251 has been demonstrated in other studies (171, 282); therefore, it is likely that in this model some degree of microglial activation was present

The other possibility is that the microglia of these macaques are exhibiting an antiinflammatory, neuroprotective phenotype as microglia can display different activation phenotypes in response to brain injury. The presence of an antiinflammatory phenotype chemokine profile in this cohort of SIV-infected macaques is evidenced by the low concentrations of CSF inflammatory chemokines associated with monocyte activation (eotaxin, IP-10, MCP-1) and the increase concentrations of anti-inflammatory mediators such as IL-8. In SIV-disease increases of IL-8 and eotaxin have been associated with resistance to SIV-infection (283). Also there is evidence that inflammatory chemokines can modulate each other. Eotaxin is a natural ligand of the CCR3 receptor and an antagonist for the CCR5 and CCR2 receptors (284). CCR2 is a major proinflammatory chemokine receptor expressed by macrophages, including microglia. Eotaxin has been shown to block monocytes responses to MCP-1 a major ligand for CCR2, and to interfere with HIV-entry into microglia (285). In this context, it is possible that eotaxin may have a modulatory role in inflammation through CCR2, controlling the expression of MCP-1 and MCP-4 as it

was seen in the plasma of infected animals following SIV-infection. Finally, greater concentrations of plasma IP-10 correlated well with CSF IP-10 and SIV-RNA during acute and chronic stages of SIV-infection, suggesting the potential of this chemokine as a biomarker of monocyte activation.

Finally, it is also possible that chronic immunosuppression and the breakdown of the gut-blood barrier are needed to reproduce the finding in humans. Future work should include the measurement of markers of microbial translocation such as ribosomal 16s in NHP models of CNS infection.

This study has several limitations. First, CSF-SIV RNA measurements were not available; therefore, it was not possible to evaluate the association between CSF chemokines and CSF-SIV viral load. Secondly, some of these animals took part in studies evaluating novel HIV vaccines that could have altered the immune responses to SIV within the brain. It is important to state that all the vaccine candidates tested on these animals were unsuccessful in effectively controlling plasma SIV RNA and as a result the effect that they could have in the brains of the macaques is likely to be minimal. Third, although the neuropathology of this animal model is similar to the one found in treated HIV-infected patients, it is not reflective of the clinical situation observed in patients on cART as most patients on treatment have undetectable HIV RNA during chronic infection.

In summary, in this cynomolgus SIV-model, I observed significant differences in CSF and plasma proinflammatory chemokines. The chemokine pattern described here, suggests a degree of disease control by the host, possibly limiting CNS injury. Immunologic or genetic variations that affect chemokine levels might also affect the susceptibility of the brain to HIV-infection and the rate of progression once the infection is established. This could explain why most of the inflammatory chemokines were not upregulated in infected primates.

These findings have given me an insight into the mechanisms involved in the fine-tuning modulation of CNS monocyte chemokine expression and activation in the presence of SIV-infection. Also, this study supports my second hypothesis, that microglial activation is associated with changes to CNS biomarkers of inflammation.

The cynomolgus macaques animal model described in this chapter displays inflammatory changes, and gradual neuropathology similar from those reported in patients on cART following infection with attenuated SIV-strains. The development of novel attenuated molecular clones derived from SIV-strains that are dependent upon the presence of an antibiotic switch to replicate, could potentially be used to investigate associations between viral replication and microglial activation (286) in a NHP model that reflects both the neuropathological and neuroinflammatory changes of patients with HIV-associated brain disease.

Future work is currently undergoing to examine the expression of TSPO 18kda in brain tissue of the cynomolgus macaques involved in this study. This can be done either by autoradiography or immunoblotting using anti-TSPO antibodies (287). In this study, animal samples were fixed with formaline, which makes brain tissue unsuitable for autoradiography analyses. However, immunoblotting with anti-TSPO is possible with fixed brain samples. These experiments will provide important information about the degree of expression of TSPO in cynomolgus macaques and the relationships between plasma/CSF chemokine concentrations and expression of TSPO.

Table 6.1. Descriptive statistics of CSF chemokine data

Chemokines	SIV-infected		Control	
	Median (IQR)	% Detectable	Median (IQR)	% Detectable
Eotaxin	24 (1.8-260)	90%	3.17 (1.2-20)	70%
IP-10	0.317 (0.1-52)	75%	299 (70-1730)	75%
IL-8	329 (2.2-694)	100%	0.2 (0.1-6.304)	75%
MCP-1	125 (6.7-390.5)	80%	303(145-994)	90%
MCP-4	6.67 (1.5-20)	90%	18.5 (2.8-59.7)	75%
MDC	7.04 (1.5-390)	90%	128 (36-713)	70%

[Legend Table 6.1: Median (Pg/mL), IQR (interquartile range)]

Table 6.2 Chemokine profiles of cynomolgus macaques following SIVmac251 infection

Chemokine assessed	Acute SIV infection (plasma)	Chronic SIV infection (plasma at termination)	¹ CSF (at termination)
Eotaxin	↑	↑ ↑	↓
MCP-1	↓	↓	↓
IP-10	-	-	↓
MDC	-	-	↓
MCP-4	-	↑	↓
IL-8	-	↓	↑

[Legend Table 6.2: Chemokine profiles during acute and chronic infection when compared with chemokine concentrations from the same macaques before infection with SIVmac251.

¹ CSF: chemokine profile in the CSF when compared to chemokine concentrations from HIV-negative macaques]

Chapter 7

Discussion

Chapter 7: Overall conclusions

7.1 Conclusions	156
7.2 First Hypothesis	156
7.3 Second Hypothesis	157
7.4 Third Hypothesis	160
7.5 Summary	161

7.1 Conclusions

It has been proposed that microglial activation is the main pathogenic mechanism for the development of HIV-associated brain disease in HIV-infected individuals on stable cART. This thesis sought to examine the following three hypotheses:

- 1. Microglial activation will be greater in neuroasymptomatic HIV-infected individuals stable on cART compared to HIV-negative controls**
- 2. Microglial activation in neuroasymptomatic HIV-infected individuals stable on cART will be associated with poorer cognitive function, increase biomarkers of inflammation and abnormalities of functional, structural and metabolic imaging parameters on cerebral MRI**
- 3. Microglial activation in neuroasymptomatic HIV-infected individuals stable on cART will be greater in subjects with low nadir CD4 counts compared to those with high nadir CD4 counts**

7.2 First Hypothesis

Microglial activation will be greater in neuroasymptomatic HIV-infected individuals stable on cART compared to HIV-negative controls

This hypothesis was tested in a clinical study assessing *in vivo* microglial activation in HIV-infected individuals stable on cART compared to HIV-negative controls. In Chapter 3, *in vivo* microglial activation was evaluated using cerebral PET imaging with [¹¹C] PBR28. This study found evidence of microglial activation in individuals with chronic HIV-infection stable on cART without cognitive or neurological symptoms, proving the first hypothesis. The signal for microglial activation was global involving both cortical and subcortical brain regions; however, the strongest signal was observed in subcortical brain regions such as the basal ganglia. Interestingly, this study also revealed another finding regarding expression of TSPO in HIV-infected individuals stable on cART. Although, overall there was greater microglial activation in the brain of HIV-infected individuals, I identified brain regions with lower microglial activation (temporal lobe, midbrain, and the right amygdala) compared to

HIV-negative individuals. This to my knowledge is the first time that a reduction of TSPO expression has been identified in the CNS of HIV-infected individuals stable on cART. Evidence of brain regions with both increased and reduced microglial activation suggests several possibilities. First, in response to HIV-infection, microglial activation expresses distinctive activation patterns (protective vs inflammatory) in different brain regions. Second, that HIV-infection may have a preference for specific brain regions such as the basal ganglia. The latter has been observed in neuropathological and imaging studies of HIV-infected individuals with HIV-associated brain disease (12, 228). Future work to assess the evolution of microglial activation in longitudinal studies involving both neurosymptomatic and asymptomatic HIV-infected individuals on cART are needed, as well as studies that can determine the type of microglial activation phenotype present in HIV-infected individuals with and without cART.

7.3 Second Hypothesis

Microglial activation in neuroasymptomatic HIV-infected individuals stable on cART will be associated with poorer cognitive function, increased biomarkers of inflammation and abnormalities of functional, structural and metabolic imaging parameters on cerebral MRI

To examine the second hypothesis a series of cross sectional studies were performed to evaluate the relationship between microglial activation measured by cerebral [^{11}C] PBR28 PET CT and functional, structural, metabolic, and immunological cerebral parameters in individuals with chronic HIV-infection stable on cART.

In Chapter 3, HIV-infected individuals participating in the [^{11}C] PBR28 PET CT study had neurocognitive testing to detect the presence of NCI. Although, HIV-infected individuals had no evidence of NCI, I found an association between poorer performance in cognitive tasks assessing verbal learning and memory and increased microglial activation in several brain regions including the hippocampus, amygdala, putamen and occipital lobe. Poor performance in tasks evaluating verbal learning and memory has been associated with abnormalities in subcortical brain networks

(288), and has been reported in HIV-infected subjects (31). The association between microglial activation in subcortical brain regions and poorer cognitive performance supports my second hypothesis and suggests that microglial activation could be detrimental to cognitive function and potentially lead to NCI.

Chapter 4, sought to further examine the second hypothesis evaluating the associations between microglial activation and structural, functional, and metabolic cerebral MRI parameters in stable HIV-infected individuals. HIV-infected subjects who participated in the [^{11}C] PBR28 PET CT study were investigated with a series of MRI imaging modalities including fMRI, DTI and MRS. The results of this study did provide evidence to support the second hypothesis as overall, greater microglial activation was associated with disturbances in brain activation, white matter integrity, and cerebral metabolism. Although, brain regions with an increase in microglial activation were not associated with disruption of resting brain connectivity networks, reduce brain activation in the basal ganglia was associated with increase microglial activation in the globus pallidus and the caudate.

Interestingly, this study also demonstrated a strong correlation between regions with greater microglial activation and increased mean diffusivity in all white matter tracts examined. Inflammation has been demonstrated to increase tissue water, directly affecting water diffusivity in animal models of brain inflammation (258). Water diffusivity disruption increases mean diffusivity and reduces fractional anisotropy (259) on DTI, and this pattern of white matter injury is consistent with the one observed in the study presented in chapter 4, and the pattern reported in DTI studies of HIV-infected individuals of stable cART (180).

Also in Chapter 4, MRS was used to assess the relationship between microglial activation and cerebral metabolites. Remarkably, increased microglial activation was associated with reduced ml/Cr ratio in the right basal ganglia. These results were unexpected as increases in ml/Cr are suggestive of cerebral inflammation. This result could be explained by the fact that the voxel investigated was placed in the right thalamus, which was a brain region that did not show evidence of greater microglial activation compared to controls. Similarly, it could indicate that microglial activation does not affect brain regions equally, with some brain regions more susceptible to the effects of inflammation than others.

Importantly, the results presented in Chapter 3 have several limitations. First, control data for fMRI and MRS analysis were not available; therefore, I could not determine if brain activation and cerebral metabolite concentrations in HIV-infected individuals were different from HIV-negative subjects. Second, multiple comparisons during correlation analyses can lead to errors of inference. To limit this effect I tried to adjust for multiple comparisons when appropriated. All these limitations must be considered when interpreting the data.

In Chapter 5 and 6 associations between microglial activation and biomarkers of inflammation were assessed in order to address the second hypothesis. In Chapter 5, blood and CSF biomarker analyses were performed in HIV-infected individuals who participated in the [^{11}C] PBR28 PET CT study. Interestingly, although no correlations between increase microglial activation and two markers of immuneactivation, sCD14 and sCD163 in plasma or CSF were observed, significant associations between plasma ribosomal 16s, a marker of microbial translocation and increased microglial activation were found. This is the first study to describe a direct association between *in vivo* microglial activation and microbial translocation in neuroasymptomatic HIV-infected individuals stable on cART. Microbial translocation from the gastrointestinal tract has been strongly associated with persistent immuneactivation in HIV-infected individuals (117) and HAD (116). I found that increase concentration of IL-8 in the CSF correlated positively with plasma ribosomal 16s. Thus, It is feasible that the effects of microbial translocation on microglial activation are mediated by circulating inflammatory chemokines.

Evidence from several studies supports the notion that systemic inflammation can lead to microglial activation. For instance, peripheral challenge with microbial products such as LPS has been found to induce microglial activation in animals and humans (268, 289). Several routes of communication between peripheral inflammation and the brain have been proposed. First, inflammatory markers such as cytokines or chemokines synthesised at the site of inflammation, enter the blood, and communicate with perivascular macrophages in areas that lack the BBB. The signals communicated can activate the microglia and spread through the CNS parenchyma (290). Second, inflammatory chemokines/cytokines or the microbial products

themselves can cross the blood brain barrier and activate directly the microglia (291). Finally, there are evidence that inflammatory events in the abdominal cavity can trigger signals from the vagal-nerve sensory system to the CNS, which can activate resident macrophages and astrocytes (292). Immuneactivation cause by the continuous release of microbial products from a disrupted gut-blood barrier could lead to persistent microbial translocation, neuronal injury, and eventually the development of HIV-associated NCI.

In chapter 6, a non human animal model was used to explore changes to plasma and CSF inflammatory chemokines associated with microglial activation in cynomolgus macaques infected with SIVmac251 during different stages of SIV-infection. In this study, I observed significant differences in CSF and plasma proinflammatory chemokine concentrations in SIV-infected animals compared to controls. Unexpectedly, I found lower concentration on inflammatory chemokines in SIV-infected animals compared with SIV-negative controls. This may be owed to the host exhibiting a degree of disease control, limiting neuronal injury, possibly with microglia expressing a neuroprotective instead of an inflammatory phenotype. It is also possible that there might not have been enough time for microglial activation to occur within the brain.

The studies presented in Chapters 5 and 6 paves the way to design future studies using imaging, and systemic inflammatory biomarkers to examine in detail the effects of microglial activation on brain function, and to develop new strategies to guide risk assessment and novel therapies for the management of HIV-associated brain disease in the cART era.

7.4 Third Hypothesis

Microglial activation in neuroasymptomatic HIV-infected individuals stable on cART will be greater in subjects with low nadir CD4 counts compared to those with high nadir CD4 counts

This hypothesis was tested in the clinical study presented in Chapter 3. Here, individuals with chronic HIV-infection without cognitive or neurological symptoms stable of cART undertook imaging with [¹¹C] PBR28 PET CT. HIV-infected subjects for this study were recruited with a range of nadir CD4 counts to be able to prove the

hypothesis. This study found no evidence to support the third hypothesis of the thesis. Nadir CD4 count was not associated with microglial activation in any of the brain regions examined. On the converse, microglial activation in several brain regions was correlated with lower CD4/CD8 ratio and greater pre-treatment HIV RNA. Lower CD4/CD8 indicates persistence of high circulating CD8 T cells, which can remain even after effective cART. In HIV-negative populations this immune risk profile is characteristic of immunosenescence (293, 294). In HIV-infected individuals a lower CD4/CD8 ratio is considered a strong predictor of disease progression (295), non-AIDS comorbidities (232) and persistent immuneactivation (231). Thus, it is possible that the strong correlation between microglial activation and lower CD4/CD8 ratios indicates an effect of systemic inflammation within the CNS. Measuring pre-treatment HIV RNA has been proposed as an indicator of HIV disease activity and a predictor of non-AIDS comorbidities (234, 235). In this study, I found an association between brain regions with increasing microglial activation and greater pre-treatment HIV RNA. This observation has not been described previously, and it is important as it might suggest a legacy effect, where high HIV RNA before treatment is established potentially leading to both persistent microglial activation and early brain injury. Although I found not evidence to support my third hypothesis, I have made interesting observations that have potential clinical implications for therapeutic strategies.

7.5 Summary

Mild forms of cerebral impairment affecting HIV-infected individuals on stable cART have become increasingly apparent in recent years and the reasons for its development are unclear. Microglial activation has been proposed as the main pathogenic mechanism associated with HIV-associated brain disease. In this thesis, I demonstrated that *in vivo* microglial activation is present in individuals with chronic HIV-infection on effective cART, without any cognitive or neurological deficit, and that microglial activation is associated with disturbances on cerebral function including, brain activation, white matter integrity, cerebral metabolites and cognitive performance. I also identified a correlation between biomarkers of microbial translocation and microglial activation. This association could be mediated by the expression of inflammatory chemokines, suggesting a link between peripheral inflammation and microglial activation in treated HIV-infected individuals. Individual

analyses of HIV-infected individuals revealed differences in the degree of PBR28 binding across subjects. Subjects with the greatest PBR28 binding were associated with increase levels of microbial translocation and CSF/plasma biomarkers of inflammation.

Although this thesis did not demonstrate a relationship between nadir CD4 T-cell counts and microglial activation, it showed a significant association with other markers of disease activity and progression such as pre-treatment HIV RNA and CD4/CD8 ratio.

It is possible that greater HIV viraemia both in the periphery, and the CNS compartment leads to an exaggerated microglial response, and this process is likely to occur early during the disease process. The microglia becomes primed by the systemic inflammation generated by HIV infection despite effective control of the HIV virus with cART. Persistent systemic inflammation in the presence of cART is a well recognised process in HIV disease (296), which is driven by many factors including the translocation of microbial products from the gut (297)

In this context, the exaggerated response of primed microglia to persistent systemic inflammation, might lead to disturbances in neuronal function, which contributes to the pathogenesis and progression of HIV-associated brain disease. Uncovering the mechanisms by which microglial activation affects the CNS of treated HIV-infected individuals is critical, as this could allow the identification of novel biomarkers for the early detection of patients at risk of HIV-associated NCI, and to develop therapeutics for the management of patients with established HIV-associate brain disease.

List of communications

Data contained in this thesis published in scientific journals or presented at scientific conferences are listed below

Publications:

Can antiretroviral therapy prevent HIV-associated cognitive disorders?

Curr Opin HIV AIDS, 2014. Jan;9(1):11-6

Winston A, Vera JH

Scientific conferences:

Poster presentations

Imaging neuroinflammation in HIV-1 infected individuals on stable cART with PET using [¹¹C] PBR28

Vera JH, Guo Q, Owen D, Matthews P M, Rabiner E A, Winston A, Gunn R N

Society of nuclear medicine and molecular imaging meeting, St Louis, USA, 2014

Imaging brain TSPO availability with [¹¹C] PBR28 PET in patients with retroviral (HTLV1 and HIV-1) infection

Guo Q, Vera JH, Dimber R, Adonis A, Bishop C, Owen D, Kalk N, Newbould R, Matthews P, Taylor GP, Gunn R, Winston A, Rabiner E

International symposium on functional neuroreceptor mapping, Amsterdam, 2014

Neuroinflammation in asymptomatic HIV-infected subjects on effective cART

Vera JH, Guo Q, Matthews P, Rabiner E, Gunn R, Winston A

20th conference on retroviruses and opportunistic infections (CROI), Boston, USA, March 2014

Cerebrospinal fluid chemokines are associated with both maraviroc exposure and cerebral metabolites measured via magnetic resonance spectroscopy

Vera JH, Garvey L, Allsop J, Kaye S, McClure M, Back D, Taylor-Robinson S, Winston A

14th European AIDS conference, Belgrade, 2011

Data from academic work undertaken during this PhD published in scientific journals or presented at scientific conferences:

Publications:

The profile of raltegravir-containing antiretroviral therapy in HIV-infected individuals over 60 years of age

Vera JH, Jackson A, Dickinson L, Else L, Barber T, Mora-Peris B, Back D, Boffito M, Winston A.

HIV Clin Trials, 2014,(In press)

The safety of Flucloxacillin in HIV-infected subjects with positive HLA-B*5701 genotype

AIDS, 2013, Jan 28;27(3):484-5

Vera JH, Naous N, Mackie N, Winston A, Cooke G

Alterations in cerebrospinal fluid chemokines are associated with maraviroc exposure and in vivo metabolites measurable by magnetic resonance spectroscopy.

HIV Clin Trials, 2012, Jul-Aug;13(4):222-7

Vera JH, Garvey L, Allsop J, Kaye S, McClure M, Back D, Taylor-Robinson S, Winston A

Poster presentations

Raltegravir-containing antiretroviral therapy in HIV-infected individuals over 60 years of age: a phase 1 pharmacokinetic study

Vera JH, Jackson A, Dickinson L, Else L, Barber T, Mora-Peris B, Back D, Boffito M, Winston A.

British HIV association spring conference, Liverpool, 2014

Evidence of premature atherosclerosis in HIV-infected individuals with low cardiovascular disease risk factors

Vera JH, Rose K, Banya W, Drivas P, Keenan N, Pennell D, Winston A.

15th International Workshop on Co-morbidities and Adverse Drug Reactions in HIV, Brussels, 2013

A past history of syphilis is associated with poorer performance in the cognitive domains of memory and learning in HIV-1 infected subjects on stable cART

Vera JH, Garvey L, Tipple C, Goldmeier D, Winston A

British HIV association spring conference, 2012

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